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BEYOND POLLINATOR REWARD: STEPS FORWARD AND KNOWLEDGE GAPS ON THE ROLE OF FLORAL NECTAR IN PLANT-ANIMAL INTERACTIONS

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«Dobbiamo imitare le api che
svolazzano qua e là e suggono i
i fiori adatti a fare il miele […]
distinguere quello che abbiamo
ricavato dalle diverse letture […].
Fondere poi, in un unico sapore,
valendoci della capacità e della
diligenza della nostra mente, i
vari assaggi, così che, anche se
ne è chiara la derivazione,
appaiono tuttavia diversi dalla
fonte».

Seneca

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ABSTRACT

The work done within the framework of my PhD project has been carried out between November 2019 and January 2023 at the Department of Biological, Geological and Environmental Sciences of the University of Bologna, under the supervision of Prof. Marta Galloni and PhD Gherardo Bogo. A period of three months was spent at the Natural History Museum of Rijeka, under the supervision of Prof. Boštjan Surina. The main aim of the thesis was to investigate further the so-called pollinator manipulation hypothesis, which states that when a floral visitor gets in contact with a specific nectar chemistry, the latter affects its behavior of visit on flowers, with potential repercussions on the plant reproductive fitness. To the purpose, the topic was tackled by means of three main approaches: field studies, laboratory assessments, and bibliographic reviews. This research project contributes to two main aspects. First, when insects encounter nectar-like concentrations of a plethora of secondary metabolites in their food-environment, various aspects of their behavior relevant to flower visitation can be affected. In addition, the results I gained confirm that the combination of field studies and laboratory assessments allows to get more realistic pictures of a given phenomenon than the single approaches. Second, reviewing the existent literature in the field of nectar ecology has highlighted how crucial is to establish the origin of nectar biogenic amines to either confirm or reject the multiple speculations made on the role of nectar microbes in shaping plant-animal interactions.

INTRODUCTION

GENERAL FRAMEWORK: WHAT DO WE KNOW ABOUT NECTAR?

Nectar is defined as the secretion of specific organs, the nectaries (Linnaeus 1735), which rewards animal consumers whose visits somehow benefit the plant (Pacini et al. 2003). To date, its evolutionary appearance remains obscure (Pacini and Nicolson 2007), however, one of the first hypothesis on its origin states that nectar has evolved independently of any interaction with animals. For Lorch (1978), nectaries originated to rid the flower of exceeding liquid, an idea that was later revived by de la Barrera and Nobel (2004) who turned it into the better known "leaky phloem" hypothesis. In this view, nectar secretion originally had a physiological significance, nothing more than a phloem leakage occurring at structural weakness points of the plant tissues.

Beyond its evolutionary origins, nowadays nectar can either mediate indirect defense against herbivores by attracting protective ants to the plant vegetative parts (Nepi et al. 2018) or the entomophilous pollination process, by guaranteeing pollen removal and deposition on receptive female organs (Nepi 2017). Nectar mediating these two types of interactions is generally addressed as extra-floral and floral, respectively (Pacini et al. 2003). Nectaries secreting both types of nectar were already existent in the Late Cretaceous (Friis and Endress 1990). Such distinction, however, is not always precise, and the terminology should be rather adopted for flowering plants. In evolutionary history, though, structures similar to extra-floral nectaries appeared at first in pteridophytes. The most ancient currently living plant with nectaries, *Pteridium aquilinum* (L.) Kuhn (Dennstaedtiaceae), is a fern which provides nectar to reward ants in exchange for predator defense (Heads and Lawton 1985 and references therein). Ferns do not have flowers, though, thus addressing such nectaries as extra-floral is not correct, and the term "foliar" nectaries should be rather used (Koptur et al. 2013). Similarly, despite the presence of nectar in gymnosperms is debated, several Gnetophyta present pollination drops, sugary secretions produced by ovules (Nepi 2017 and reference therein). The primary function of such drops is to capture pollen grains and to nourish them, however, there are several reported cases of insects contributing to gnetophyte pollination, and in all such cases pollination drops are exploited by insects as food source and "reward" (e.g. Procheş and Johnson 2009, Gong et al. 2016).

In this thesis I exclusively refer to floral nectar and to its role in mediating entomophilous pollination in angiosperms. Why and how nectar started mediating plant-pollinator interactions remains to clarify. According to Takhtajan (1980) the original food source exploited by insects in these early relationships was the pollen. In this view, nectar took the role of floral reward later, representing an alternative alimentary resource to economize pollen grains. However, in open contrast with this perspective, Endress (1994) suggested an opposite hypothesis, for which the main reward to

pollinating insects in early plant-pollinator interactions were secretions similar to nectar, something comparable to the pollination drops and the stigmatic exudates still observable nowadays (Pacini and Nicolson 2007). However, it is worth mentioning that mesozoic flies involved in the pollination of nowadays extinct cycad-like gymnosperms (Bennettitales) showed clear morphological adaptations to nectivory (Peñalver et al. 2015).

Floral nectar is originally defined as the secretion of specific organs, the nectaries (Beutler 1953 and reference therein), associated with plant reproductive structures (Nepi, 2017) and which reward animals that may perform pollination while visiting the flower (Nepi et al. 2018). Due to its chemical composition, dominated by high concentrations of simple sugars such as sucrose, glucose and fructose (Nicolson and Thornburg 2007), nectar is considered as an easily absorbable, very cost-effective, alimentary resource for many animals (Nicolson 2007, González-Teuber and Heil 2009). Besides sugars, amino acids are the most abundant nectar solutes detectable in floral nectar, and all twenty protein amino acids are present in nectar (Nicolson and Thornburg 2007). By virtue of its sugar and amino acid content, it has been long considered a simple reward offered by plants to attract animals and ensure the pollination service.

Starting from the ’70s, though, the discovery of hundreds of secondary metabolites such as alkaloids, phenolics, terpenoids, and non-protein amino acids (e.g. Baker and Baker 1977, 1986), has challenged the traditional view of floral nectar. At first, the occurrence and maintenance of such compounds in floral nectar has been explained through the pollinator fidelity hypothesis (Baker and Baker 1975), stating that “toxic” nectar could result beneficial to the plant by deterring the less specialized floral visitors which are likely to carry a smaller amount of co-specific pollen. Nowadays, it is well established that these compounds can play a variety of different roles which can turn out to be potentially beneficial to both parties (Stevenson et al. 2017). For example, research has demonstrated that insect pollinators benefit from the intake of alkaloids which reduce their pathogen load (Manson et al., 2010; Gherman et al., 2014), and nectar chemistry modulates floral visitor behavior in several ways, for example by affecting feeding, locomotion, learning, and flight muscle performance (e.g. Petanidou et al. 2006, Wright et al. 2013, Nepi 2014, Baracchi et al. 2017, Bogo et al. 2019, Carlesso et al. 2021).

The accumulation of such evidence led Pyke, in 2016, to introduce the so-called manipulation hypothesis. The idea beyond this is that trough nectar, a plant can manipulate the foraging behavior of nectar-feeding pollinators during their visits, positively influencing its own reproductive fitness (Bailey et al. 2007). This can be particularly well understood in those cases where potentially

disadvantaging behaviors emerge in the pollinator after nectar consumption. For example, the onset of addiction leads bumblebees to consistently return to food sources containing nicotine, even when such sources become suboptimal (Baracchi et al. 2017).

The chemical composition of floral nectar can be shaped by both ecological and phylogenetic constraints (Nepi et al. 2010). Among the first, interactions with specific guilds of pollinators may drive selection towards convergent nectar chemistry in unrelated taxa (Fenster et al. 2004, Pozo et al. 2015). On the other hand, phylogenetic conservatism may result in similar nectar chemistry in related taxa regardless of their pollinators (Nicolson and Thornburg 2007, Nepi et al. 2010).

Moreover, complex and frequent processes of post-secretion modification affect floral nectar in most cases. On one hand, some of these post-secretion changes are determined by microclimatic factors: these fluctuations in the nectar constituent concentrations can be due to changing water availability, changing environmental humidity and temperature, soil-related factors or atmospheric CO_2 concentration (Corbet et al. 1979, Plowright 1981, Nicolson 2002, Chalcoff et al. 2017, Parachnowitsch et al. 2019). On the other hand, mechanisms of nectar homeostasis which actively maintain constant nectar sugar concentrations have been also described (Nepi and Stpiczyńska 2008, Nepi et al. 2011), as well as mechanisms of active sugar reabsorption when nectar remains unconsumed (Pacini and Nicolson 2007, Nepi and Stpiczyńska 2008).

Among the post-secretion processes determined by biotic variables, instead, it is nowadays known how crucial the influence of floral visitors can be in modifying nectar chemistry through the introduction of external contaminants (Canto and Herrera 2012, Chappell and Fukami 2018, Vannette and Fukami 2018, Yang et al. 2019). This can happen when pollen grains and/or microbes are introduced and transferred through the visitor's mouth parts or bodies (Herrera et al. 2009, Mittelbach et al. 2015, Pozo et al. 2015), making some of the primary and secondary metabolites characterizing the chemical profile of the nectar coming from contamination processes (Vannette and Fukami 2018, Bogo et al. 2021). Yeasts, for example, can modify the nectar sugar profile (Canto and Herrera 2012, Chappell and Fukami 2018), alter the abundance of some amino acids (Vannette and Fukami 2018) or change composition and concentration of some secondary compounds (Vannette and Fukami 2016). Pollen grains can either spontaneously fall into the nectar from the anthers or be brought in by floral visitors, in both cases they can modify the nectar chemical profile both quantitatively and qualitatively (Bogo et al. 2021). For long, the presence of microbes in floral nectar has been mainly pointed as detrimental for the quality of nectar (Eisikowitch et al. 1990, Vannette et al. 2013), leading to the conviction that their presence weakened or negatively interfered with the plant-pollinator

mutualism. This view, though, has been challenged by a series of recent studies. For example, it is nowadays believed that yeast cells may supplement insects for important nutritional elements such as vitamins, sterols, and minerals (Vega and Dowd 2005, Stefanini 2018), representing an important nutritional component themselves (Jacquemyn et al. 2021). Moreover, recent findings demonstrated that not only bumblebees can detect microbial presence in artificial nectars (Fouks and Lattorff 2011), but also show a preference for yeast-containing flowers (Herrera et al. 2013, Schaeffer et al. 2014, Schaeffer et al. 2017). This could be also related to the fact that the presence of determined yeast or bacteria species in the floral nectar can positively affect bee fitness (Rima et al. 2012, Jacquemyn et al. 2021), by reducing pathogen growth inside the gut (Pozo et al. 2020) or increasing the insect reproductive success (Pozo et al. 2021).

This long series of breakthroughs in the field has brought the research to consider novel perspectives on both the evolutionary and ecological meaning of floral nectar in its interacting with the surrounding environment. Several questions, though, remain unanswered and further investigation is crucial to support revolutionary perspectives that are starting to loom in the distance.

GENERAL AIM OF THE THESIS

The general aim of my thesis is investigating how nectar-like concentrations of naturally occurring secondary metabolites affect animal behavior, perception, and physiology. In this regard, an extensive review of literature regarding the already available information about different classes of nectar chemicals was carried out for the entire duration of the project.

The aim of studying the effects of nectar secondary metabolites on pollinators was pursued by tackling the subject from several points of view and by means of different approaches. The work was organized in experimental essays and reviews: within the former, some were laboratory assessments, whilst some were field studies. This choice was driven by the conviction that a better comprehension of the phenomenon is possible if both methods are applied, and results are interpreted together.

Laboratory assessments are generally more feasible than field studies because they allow an easier identification of the behavioural effects produced by different *ad hoc* artificial nectars (Muth et al. 2017). This is done by removing the impact of several variables and considering the effects of only one (or few) chemical at a time. In this regard, they allow a more reliable measurement of a consumer's learning performance, preference, and motivation. Nevertheless, they present some limitations: observations on captive animals, for example, may not always yield a realistic picture of how behavior is affected. Harnessed bees can behave differently compared to free-moving bees: for

instance, they may accept different sucrose concentrations (Mujagic and Erber 2009) or be more likely to ingest toxic substances (Ayestaran et al. 2010). Similarly, the administration of single compounds – even though at nectar-like concentrations – can yield a distorted picture, since it is nowadays well-established that different chemical compounds may interact synergically or antagonistically (e.g. Muth et al. 2022). On the contrary, despite being subjected to a series of uncontrollable variables as well as not always bringing a control for comparison, field studies allow individual pollinators to decide the extension and frequency of their exposure to the natural nectar chemistry.

In addition, most behavioural studies are often conducted on few insect model species, generally coinciding with those commercially available (e.g. in our continent: *Apis mellifera* Linnaeus, 1758; *Bombus terrestris* Linnaeus, 1758*; Osmia bicornis* Linnaeus, 1758). However, wild bees vary in several characteristics such as life cycle, sociality, and dietary specialization (Muth et al. 2017) highlighting the urgency to extend the investigations on the effects of nectar secondary metabolites to a higher number of species.

Conclusive reviews of the information available in the broad body of literature then highlight novel perspectives and knowledge gaps still existent in the field.

STRUCTURE OF THE THESIS

The thesis consists of seven Chapters, two Appendix studies, list of publications and undertaken activities, and a Supplementary material section which recalls each Chapter, whose content is summarized below.

Part 1: experimental essays

Chapter 1 consists of a laboratory assessment finalized to test how the amino acids proline and β-alanine at concentrations similar to those found in the floral nectar of *Gentiana lutea* subsp. *symphyandra* (Murb.) Hayek affect bee preference and consumption. The species offers a hexose-dominant nectar known to attract mainly dipterans. The presence of high concentrations of both proline and β-alanine has been hypothesised to be the cause of the abundant number of bee visits observed instead.

Chapter 2 consists of a field study conducted on the wide-spread species *Echium vulgare* L.. Nectar was collected and analyzed considering the two floral sexual phases (functionally female and functionally male flowers). The proportion of protein amino acids appeared to be significantly higher in male-phase flowers compared to the female-phase flowers, which could explain the significantly higher number of visits performed by bees on male flowers compared to the expected one. Results of

this study are reported as in the article published on *Plant Ecology* (Barberis et al. 2021, https://doi.org/10.1007/s11258-020-01101-5).

Chapter 3 reports a study under the controlled conditions of a greenhouse were the effects of the nectar biogenic amine tyramine found in *Echium vulgare* were investigated on the behavior of visit of bumblebee workers. *Ad hoc* artificial sucrose solutions enriched with tyramine were injected into the nectar chambers of zucchini flowers previously emptied of their natural nectar and bees were set free to visit the flowers. Bees fed control spent more time on a single flower than those fed tyramine-enriched solution, suggesting a less dynamic behavior. Results of this study are reported as in the version accepted for publication by *Arthropod-Plant Interactions*.

Chapter 4 reports a second field study conducted on the same species, *Echium vulgare*. The species exhibits a long bloom that, at our latitudes, start in June and ends in October. Nectar samples were collected from functionally female flowers in early and late summer, in two distinct populations, and so were the insect behavioral observations. Nectar chemistry changed substantially as the season proceeded. This study set a baseline for future research, and highlighted an interesting point: since long flowering plants face changing surroundings during the unfolding of their blooming season, do they express chemical constraints to regulate their attractiveness?

Chapter 5 reports a study on the effects of two nectar biogenic amines, namely tyramine and octopamine, on bumblebee locomotion, consumption and gustatory responsiveness. Our results suggest a preference for octopamine over tyramine and control, as well as a dose-dependent effect on flight, confirming that even nectar-like concentrations of the biogenic amines produce biological effects in various bumblebee behaviors which are relevant to flower visitation.

Part 2: reviews

Chapter 6 represents a review on the effects of nectar secondary metabolites on pollinator behaviour. Other than treating the main classes of nectar chemicals, such as phenols, terpenoids, alkaloids, non-protein amino acids and the recently discovered class of biogenic amines, the review gives evolutionary insights and future perspectives. The review is reported as in the article published on *Plants* (Barberis et al. 2023, https://doi.org/10.3390/plants12030550).

Chapter 7 consists of a conclusive mini-review briefly reviewing the ecological roles played by floral nectar that have been succeeding over the past fifty years, since the first discovery of nectar secondary metabolites. The paper also highlights the current gaps in our knowledge and ends with a question open to debate regarding the proposal to expand the traditional definition of floral nectar.

Appendix.

Appendix 1 is a data paper born from the joint effort of ecologists from all over the world who, during the pandemic lockdown of 2020, collated data on plant-pollinator interactions in their garden, making them available for students and researchers. The dataset is reported as in the article published on *Journal of Pollination Ecology* (Ollerton et al. 2022, https://doi.org/10.26786/1920-7603(2022)695). *Appendix 2* reports an application of the species-based approach named SHARP (Systematic Hazard Analysis of Rare-Endangered Plants; Aronne, 2017) proposed by a group of European experts committed to plant conservation within the framework of the COST Action ConservePlants 18201, of which I hold the Working Group 1 membership. The study aims to assess whether the already available information on the biology of endangered plant species is adequate to identify bottlenecks in their generation turnover and plan executive actions, highlighting that the goals of biological research studies often diverge from conservation purposes.

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PART 1: EXPERIMENTAL ESSAYS

1. PROLINE AND B-ALANINE INFLUENCE BUMBLEBEE NECTAR CONSUMPTION IN DIFFERENT WAYS WITHOUT AFFECTING SURVIVAL

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Abstract

Nectar composition is an important driver of insect attractiveness. Although bumblebees prefer sucrose-rich nectar, they were found to be the main pollinators of *Gentiana lutea*, whose nectar is low on sucrose. Here we test the hypothesis that bumblebees are attracted to proline and β-alanine, two amino acids naturally occurring at high concentrations in the nectar of *G. lutea*. We analysed the preference and survival of *Bombus terrestris* workers fed with artificial nectars enriched with proline, β-alanine or both, at natural and double concentrations. Bumblebees consumed less proline-enriched nectar at twice the natural concentration (P2) than all other combinations. Nectar consumption significantly increased with bumblebee weight when bumblebees were fed with the control solution or P2, suggesting that these solutions satiate them less. Bumblebee survival was not affected by any nectar composition. Our results indicate that bumblebees are able to perceive proline and, contrary to honey bees, they don't have a preference for it. β-alanine, on the other end, did not increase consumption but it seems to contrast the negative effect of proline on preference. We therefore concluded that the high visitation rate of bumblebees on flowers of *G. lutea* could be partially due to the nectar amino acidic composition.

Keywords

artificial nectar; *Bombus terrestris*; bumblebee survival; nectar consumption; nectar preference

1.1 INTRODUCTION

Nectar is an aqueous solution mainly composed of mono- and disaccharides, namely glucose and fructose, and their combination into sucrose (Nicolson and Thornburg 2007). However, less abundant components such as amino acids, lipids, phenols, alkaloids and volatile organic compounds are commonly found in floral nectar (Kessler and Baldwin 2007, Nicolson and Thornburg 2007, González-Teuber and Heil 2009, Roy et al. 2017). All nectar components, including primary and secondary compounds, may affect the attractiveness of nectar to pollinators: as a consequence, their amount and concentration are often related to a specific pollinator type (Baker and Baker 1977, Faegri and van der Pijl 1979, Baker and Baker 1983a).

Many studies have demonstrated that clear differences in nectar sugar composition are correlated with different pollinator functional groups: for example, flowers with a high sucrose/hexose ratio are preferably pollinated by hummingbirds, Megachiroptera and long-tongued bees (e.g. honey bees and bumblebees), while a low sucrose/hexose ratio is tendentially preferred by passerine birds, Microchiroptera and short-tongued bees (Baker and Baker 1983b, Kress 1985, Baker and Baker 1990, Baker et al. 1998). In addition to sugar composition, other nectar components can influence the attractiveness towards different pollinators. Among these components, several studies have investigated the role of amino acids, the second most concentrated solute in nectars (Alm et al. 1990, Nicolson and Thornburg 2007, Barberis et al. 2021). A unique aspect related to amino acids is their potential contribution to nectar taste (Gardener and Gillman 2002). Amino acids have much more diverse chemical structures than sugars and their concentration may be highly variable, producing a wide range of tastes (Birch and Kemp 1989).

Several insect groups have been found to detect and show preferences for certain amino acids dissolved in nectars, including ants (Bluthgen and Fiedler 2004), flesh flies (Potter and Bertin 1988), butterflies (Alm et al. 1990, Erhardt and Rusterholz 1998) and fruit flies (Croset et al 2016, Ganguly et al. 2017). Among bees, honey bees displayed a clear preference for solutions enriched with proline and phenylalanine, while disliking solutions enriched with serine (Inouye and Waller 1984, Alm et al. 1990, Bertazzini et al. 2010). Recent work showed that bumblebee (*Bombus terrestris*) workers preferred sugar solutions enriched with β-alanine at concentrations commonly found in nature over solutions presenting sucrose and γ-amino butyric acid, while survival was not affected by either solution (Bogo et al. 2019). In addition, bumblebees were able to perceive a variety of amino acids (excluding proline) and to differentiate among different concentrations of the same amino acid, while they were not able to discriminate between different amino acids (Ruedenauer et al. 2019).

Bumblebees are the main pollinators of the perennial plant *Gentiana lutea* L. (Gentianaceae), despite the fact that its nectar is almost sucrose-free (Rossi 2014). Among other solutes, proline and β-alanine are the single most abundant amino acids in the nectar of *G. lutea*, reaching up to 64% of the total amino acid content (Rossi 2014). Proline is a non-essential protein amino acid (de Groot 1953) commonly found in nectar (Nicolson and Thornburg 2007), which can stimulate insect salt cells increasing the intensity of feeding behaviour (Hansen et al. 1998, Wacht et al. 2000). In addition, proline is the most abundant amino acid found in honey bees' haemolymph (Crailsheim and Leonhard 1997, Hrassnigg et al. 2003), where it is selectively degraded during the initial stages or lift phase of flight (Micheu et al. 2000), acting as a more efficient short-term fuel than sugar and resulting in short-term bursts of energy production (Carter et al. 2006, Teulier et al. 2016). β-alanine is a non-protein, non-essential amino acid commonly found in nectar (Nepi et al. 2012, Nepi 2014), which is apparently involved in the regulation of muscular activity as a precursor of the dipeptide carnosine (Harris et al. 2006). Carnosine is found in both vertebrate and non-vertebrate skeletal muscles, and is known to increase isometric endurance in humans (Harris et al. 2006).

Here we investigate the role of the two amino acids proline and β-alanine on nectar preference and survival of worker bumblebees (*B. terrestris*). To do so, we conducted a laboratory experiment by presenting different solution combinations to bumblebees, including amino acid concentrations at levels naturally found in nectar of *G. lutea* and twice the natural levels, in order to stimulate different responses. We hypothesise that bumblebees prefer artificial nectars containing amino acids over those with only sugar, with responses dependent on amino acid combinations, while we do not expect significant effects on their survival. Results will improve our knowledge about the effects of amino acids on bees, and their importance as an interface between plants and pollinators.

1.2 MATERIALS AND METHODS

1.2.1 Study species and experimental conditions

We performed this study on 229 worker bumblebees (*Bombus terrestris* L.) obtained from commercial colonies (Bioplanet S.r.l., Cesena, Italy), maintained at 25 ± 1°C and 40 ± 5% relative humidity (RH) in continuous darkness and fed *ad libitum* with fresh frozen pollen (multi-floral pollen collected from honeybees by pollen traps) and sugar syrup, for 3-4 days before the experiment started. Colonies contained around 80 workers, brood in all stages of development and a laying queen.

Workers were collected from three colonies under red light, caged individually into Nicot cages (7.1 × 2.0 cm) and weighed. Bumblebee weight and colony of origin were used as gauge of an equal distribution of individuals among the different treatment groups. We used a minimum of 30

bumblebees (each individual representing a replicate) for each treatment and concentration. Bumblebees were maintained in a dark climate room at 25 ± 2°C and 40 ± 10% RH. Since large variation in body size exists among workers of *B. terrestris*, very small (< 0.10 g) and very large (> 0.35 g) individuals were excluded from the experiment to standardize the samples. Newly emerged and old bumblebees (visually discriminated on the basis of whitish colour and lack of hairs, respectively) were also excluded from the experiment (Hanewald et al. 2014).

1.2.2 Experimental design

We followed the OECD guideline for the testing of chemicals on bumblebees (OECD 2017) as modified by Sgolastra et al. (2017). Bumblebees were acclimatised to the test conditions over night (12-24 h), fed with a sugar solution (1:2 w/v) provided *ad libitum* in 2.5 mL nozzle-cut syringes. The first day of the experiment the syringes with sucrose solution were replaced by syringes with treatment solutions, previously weighed. For the whole duration of the experiment (23 days), syringes were weighed daily to calculate consumption with evaporation correction, mortality was checked daily, and the solutions were replaced at least twice a week.

1.2.3 Artificial nectar solutions

We tested the effect of artificial nectar solutions enriched with proline and β-alanine. The solutions used in this study were based on the sugar and amino acid composition of floral nectar of wild yellow gentian (*G. lutea*) produced in natural conditions (Rossi 2014, Table 1).

Concentration [a]	Solution	Proline		β-alanine		Total concentration	
		mg/L	mM	mg/L	mM	mg/L	mM
C1	Proline (P)	138	1.2	-	-	138	1.2
	β-alanine (B)	-	-	205	2.3	205	2.3
	Proline + β-alanine (PB)	138	1.2	205	2.3	343	3.5
C2	Proline (P)	276	2.4	-	-	276	2.4
	β-alanine (B)	-	-	410	4.6	410	4.6
	Proline + β-alanine (PB)	276	2.4	410	4.6	686	7.0

[a] C1: same concentration of amino acids as found in nectar of *G. lutea* in natural conditions. C2: amino acids at twice the concentration of C1

Table 1. Amino acid concentrations used in the artificial nectar solutions administered to worker bumblebees. All nectar solutions contained the same amount of sugars of the sugar-only solution (S: glucose = 987 mM, fructose = 915 mM, sucrose = 5.55 mM)

We prepared one control solution (S) that contained only sugars in the same concentration as the natural nectar of *G. lutea* (glucose: 987 mM; fructose: 915 mM; sucrose: 5.55mM), and six analogous

solutions that contained the same amount of sugars and were enriched with amino acids (Sigma-Aldrich, Milano, Italy) in different combinations and concentrations. Three solutions were prepared using the concentration of amino acids found in natural conditions (C1, Table 1): one solution was enriched only with proline (P), one only with β-alanine (B), and one with both proline and β-alanine (PB). We also prepared three artificial nectar solutions with the same solutes as C1 (i.e., P, B, PB) at twice the natural concentration (C2, Table 1), to increase the likelihood of stronger effects on bumblebee consumption and survival.

1.2.4 Data analysis

All analyses were performed in R version 4.1.1 (R core Team 2021). To evaluate differences in solution consumption by bumblebees, we fitted a linear mixed-effect model (LMMs) using the R package *nlme* (Pinheiro et al. 2021). We set the log-transformed consumption as response variable, and treatment (i.e., amino acid type), log-transformed bumblebee weight, and their interaction as explanatory variables. Treatment comprised the control solution and the six amino acids (proline, β-alanine, proline and β-alanine) × concentration (natural and twice the natural concentration) combinations. We included bumblebee ID nested within colony ID as random effect to account for individual and colony variability. Moreover, because consumption was measured on the same individuals on consecutive days, we included an autoregressive process of order 1 to account for temporal autocorrelation (Box et al. 1994). Pairwise contrast effects and the significance of interaction effects were estimated using the R package *emmeans* (Lenth 2021).

We evaluated the effects of log-transformed solution consumption, treatment (i.e., amino acid type), and log-transformed bumblebee weight on bumblebee survival by means of Cox proportional hazard regression mixed-effects models (Ripatti and Palmgren 2000; Therneau et al. 2003), using the R package *coxme* (Therneau 2020). First, we fitted the full model including each response variable as additive factors and the interaction between treatment and consumption. Then, we fitted different models by sequentially excluding the interaction effect, the interaction and the treatment effects, and the interaction, treatment and weight effects, respectively. In each model we included bumblebee ID nested within colony ID as random effect. We fitted type II ANOVAs using the R package *car* (Fox et al. 2019) to evaluate the contribution of every factor to the model's variance (each variable was tested against the model without it and without any interactions with other variables). We tested for significance of explanatory variables by means of analysis-of-deviance between pairs of nested models, using the R package *lmerTest* (Kuznetsova et al. 2017). Model effects were compared using the R package *emmeans* (Lenth 2021). Figures were drawn using the R packages *ggplot2* (Wickham et al. 2016) and *survminer* (Kassambara et al. 2021).

1.3 RESULTS

1.3.1 Artificial nectar consumption

Bumblebees consumed significantly lower amounts of the solution containing proline at twice the natural concentration (P2) than all other solutions, except solutions containing proline at natural concentration (P1) and both proline and β-alanine at twice the natural concentration (PB2), for which differences were only marginally significant (Figure 1, Tables S1-S2).

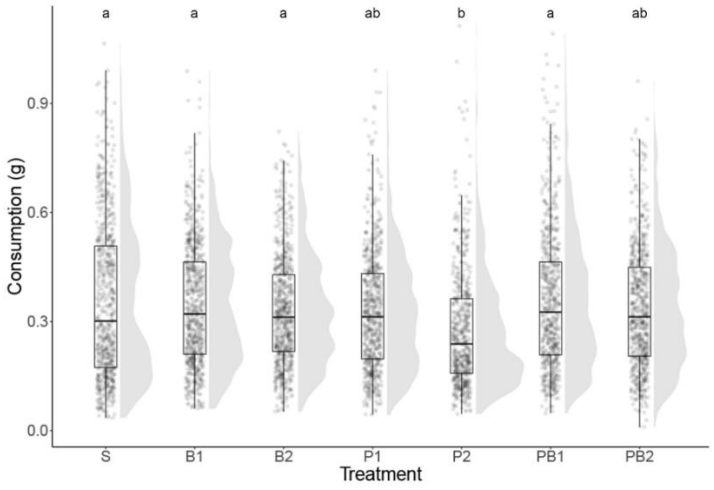

Figure 1. Consumption of the seven treatment solutions by bumblebees. S: control solution (i.e., only sugars), B: β-alanine, P: proline, PB: proline and β-alanine, 1: natural amino acid concentration found in nectar of *G. lutea*, 2: twice the natural amino acid concentration. Different letters above boxplots indicate significant differences between solutions at 0.95% confidence level.

There was a strong positive correlation between bumblebee weight and solution consumption (Table S1). Solution consumption significantly increased with bumblebee weight in a similar way when bumblebees were fed with the control (S) or the proline solution at twice the natural concentration (P2) (Table S1, Figure 2a-b). Intake of all other amino acid solutions increased less markedly with increasing bumblebee weight (Table S1, Figure 2a), and effects were only significant for solutions containing β-alanine at twice the natural concentration (B2) and both proline and β-alanine at natural concentration (PB1) (Figure 2b).

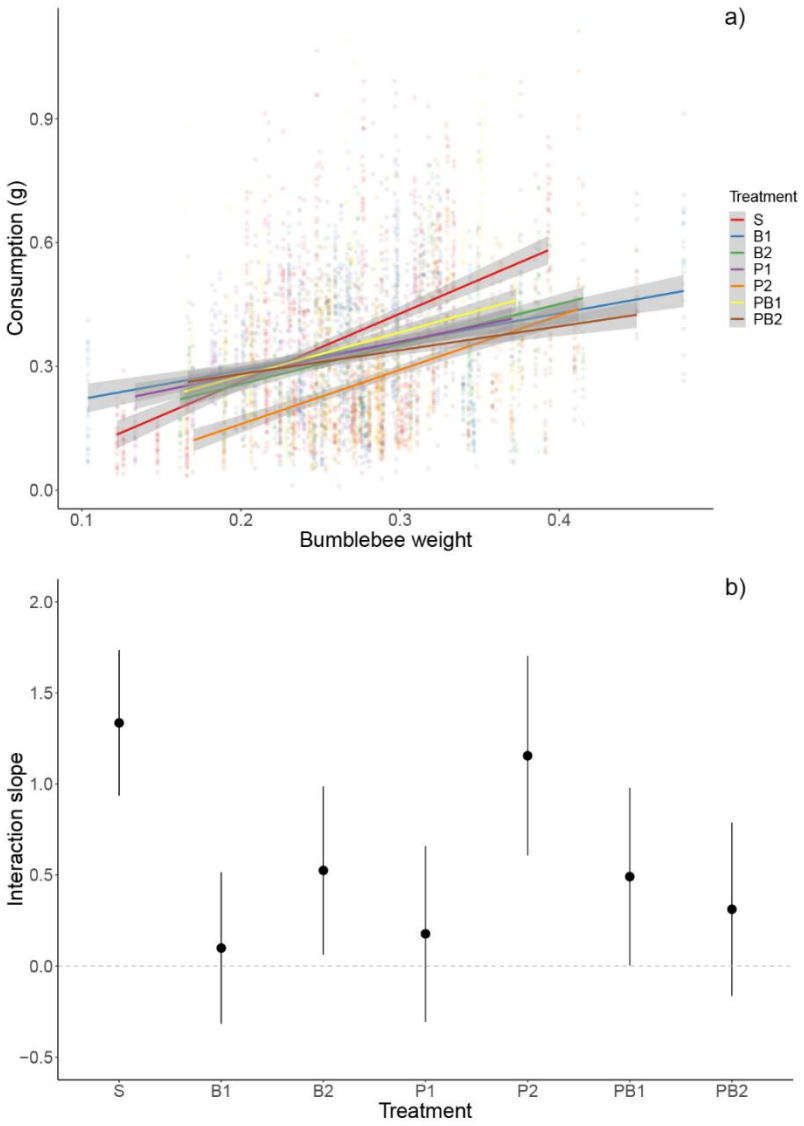

Figure 2. Interaction between log-transformed bumblebee weight and solution consumption (a), and slope of each interaction with related 95% confidence levels (b). Confidence levels that do not cross zero in panel (b) indicate significant positive effects of treatment × bumblebee weight interactions. S: control solution (i.e., only sugars), B: β-alanine, P: proline, PB: proline and β-alanine, 1: natural amino acid concentration found in nectar of *G. lutea*, 2: twice the natural amino acid concentration.

1.3.2 Bumblebee survival

At the end of the 23-days observation period, more than 80% of bumblebees survived under all amino acid treatments (Figure 3). The probability of bumblebee survival increased with increasing solution intake (estimated coefficient = −2.61, SE = 0.39, z = −6.78, p = 1.2e−11), while survival was not significantly related to either the type of amino acid solution consumed or bumblebee weight (Tables S3-S5).

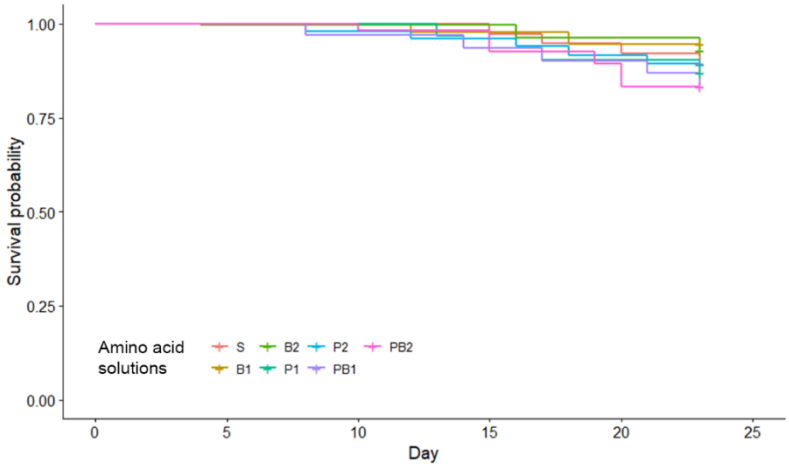

Figure 3. Kaplan-Meier survival curves for bumblebees fed with six amino acid combinations. S: control solution (i.e., only sugars), B: β-alanine, P: proline, PB: proline and β-alanine, 1: natural amino acid concentration found in nectar of *G. lutea*, 2: twice the natural amino acid concentration.

1.4 Discussion

We tested the effects of the protein amino acid proline and of the non-protein amino acid β-alanine on bumblebee nectar preference and survival. We observed a marked negative effect of the proline-enriched solution at twice the concentration naturally found in the nectar of *G. lutea* on bumble bee consumption, while we did not find any solution-dependent effects on bumblebee survival.

Our findings suggest that proline acted more as a deterrent than phagostimulant for bumblebees, especially at high concentrations. In fact, the solution containing proline at twice the concentration found in natural conditions was the least consumed. A similar result was found for honey bees feeding at very high proline concentration (100mM, Simcock et al. 2014). However, Bertazzini et al. (2010) found that honey bees preferred solutions containing proline at four times the highest concentration used in our study (10 mM vs 2.4 mM) over alanine and serine. Such different responses suggest that the composition of flower nectar plays a pivotal role in driving attractiveness to wild bees and defining

diet specialisation, since both the positive and negative effects of amino acids on bee preferences appear to be species specific (Felicioli et al. 2018; Bogo et al. 2019). Although Ruedenauer et al. (2019) found that workers of *B. terrestris* were not able to perceive proline and α-alanine through their antennae, this is not in contrast with our results, as the chemotactile and gustatory perceptions can be complementary and very different from each other.

Because bumblebees consumed less solution containing proline at high concentrations than solutions containing both proline and β-alanine together, we hypothesise that β-alanine plays a positive role on bee preference and mitigates proline deterrence. β-alanine has multiple functions in insect physiology and is a precursor of the dipeptide carnosine (Harris et al. 2006) and, like taurine, could be associated with fully functional flight muscles (Whitton et al. 1987). Social Apidae use flight muscles not only to fly but also to increase their thorax temperature to regulate the temperature inside the colony (Heinrich 1975). In addition, a high concentration of β-alanine was found in the retinal interstitial fluid of male honey bees, and neurons can use it as substrate for energy metabolism (Cardinaud et al. 1994). Therefore, bumblebees could actively consume nectar containing β-alanine to increase its intake or to meet their own requirements.

Our results suggest that flowers with nectar naturally rich in specific amino acids such as β-alanine may appear more attractive to bumblebees, possibly affecting their foraging choices. Plant species with a higher content of specific amino acids may therefore be more competitive than co-flowering species in terms of pollination service, potentially increasing pollinator visits and conspecific pollen transfer, thus promoting reproductive fitness (Nattero et al. 2011; Brosi and Briggs 2013).

Bumblebee survival was not affected by amino acid composition nor by amino acid concentration. These results are in accordance with those found in similar studies on honey bees, which showed longer survival when fed on low protein:carbohydrate ratios or low concentration of essential amino acids (Pirk et al. 2010; Paoli et al. 2014a, b). Although Stabler et al. (2015) found a negative correlation between survival and amino acid concentration, the authors used significantly lower amino acid:carbohydrate ratios than in our study. The fact that we found a positive correlation between bumblebee survival and solution intake confirms that worker bumblebees prioritize carbohydrate intake over specific amino acid preferences (Stabler et al. 2015).

The results of the present study combined with those previously obtained by Felicioli et al. (2018) and Bogo et al. (2019) outline a complex picture concerning the involvement of protein and non-protein amino acids in affecting nectar consumption, survivorship, and behaviour in bees. Since non-

protein amino acids are quite common in nectar but rarely studied (Nepi 2014), our findings highlight that these highly overlooked compounds deserve further investigation to untangle plant-pollinator interactions.

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2. GENDER-BIASED NECTAR TARGETS DIFFERENT BEHAVIOURAL TRAITS OF FLORAL VISITORS

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Gender-biased nectar targets different behavioural traits of flower visitors

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Abstract

Floral nectar is a chemically complex aqueous solution within which several secondary metabolites have been identified 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 hypothesize 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 min of behavioural observations on foraging insects was 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 hypothesize 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 interaction

2.1 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 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, 1982, Faegri and van der Pijl 1979, 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 c-aminobutyric acid, a phagostimulant function has been reported in some caterpillars and adult beetles (Mitchell and Harrison 1984, Schoonhoven 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, Pyke 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 Masierowska 2020) and few reported the observation of insect visit bias (Carlson and Harms 2006 and references therein). In this study we focused on the many-flowered hermaphrodite species *Echium vulgare* L., a self-compatible plant which shows both herkogamy and incomplete protandry, that avoids self-pollination within the same flower, but within which geitonogamy can still occur (Rademaker et al. 1999). Melser et al. (1999) reported evidences of inbreeding depression in *E. vulgare*, finding a significant decline in siring success when selfing occurs. A study on geitonogamy conducted by Rademaker et al. (1999), though, found a consistently lower percentage of selfing rate than expected. Also, they reported that bumblebees visited only a small fraction of the flowers on *E. vulgare* as a result of the presence of different flower stages simultaneously occurring on a single individual plant. *E. vulgare* represents an important food resource for many insect visitors, despite containing toxic pyrrolizidine alkaloids in both nectar and pollen (Lucchetti 2017). The pollen contains high concentrations of pyrrolizidines, whilst more than 500 times lower concentrations are found in nectar (Lucchetti et al. 2016). For this reason, only a few taxa show oligolecty or floral constancy on *E. vulgare* by actively collecting pollen for larval nourishment (Cane and Sipes 2006, Burger et al. 2010, Filella et al. 2011), even if its flowers are visited by a wide spectrum of insect taxa among which bumblebees have often been reported as main pollinators (Corbet 1978, Klinkhamer and de Jong 1990, Pappers et al. 1999, Rademaker et al. 1999). Here, we examined if floral visitation pattern may be influenced by variations in the chemical composition of nectar through different floral stages, and thus we investigated (i) whether *E. vulgare* produces a gender-biased nectar for volume, sugar and amino acid composition and (ii) if flower visitation rates of insects looking for nectar varied among different floral stages.

2.2 MATERIALS 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 (an area of approximately 600 m^2) 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 patches were alternatively considered: the first one was a single plant carrying 6 inflorescences while the second one was made up of 6 plants carrying one or two inflorescences each.

2.3 NECTAR QUALITY

2.3.1 Sampling

We collected nectar samples by means of Drummond Microcaps (3–5 μL; Drummond Scientific Co., Broomall, PA), we transferred samples to Eppendorf tubes filled with 100 μL of pure ethanol, and then we took them to the laboratory in thermal bags where they were kept at 5 °C until analyses. We collected each sample from multiple flowers at the same floral stage in order to reach a minimum volume of 2 μL needed for the sugar and amino acid analyses. In order to let the nectar accumulate, flowers were bagged in the morning for 2 h prior to sampling; all nectar present in the selected flowers was collected. We collected a total of 8 nectar samples, each one from 3 to 13 male flowers belonging

to 1–7 plants, and a total of 8 samples from 2 to 9 female flowers belonging to 1–3 plants. Both sugar and amino acid compositions were investigated on these samples. We then collected 14 additional samples from 1 to 22 buds belonging to 1–10 plants. Since the amount of nectar presents in the buds was very low, the minimum volume of 2 μL needed for amino acid analysis could not be reached and thus these samples were tested for sugar composition only.

2.3.2 Sugar analysis

Sugar content was analysed by HPLC technique through a Waters LC1 with refractive index detector (Waters 2410) connected to the output of a REZEX RCM Monosaccharide column (Phenomenex, 300 mm 9 7.8 mm, grain 8 lm) 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).

2.3.3 Amino acid analysis

Amino acid analysis was performed by gradient HPLC with an ion exchange Novapack C18 (15 mm 9 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).

2.3.4 Flower visitor 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-min periods separated by 10 min 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 min 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-min 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.

2.3.5 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 ni is the abundance of the ith 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 ith 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; Hubalek 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.

2.4 RESULTS

2.4.1 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.

2.4.2 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).

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

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

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_{15} = 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).

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

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

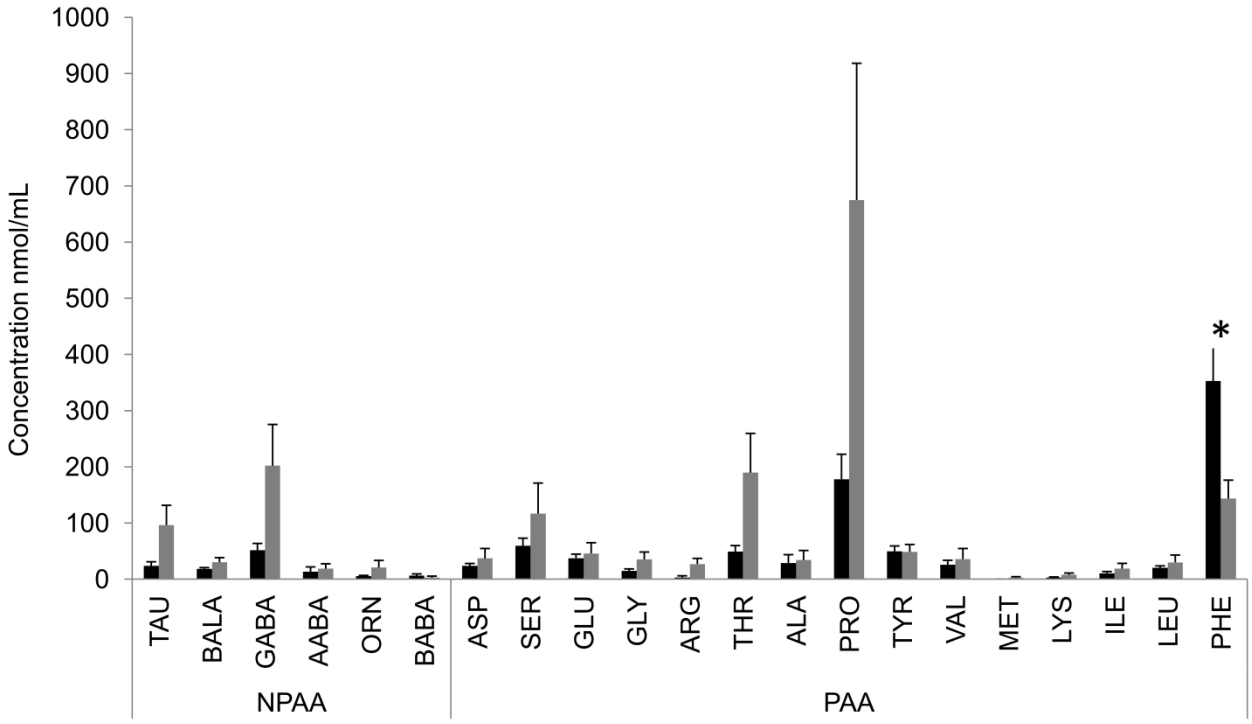

Figure 1. Amino acid concentrations (nmol/mL) detected in functionally male (dark bars) and in functionally female (light bars) flowers (mean ± SE). Amino acids hydroxyproline, homoserine, citrulline, cysteine, histidine, glutamine, asparagine and L-thyronine were not detected in either floral stages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

Insect visit analysis

Flower visitor's abundance

A total of 215 insect visits were recorded on Echium vulgare during 200 min of field surveys (Table 3). Visitors belonged to three orders: Hymenoptera (87.4%), Lepidoptera (9.8%) and Diptera (2.8%). The order Hymenoptera was mainly represented by individuals belonging to the family Megachilidae (59%), followed by the family Halictidae (26.5%) and Apidae (14%). The order Lepidoptera was represented mainly by individuals belonging to the species *Macroglossum stellatarum* (43%) and the family Pieridae (43%). The order Diptera was represented only by 6 individuals belonging to the families Bombyliidae and Syrphidae. The most frequent visitors were solitary bees of the species *Hoplitis adunca* (42%).

Flower visitor observations

Among the 215 insects visiting the plant, we fully recorded data for 189 individuals. Statistical analyses were carried out only on the 112 individuals which were looking for nectar and for which the number of total visits exceeded 5 (*Macroglossum stellatarum*, Pieridae, *Anthidium florentinum*, *Apis mellifera* and *Hoplitis adunca*). The family Pieridae was analysed as a single taxon in order to reach a total number of visits above 5. Since *Hoplitis adunca* was the most abundant taxon and the only species strongly oligolectic on *Echium*, we therefore decided to analyse the sexes separately. Although nectar is produced before flower opening and insects can force the bud searching for nectar (personal observation), this event occurred very rarely. Consequently, we did not consider the phenological stage bud in these analyses. For each insect taxon, we compared the number of visits to male and female flowers with the expected ones, calculated according to the ratio 1:9 between male and female flowers registered in the studied population. Regarding the number of male flowers visited, no significant difference was reported for lepidopterans (Pieridae spp., *Macroglossum stellatarum*) and for females *Hoplitis adunca*, while *Anthidium florentinum*, *Apis mellifera* and *Hoplitis adunca* males visited more male flowers than expected (Table 4).

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 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

[a]value calculated only on individuals with fully recorded data (n=21)

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

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

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

The number of female flowers visited was never statistically different from that expected. The frequency of male flowers visited in relation to the total number of flowers visited among taxa was statistically different ($H_4 = 14.01$, $p = 0.016$). Statistical analyses confirmed that the female *Hoplitis adunca* visited fewer male flowers than did *Anthidium florentinum* ($U = 65$, $p = 0.002$), *Apis mellifera* ($U = 48$, $p = 0.002$) and *Macroglossum stellatarum* ($U = 28.5$, $p = 0.043$; Fig. 2).

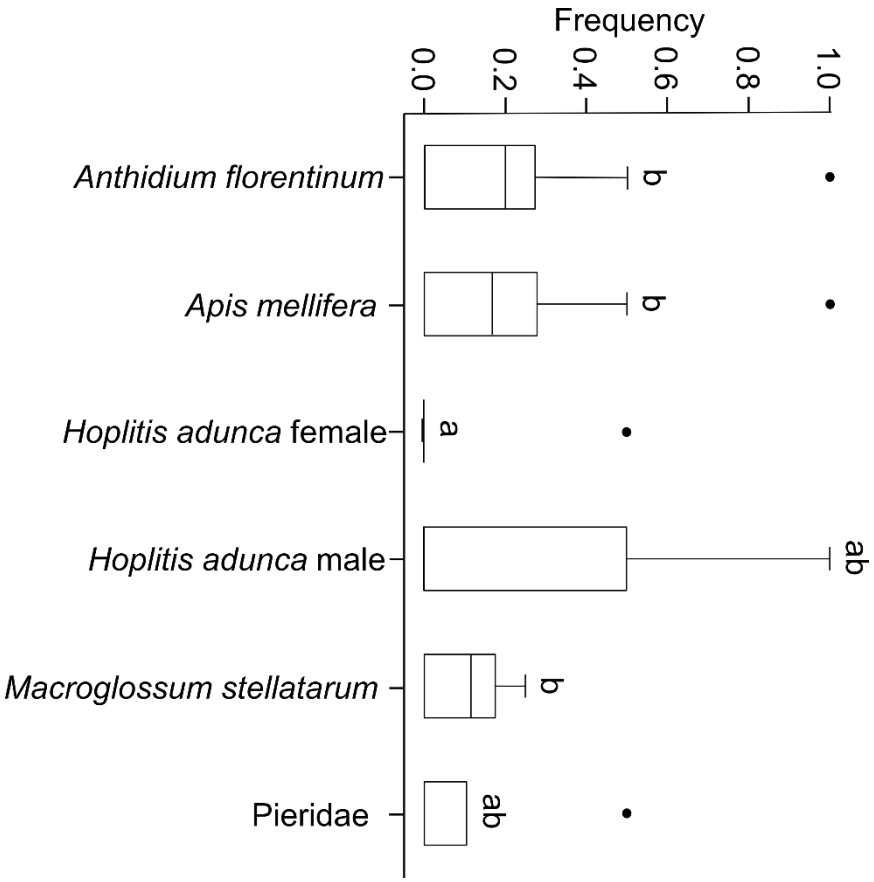

Figure 2. Frequency of male flowers visited by each taxon. Different letters denote statistical differences according to Kruskal Wallis H-test followed by Mann-Withney pairwise comparison with Benjamini-Hochberg correction ($p < 0.05$).

2.5 DISCUSSION

Our studied population did not show the phenomenon of gynodioecism, as all flowers were hermaphrodite, and our data confirmed the ratio of 1:9 found by Rademaker et al. (1999) between functionally male and functionally female flowers. Our analyses confirmed that nectar is secreted in the bud, as reported by Chwil and Weryszko-Chmielewska (2011). Contrary to Klinkhamer and de Jong (1990), we found that nectar volume, as well as sugar quantity per flower, increased with the age of the flower (from bud to female phase), although the positive trend between male and female phases was not statistically significant. Both quantity of hexose sugars and sucrose per flower increased with the age of the flower, the latter reaching a mean almost sevenfold higher in functionally female flowers than the mean amount found in the bud stage and almost twice the amount found in functionally male flowers. At the same time, the mean percentage of sucrose per flower appeared to be lower in male-phase flowers, even though not significantly, meaning that the total sugar increase in relation to floral age is due to the rise of nectar volume, since total sugar concentration and composition remained constant during the entire flower phenology. 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 Wackers (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 insects' physiology and behaviour, feeding rate and flight muscles performances (Shelp et al. 2017, Felicioli et al. 2018, Bogo et al. 2019). Besides GABA, or possibly the combination of GABA and NaCl, can constitute an important nectar phagostimulant and its presence correlates with visits by an array of pollinators such as long tongued bees, ex-anthophorid and andrenid bees, as well as anthomyiid and syrphid flies (Petanidou 2007 and reference therein). The spectrum of visitors recorded through our observations confirm that reported by previous studies stating that flowers of *E. vulgare* are visited by hummingbird hawkmoths (Aguado Martín et al. 2017), bees, bee flies (Proctor et al. 1996) and syrphids (Willmer and Finlayson 2014). Also, even

though the species has often been reported as mainly pollinated by bumblebees (Corbet 1978, Klinkhamer and de Jong 1990, Pappers et al. 1999, Rademaker et al. 1999), we observed only one individual of *Bombus pascuorum* visiting the flowers. Pollinators of widespread plant species can vary in relation to their geographical distribution (Armbruster 1985, Thompson 2006, Pérez-Barrales et al. 2007) and, moreover, as reported by Lazaro et al. (2010), the plant and pollinators assemblages of an entire community may also influence the composition of visitors of a particular species by determining, for instance, the strength of competition or the intensity of attraction to that species rather than another. Thus, the scarcity of bumblebees observed on *Echium vulgare* in 2018 may either depend on several factors and/or reflect a temporal fluctuation in the species composition of the pollinator community, as previously reported by many studies (Cane et al. 2005, Petanidou et al. 2008, Dupont et al. 2009).

2.6 CONCLUSIONS

The inbreeding avoidance hypothesis states that some mechanisms develop within a species in 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 behavior (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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3. NECTAR TYRAMINE DECREASES THE DURATION OF BUMBLEBEE VISITS ON FLOWERS

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Abstract

Several studies on floral nectar demonstrated that the behaviour of visit performed by pollinators is influenced by nectar chemistry. Biogenic amines act as neurotransmitters in invertebrates and recently have been reported in the floral nectar of 15 plant species for the first time. However, both their occurrence in floral nectar and the effects of their nectar-like concentrations on bee behaviour remains largely unsubstantiated. To increase knowledge on the role of biogenic amines on plant-pollinator interactions, here we i) investigated the biogenic amine composition of *Echium vulgare* nectar in relation to its floral sexual phases, and ii) studied how an artificial solution enriched with nectar-like concentrations of tyramine affects the visit on flowers of bumblebees under semi-controlled conditions. The chemical analysis reported the presence of tyramine in *E. vulgare* nectar and no difference in concentration between the two sexual phases. To explore potential effects of tyramine on bee behaviour, we designed a new method consisting in zucchini flowers emptied of their natural nectar and refilled with artificial tyramine-enriched nectar, and we used bumblebee workers as pollinator model. We found that bees fed tyramine enriched solution spent less time foraging on a single flower than those fed control, suggesting that their behaviour of visit was overall less dynamic. Our results highlight the importance of addressing further investigations on this emerging class of nectar compounds on insect cognition and behaviour, other than on its occurrence and distribution in nectar of other species.

Keywords: biogenic amines, *Bombus terrestris*, *Echium vulgare*, flight enhancer, pollinator behaviour

3.1 INTRODUCTION

An increasing number of studies on the chemistry of floral nectar shows that the frequency of pollinator visits (Pleasants 1981; Real and Rathcke 1991; Shykoff and Bucheli 1995), the duration of flower visits (Galen and Plowright 1984; Cresswell 1999) and the overall pattern of visit within a plant population (Fisogni et al. 2011; Barberis et al. 2021) are all variables influenced by both nectar volume and chemistry. In the past decades, hundreds of secondary metabolites have been found in nectar other than sugars (e.g. Baker and Baker 1977, 1986) and for some of them a direct influence on behaviour has been demonstrated (e.g. Wright et al. 2013; Barlow et al. 2017; Bogo et al. 2019; Barberis et al. 2023).

Among the chemicals most recently discovered in floral nectar, biogenic amines are nitrogenous compounds which are known to act as neurotransmitters in invertebrates (Blenau and Baumann 2001). Their presence in floral nectar has been reported for the first time by Muth et al. (2022) in 15 different plant species belonging to 6 different orders, where they were represented either by tyramine, octopamine or a combination of the two, with a maximum concentration averaging around 0.07 mM in the species *Cytrus x meyeri*. Both compounds work through their binding to G protein–coupled receptors (Roeder 2005), whose activation leads to the interaction with other proteins regulating enzymatic activity leading to changes in the levels of intracellular signaling molecules such as cAMP and Ca^{2+}. These signals can, in turn, regulate the expression of genes, the activity of ion channels, and the functioning of further proteins (Mustard 2020). As compounds that can activate or inhibit G proteins, their consumption can potentially affect pollinator behavior both in a short- and long-term way (Mustard 2020).

For this reason, far before their discovery in floral nectar, a number of studies has been performed to investigate their functioning in insects, demonstrating how their consumption modulates several behavioural traits such as locomotion (e.g. Fussnecker et al. 2006, Hardie et al. 2007), reward-seeking (e.g. Schulz and Robinson 2001, Peng et al. 2020), learning (e.g. Mercer and Menzel 1982, Hammer and Menzel 1998) and social communication (e.g. Barron et al. 2007, Peng et al. 2020). However, most of such studies tested concentrations much greater than those found in natural nectar (Barberis et al. 2023, and reference therein).

In this exploratory study, we studied the effect of the biogenic amine tyramine on the behaviour of flower visitors, using bumblebees and the plant *Echium vulgare* as models. In particular, we investigated i) the biogenic amine composition of the floral nectar of a natural population of *E. vulgare* in relation to its floral sexual phases and, on the basis of the former result, ii) how an artificial solution enriched with nectar-like concentrations of biogenic amines affects the visits on flowers

performed by bumblebees under the semi-controlled conditions of an insect net greenhouse. Though the use of artificial flowers is common in nectar experiments (e.g. Thomson et al. 2015; Felicioli et al. 2018) it is not excluded that they may potentially affect animal behaviour. For this reason, we opted for a novel experimental design under conditions as much natural as possible, adopting real flowers emptied of their natural nectar and refilled with the treatment diets to test.

3.2 METHODS

3.2.1 Biogenic amine composition of *Echium vulgare* floral nectar

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

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, at our latitudes, between June and October (Barberis et al. 2021). Anthesis lasts 3-4 days and flowers show incomplete protandry (Melser et al. 1997): the anthers often start to dehisce already at the bud stage, while the stigma becomes receptive only hours after the flower opening. In this study, we considered two phases of floral development: 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 recognized as soon as the stigma became bifid and receptive (Corbet 1978; Barberis et al. 2021).

In order to let the nectar accumulate, flowers were bagged in the morning with 1 mm mesh size tulle fabric for 2 hours prior to sampling. Due to the small volume of nectar produced per single flower (less than 0.5 μL and 0.7 μL in functionally male and female flowers, respectively) (Barberis et al. 2021), nectar was gathered from multiple flowers to reach a minimum volume of 15 μL needed for the chemical analyses. We obtained a total of 9 samples: 5 samples from functionally female flowers (pooled from 5-14 flowers, each sample collected from a single individual plant), and 4 samples from functionally male flowers (pooled from 30-63 flowers, each sample from one or two individual plants). We collected nectar samples by means of Drummond Microcaps (1-3 μL; Drummond Scientific Co., Broomall, PA), then we transferred the samples in Eppendorf tubes filled with 100 μL of pure ethanol and took them to the laboratory on the same day of field sampling with the help of thermal insulated ice containers. Samples were stored at 5°C until analyses.

We characterized the content of biogenic amines in nectar samples by high performance liquid chromatography coupled with Diode Array Detector (HPLC-DAD), A Perkin Elmer series 200 chromatographic system equipped with DAD detector and auto-sampler was used for the determination. Detection and quantification were based on UV absorption at 230 nm. The bandwidth has been set to 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. The eluent (A) consisted of 0.02 M potassium phosphate buffer (KH_2PO_4) adjusted at pH 2.5 with ortho phosphoric acid, the eluent (B) was methanol. The composition of the mobile phase was changed 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 individual analyte by calibration curve obtained with external standard. Analyte identification was achieved by comparison with the UV spectrum of the pure standards of 8 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) were reported in Supplementary Material (Table S1). LOD for each amine was calculated by adding 3 times the standard deviation to the mean of 10 blank samples.

All used standards (purity ≥ 98%) and solvents were purchased by Sigma-Aldrich.

3.2.2 Effect of biogenic amines on bumblebee duration of visit

The behaviour of visit on flowers of bumblebee workers was analysed in a greenhouse using queenless micro-colonies and *Cucurbita pepo* L. flowers previously emptied of their natural nectar and refilled with artificial solutions mimicking the biogenic amine composition of the floral nectar of *Echium vulgare* sampled in the field. Since flowers of *C. pepo* are large and nectar is easily accessible, they are particularly suitable for nectar experiments (Nepi et al. 2011).

3.2.2.1 Plants and bumblebees

Plants of *Cucurbita pepo* L. cv. Genovese were grown at CREA-AA in Bologna during April-July 2021. They were first planted in a seedbed and kept indoor at 20 ± 2 °C, then transplanted outdoor in a greenhouse where they were watered daily in the evening. Six plants were transplanted in each compartment. During the experiment, we kept an equal number of open flowers in each compartment cutting off potential supernumerary flowers, and male flowers were emasculated to avoid pollen contamination of the artificial nectar replaced. Some extra plants were kept in mobile pots in order to move them daily to one compartment or the other according to the need. Bees of the species *Bombus*

terrestris (Linnaeus, 1758) were obtained from a commercial colony (Bioplanet S.r.l., Cesena, Italy). We set up two queenless micro-colonies capturing 30 workers (15 worker each, marked with a different colour code to be individually recognizable) from the mother colony. Very small (< 0.10 g) and very large (> 0.35 g) individuals, and newly emerged and old bumblebees (visually discriminated on the basis of whitish colour and lack of hairs, respectively) were avoided (Sgolastra et al. 2017). The micro-colonies were acclimatised at 25 ± 1°C and 40 ± 5% relative humidity (RH) in continuous darkness, fed *ad libitum* sucrose syrup (20% w/v) for three days before the experiment. After the acclimatisation, each micro-colony was relocated in the greenhouse the day before the beginning of the experiment (in two different compartments). Between observations, a feeder containing the same artificial nectar offered during observations was collocated nearby the entrance of both nest boxes.

3.2.2.2 Artificial nectars

Since tyramine resulted to be the only biogenic amine found in the floral nectar of *E. vulgare*, in the behavioural essay we tested this compound only. A 20% sucrose solution (w/v) was used as control (named C), whilst an identical solution containing tyramine (Sigma-Aldrich, Italy) in the mean concentration found in the natural population (0.29 mM) was used as experimental solution (named T). Prior to each observation, nectar was removed from flowers by means of glass disposable 20 µL microcapillary tubes and a strip of absorbent paper was introduced through the nectary pores to remove the remaining natural nectar. Nectar was then replaced by 60 µL/flower of artificial solution using a micropipette. A strip of absorbent paper was introduced through the nectary pores to remove as much natural nectar as possible. After half an hour, 40 µL of solution were added into the emptied flowers so that the bees never found unrewarding flowers.

3.2.2.3 Behavioural observations

Due to the very warm weather, zucchini flowers always closed early in the morning, so that we had to perform the experiment, every day for six days, between 7:00 and 9:00 am at the latest.

During the observation periods, for every bumblebee that came out of the micro-colony to forage we recorded: the colour code of the bee, the number of flowers visited for each plant, the number of successive approaches to the nectar source on the same flower, the duration of movements between two successive flower visits, the time spent feeding or walking and the total time of the visit. We included data only for those bees that fed on nectar during flower visit.

3.2.3 Data analysis

We performed a preliminary exploration of our dataset through a first principal component analysis (PCA) to assess possible difference in the behaviour of visit of bees fed Control (C) *vs* Tyramine (T) artificial solutions. The behavioural parameters considered were the number of flowers approached during each visit, the time spent feeding and walking during the entire visit, the total duration of visit and the number of consecutive approaches to nectar performed by a worker bee on the same flower. Data were scaled and centred around the mean, and analyses were performed using the function 'dudi.pca' in the R-packages *ade4* (Venables and Ripley 2002). Subsequently, we run a one-way PERMANOVA on the same parameters.

To conclude, we focused on the behaviour exhibited by bees on single flowers. To evaluate differences between treatments on each behavioural parameter, we fitted a generalized linear mixed-effect model (GLMM) with a Poisson error structure-log-link function. We set each behaviour (feeding, walking, total permanence on flower and no. of distinct approaches to nectar on a single flower) as response variable, whilst treatment as explanatory variable. We included as random factors: i) the bee ID, to account for individual autocorrelation and variability, and ii) the progressive number of the flower visited, as previous studies demonstrated a correlation between the handling time and the increasing number of flowers visited. The nature of such correlation may depend on various variables such as, for instance, the complexity of the flower, the reachability of the nectar, or the visitor's degree of specialization (e.g. Harder 1983; Laverty 1994). All GLMMs were built through the *glmmPQL* function of the R package *nlme* (Pinheiro et al. 2022).

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

3.3 RESULTS

3.3.1 Biogenic amine composition of *E. vulgare* floral nectar

In nectar samples we found only the biogenic amines tyramine, in a mean concentration of 0.286 ± 0.034 µmol/mL, with no statistical difference between functionally male and functionally female flowers ($t_{6.86}$ = -1.845, p = 0.108; 0.238 ± 0.047 and 0.347 ± 0.035 µmol/mL, respectively).

3.3.2 Effect of biogenic amines on bumblebees' flower visit pattern

A total of 70 visits on flowers were performed by 16 individual bees during the 6 days of running experiment (Table S2). Of these visits, 4 were excluded from the dataset because one of the behavioural categories appeared to exceed 70% of the time of visit.

The PCA on the parameters selected as descriptors of the pattern of visit showed a partial separation between control and tyramine solutions, with the first two components explaining 82.8% of the variance (Figure 1). The first component was positively correlated with the total time of visit and with the time spent feeding (PC1 loadings = 0.55 and 0.49, respectively). The second component was positively correlated with the number of consecutive approaches to nectar showed on the same flower and negatively correlated with the number of visited flowers (PC2 loadings = 0.84 and -0.50, respectively; Table S3).

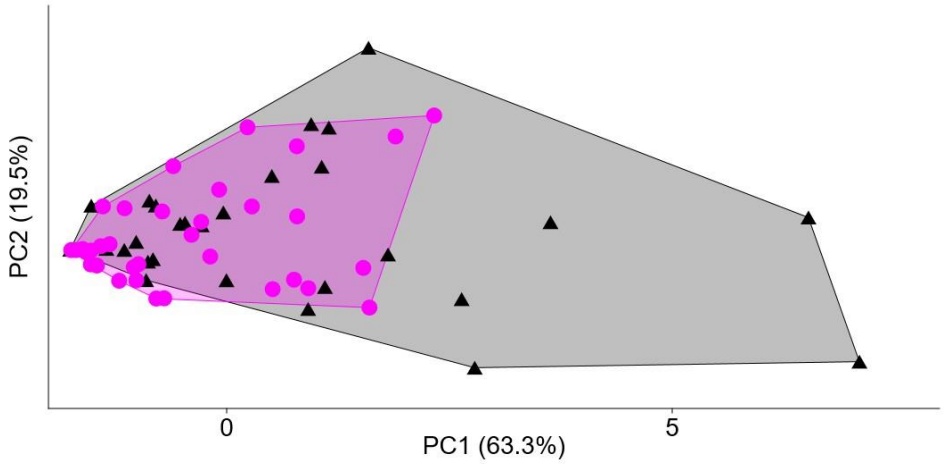

Figure 1. Principal components analysis (PCA) on the behavioural parameters describing visits performed by free-flying bumblebees. Black circles represent visits recorded for the treatment C, whilst aqua circles represent visits recorded for the treatment T.

The one-way PERMANOVA showed that the behaviour of visit significantly differed between bees visiting flowers containing C artificial nectar and those visiting flowers containing T artificial nectar ($F_{1,64} = 5.756$, $p = 0.013$).

When considering the single behavioural parameters, the total time spent on a single flower by bumblebee workers resulted higher in bees fed C than in those fed T ($t_{14} = -2.308$, $p = 0.036$, Figure 2a), as did the total time spent feeding ($t_{14} = -3.456$, $p = 0.004$, Figure 2b). All the other behavioural parameters did not show any significant difference (Table S4).

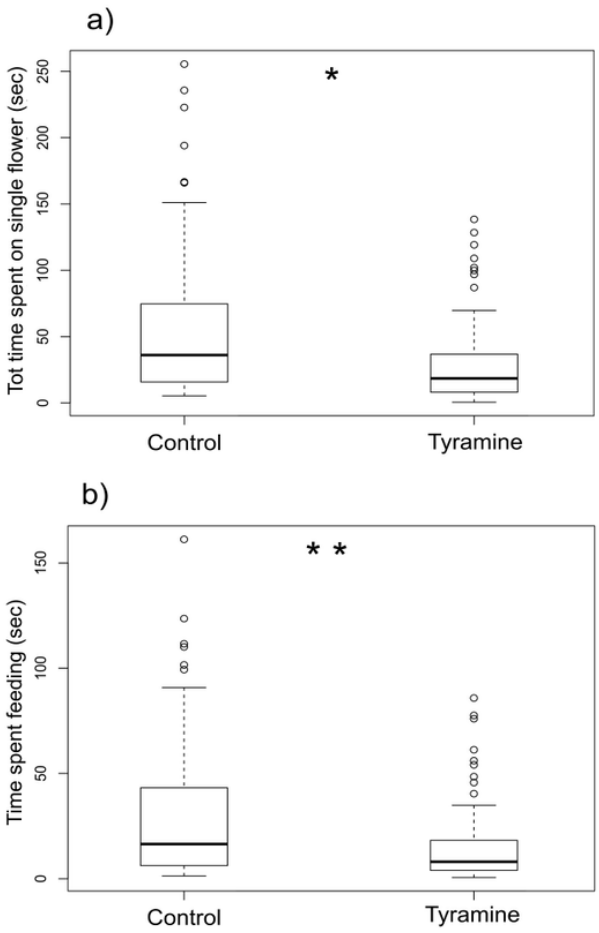

Figure 2. Total time spent on a single flower (a) and time spent feeding on a single flower (b) out of the 161 flower approaches recorded. Asterisks indicates a significant difference (* = $p < 0.05$ and ** = $p < 0.01$) between treatments according to Generalized Linear Mixed Model where Control treatment C was set as intercept.

3.4 DISCUSSION

Our finding of the biogenic amine tyramine in the floral nectar of *Echium vulgare* represents the first report of this compound in the plant order Boraginales, in concentrations which appear dozens of times greater than those reported in the only study published to date highlighting the presence of this class of compounds in floral nectar (Muth et al. 2022). Despite *E. vulgare* presents a gender-biased chemistry of its floral nectar (Barberis et al. 2021), no difference between the functionally male and female flowers were found in the current study for what concerns the concentration of tyramine. However, the lack of difference may be also imputable to the small sample size.

Given that our current knowledge on the occurrence and distribution of biogenic amines in floral nectar is still extremely limited, we consider this finding as a nonetheless valuable data for a class of nectar compounds which represents a breaking-through finding for the field.

Since tyramine is synthesized from the amino acid tyrosine through the action of the enzyme tyrosine decarboxylase and then converted into octopamine by the enzyme tyramine b-hydroxylase, for years it has been considered as the simple precursor of the better-known octopamine. For this reason, its influence on insect behaviour remains, nowadays, largely unstudied, though in recent years tyramine has been proved to function as an independent neurotransmitter (Kutsukake et al. 2000; Nagaya et al. 2002; Roeder 2004; Alkema et al. 2005; Fussnecker et al. 2006; Lange 2009).

In this sense, the current work provides preliminary results on the effects of nectar-like concentrations of tyramine on bumblebee behaviour, reporting that bees fed tyramine-enriched solution spent significantly less time foraging on a single flower compared to those fed control. This aspect needs further investigations, since tyramine may act in different ways, for example affecting palatability (acting as deterrent), or instead imparting the sensation of satiety, by interfering with the nervous system functioning. However, the presence of tyramine in nectar seems to enhance bee dynamic behaviour, as they appear more prone to leave the flower sooner and reach out for the next. This, seen from a plant's perspective, may encourage pollen transfer and thus potentially promote cross-pollination. In addition, less time spent in foraging should result in lower volumes of nectar consumed, reducing nectar depletion and increasing the number of possible insect visits.

Tyramine and octopamine represent the invertebrate counterparts of the vertebrate adrenergic transmitters (Roeder 2005), thus ruling the so-called fight or flight response, which is to say the quick adaptation to energy-demanding situations (Roeder 2005). They have physiological roles similar to adrenaline and noradrenaline, with whom they share a similar chemical structure, suggesting an early evolutionary origin of the adrenergic/octopaminergic/tyraminergic system, which points to an ancient origin of complex behavioural traits (Roeder 2005).

This, contrarily to our finding, suggested how tyramine is expected to reduce the overall bee dynamism. However, tyramine works by binding to G protein–coupled receptors (Roeder 2005), whose activation leads to a plethora of possible metabolic responses involving enzymatic activity, intracellular signalling, and gene expression (Roeder 2005, Mustard 2020).

Finally, it is well known that the nectar of *Echium vulgare* contains pyrrolizidine alkaloids (Lucchetti et al. 2016), whose toxicity has been assessed (Hartmann and Witte 1995; Boppré 2011), contrarily to their potential role in shaping animal behavior through neuroactive action. Muth et al. (2022) demonstrated that nectar biogenic amines can modulate a bee's perception of other compounds, as

caffeine. They found that biogenic amines neutralized the effects of caffeine in enhancing sucrose responsiveness and that, apparently, erased the aversion of bees towards the presence of caffeine. Moreover, the combination of the biogenic amines with caffeine decreased the time of visit on a single flower compared to that recorded for bees feeding on the biogenic amines only. Therefore, we can't exclude that the presence and maintenance of biogenic amines in the floral nectar of *Echium vulgare* may have been driven by its regulation of the tolerance threshold of nectar feeding insects for pyrrolizidine alkaloids.

3.5 KNOWLEDGE GAPS, CONCLUSIVE REMARKS, AND FUTURE RESEARCH

To date, most of our knowledge on the effects of nectar biogenic amines on floral visitors involves studies where these compounds have been tested alone and at much higher concentrations than those found in natural nectar (Barberis et al. 2023 and reference therein). Given that different compounds may exert different actions when coupled together, and can show diverse, dose-dependent effects, this lack of investigations leads to the evidence that future studies should address these aspects. Moreover, a growing number of studies has been demonstrating how microorganisms occurring in flowers can impart significant modifications in the chemistry of floral nectar (e.g. Bogo et al. 2021). As some authors have suggested that nectar biogenic amines may be by-products of the activity of nectar microbes rather than being plant exudates (Nepi 2017, Nepi et al. 2018, Barberis et al. 2023), the assessment of their origin would be of great interest for the advancement of the field. If the potential aminogenic activity of nectar microbes resulted beneficial for the plant reproductive success, then we could even hypothesize that plants do not simply passively undergo microbial-induced changes of their nectar, but rather modulate or even facilitate the settlement of specific microorganisms whose by-produced metabolites exert positive effects on pollinator visits.

Last, we want to highlight how little scientific evidence has so far proved the direct influence exerted by specific nectar secondary metabolites on the plant reproductive outputs (e.g. Kessler et al. 2012), as predicted by the "pollinator manipulation" hypothesis (Rhoades and Bergdahl 1981; Pyke 2016). In this regard, the implementation of experimental designs making use of real flowers instead of artificial feeders may allow future studies to explore further this aspect, for instance by examining difference between the ratio of self- and cross-obtained progeny in the presence and absence of specific nectar secondary metabolites.

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4. FLORAL NECTAR AND INSECT FLOWER HANDLING TIME CHANGE OVER SEASON: RESULTS FROM AN EXPLORATORY STUDY

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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 minutes. 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?

Keywords octopamine, biogenic amines, neuroactive nectar, amino acids, pollinators, *Echium vulgare*

4.1 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 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 behavior 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).

4.2 MATERIAL AND METHODS

4.2.1 Study site

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) (Figure 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 50-100 and covered an area of about 20 m^2 on open meadows along public countryside roads in full sunlight.

4.2.2 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 20-35% (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).

4.2.3 Nectar sampling

Flowers were bagged before 8:00 am with 1 mm mesh tulle fabric, 2 hours 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).

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.

4.2.4 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 Perkin Elmer 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.

4.2.5 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 per 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.

	Early		Late	
	MO	**TO**	**MO**	**TO**
No. floral visitor observations (30 mins 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*, 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

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

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.

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.

4.2.6 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 aminoacidic 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.

4.3 Results

4.3.1 Nectar analysis

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 (Figure 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).

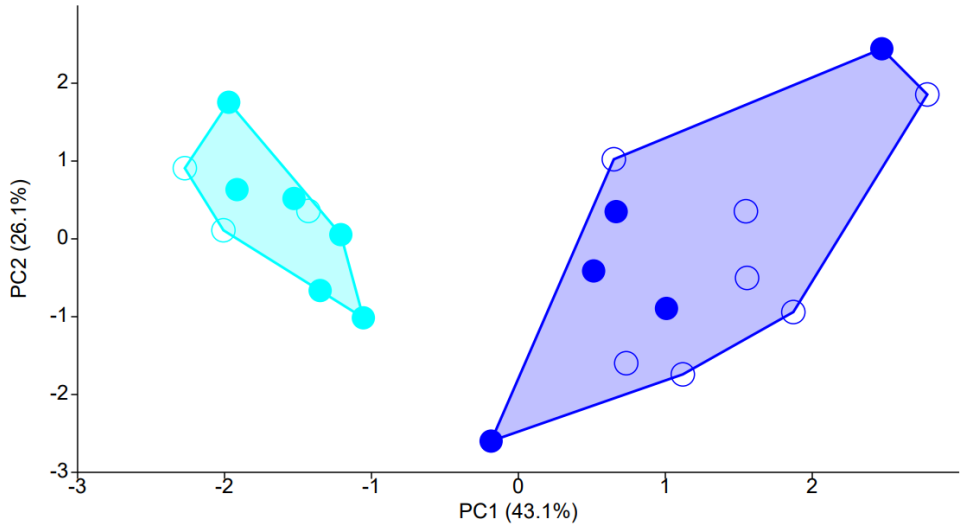

Figure 1. Principal components analysis (PCA) of nectar composition. Early and late periods are indicated in dark blue (right) and light blue (left), respectively; the MO and TO populations are indicated by empty and solid circles, respectively.

Regarding single nectar parameters, nectar volume was lower in the late than the early period (PeriodLate: t_{18} = -5.431, p < 0.001, Figure 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: $t_{18} = 1.276$, $p = 0.297$) (Figure 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; Figure 2f; Table S4).

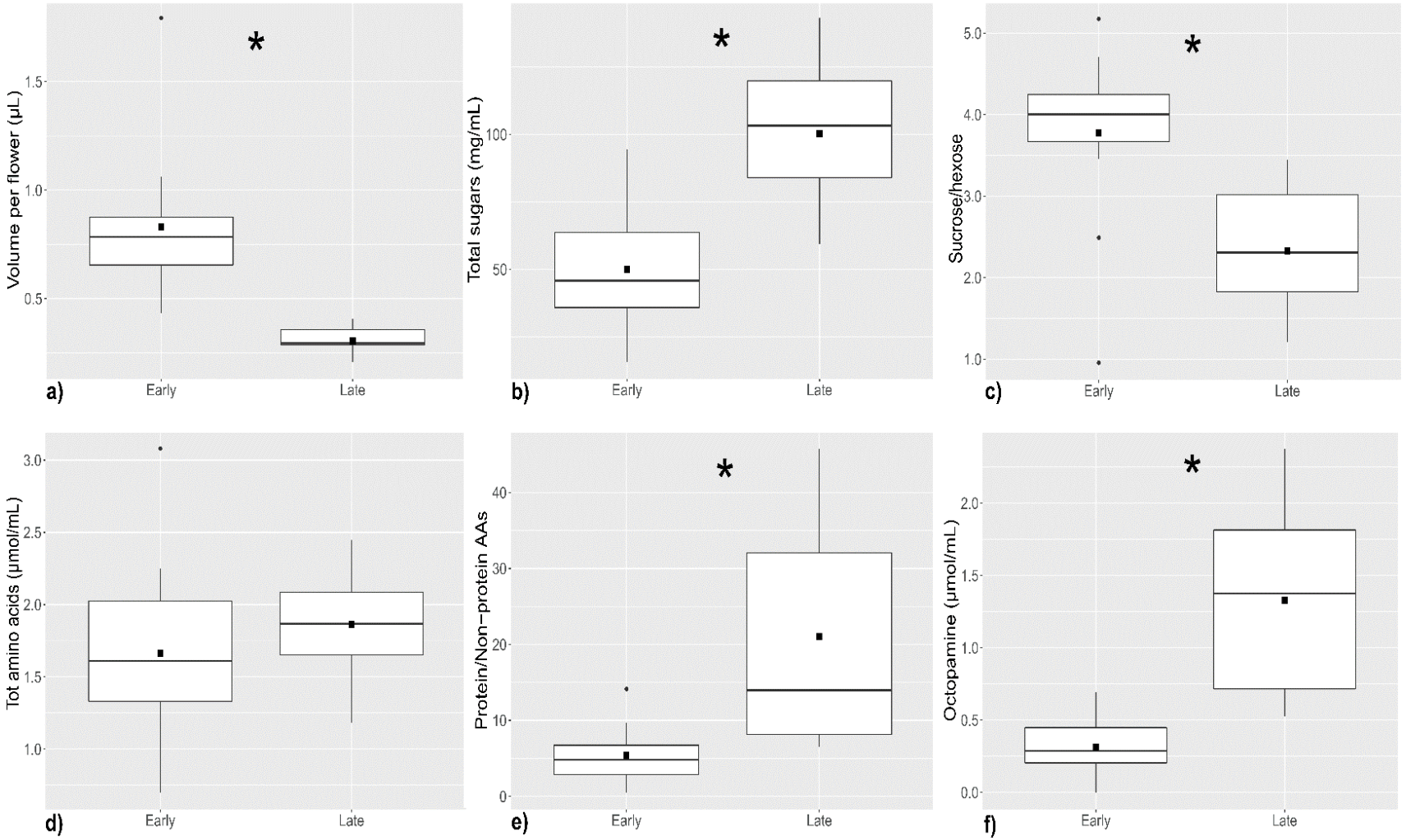

Figure 2. Nectar chemistry of the 21 nectar samples collected from two populations (TO and MO) in the two periods (early and late summer): volume (a), total sugar concentrations (b), sucrose:hexose ratio (c), total amino acid concentrations (d), PAA:NPAA ratio (e) and octopamine concentration (f). Asterisks 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.

The PCA on the aminoacid spectrum showed partial separation of the two periods, the first two components explaining 67.4% of the variance (Figure 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 Figure S2).

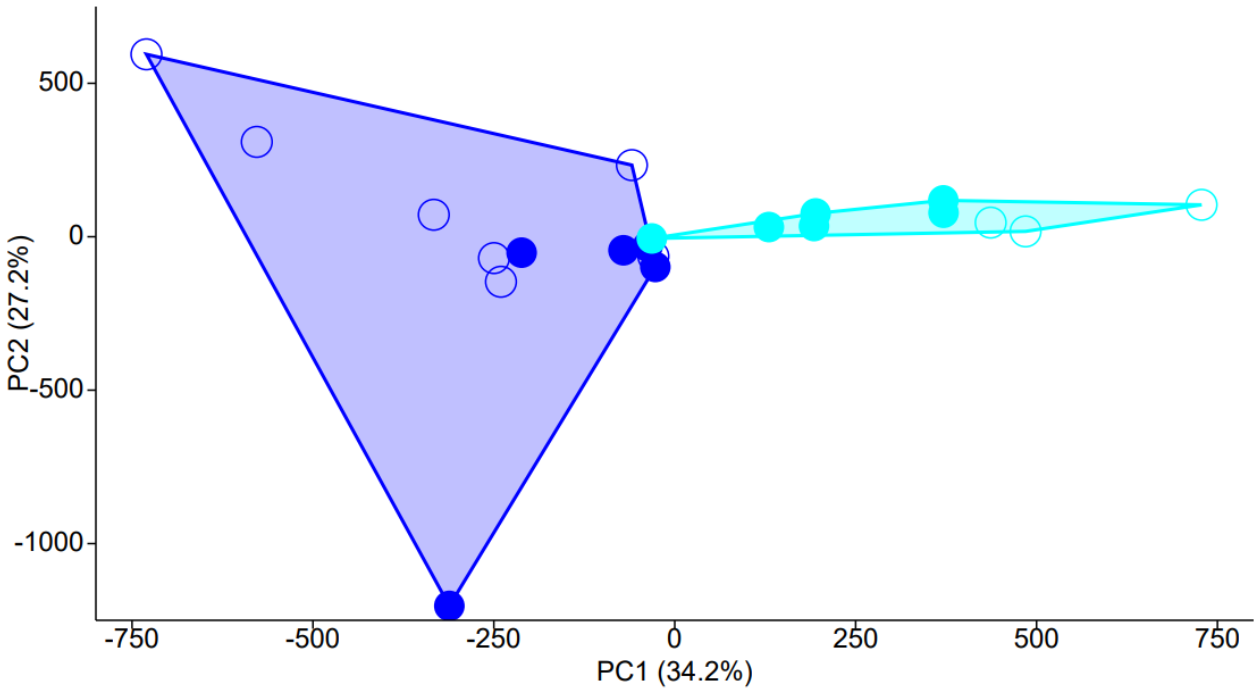

Figure 3. Principal components analysis (PCA) of amino acid spectrum. Early and late periods are indicated in dark blue (left) and light blue (right), respectively; the MO and TO populations are represented by empty and solid circles, respectively.

4.3.2 Flower visitors

A total of 319 insect visits to *Echium vulgare* were recorded during 480 minutes 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).

a)

Number of visits			
	TO	**MO**	**Tot**
early	91	142	233
late	29	57	86
Tot	120	199	**319**

b)

Number of insect taxa recorded			
	TO	**MO**	**Tot**
early	12	6	18
late	3	4	7
Tot	15	10	**25**

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).

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 (Figure 4).

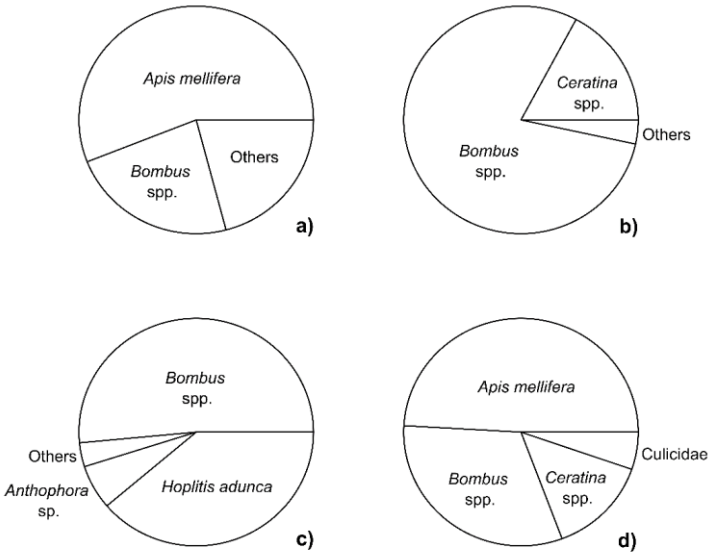

Figure 4. Abundance of visits by the main insect taxa recorded in population TO in the early (a) and late (b) periods, and in population MO in the early (c) and late (d) periods of the study.

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, Figure 5).

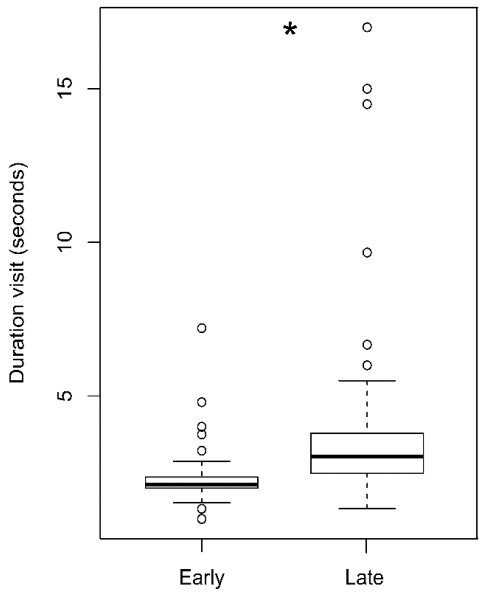

Figure 5. Duration of visits to single flowers recorded for 79 visits by individuals of the *Bombus pascuorum* group in the early and late periods of observation. The asterisk indicates a significant difference between periods according to a GLMM with a Gamma error structure-log-link function.

4.4 DISCUSSION

4.4.1 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 rather that 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 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).

4.4.2 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 and reference therein), 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.

4.4.3 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), causing the decrease of the number of the visits, since less conspicuous flowering masses may reduce attractiveness (Ohashi and Yahara 2001 and reference therein).

4.4.4 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. Nonetheless, our data on durations of visits has two critical aspects. The first concerns the moderate sample size, whilst the second regards the fact that we did not account for individual variability in the model because we did not mark individual bees. Last, a potential third aspect of concern is that the total amount of time spent performing behavioural surveys on floral visitors is relatively reduced. Altogether, these points mean that our behavioural results are only preliminary, and suggest the need for further study.

4.4.5 Future perspectives

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 raising an important question. 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.

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5. NECTAR-LIKE CONCENTRATIONS OF EXOGENOUS INSECT NEUROTRANSMITTERS GENERATE RELEVANT EFFECTS ON BEE BEHAVIOURS RELATED TO FLOWER VISITATION

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Abstract

The class of biogenic amines has been reported for the first time in floral nectar only very recently. This class is represented by nitrogenous compounds known to function as neurotransmitters in invertebrates, meaning that whilst making use of the nectar-landscape, insect pollinators encounter concentrations of exogenous neurotransmitters which may result pharmacologically active and thus may severely rule their behaviors related to flower visitation. In this study, we investigated how tyramine and octopamine at two concentrations, representing the extreme values found so far in natural nectars, affect bumblebee consumption, locomotion, and gustatory responsiveness. Our results suggest a preference for octopamine at the lower concentrations over tyramine and control. Moreover, they show that octopamine exerts a dose-dependent effect on flight, decreasing the motivation of bees to engage in flight when at lower concentration, whilst increasing the duration of flight when at higher concentrations. Tyramine, on the contrary, did not influence either the frequency or the duration of flight compared to control and so it did on the general bee dynamism. Our results confirm that nectar-like concentrations of the biogenic amines produce biological effects in various bumblebee behaviors which are relevant in the decision-making process implied in flower visitation.

Keywords

Octopamine, tyramine, gustatory responsiveness, neuroactive nectar, bee lomotion

5.1 INTRODUCTION

Starting from the ’70s, hundreds of secondary metabolites have been found in floral nectar (Baker and Baker 1986) and shortly later they have been demonstrated to play a variety of different functions, challenging the traditional view that nectar is a simple food reward or attractant (Nepi et al. 2018, Barberis et al. 2023 and reference therein). Among these nectar components, the class of biogenic amines in floral nectar has been reported for the first time recently, in 15 species covering six plant orders (Muth et al. 2022). This class of chemicals is represented by nitrogenous compounds known to function as neurotransmitters in invertebrates (Orchard 1982, Roeder 2000, Blenau and Baumann 2001, Scheiner et al. 2006, Farooqui 2012). More specifically, the nectar biogenic amines reported to date are represented by tyramine and octopamine, whose highest concentrations in floral nectar were reported for the species *Cytrus x meyeri* and *Echium vulgare*, where they average, respectively, around 0.07 mM and 0.70 mM (Muth et al. 2022, Barberis et al. unpublished data).

These compounds appear structurally related to the vertebrate adrenaline and noradrenaline, with whom they share similar physiological roles, suggesting an early evolutionary origin of the adrenergic/octopaminergic/tyraminergic system (Roeder 2005). The fight-or-flight response, a prompt adaptation to energy-demanding situations (Roeder 2005), is only one out of several examples that can be made to show the involvement of such system in ruling insect behaviour. They are products of the decarboxylation of the amino acid tyrosine and even though tyramine represents the biological precursor of octopamine, they are considered to act as independent neurotransmitters, both through their binding to G protein–coupled receptors (Roeder 2005). The activation of a G protein leads to its disassociation from the receptor and to its interaction with other proteins regulating the activity of enzymes leading to changes in the levels of intracellular signaling molecules such as cAMP and Ca^{2+}. These molecules can then regulate the activity of further proteins, which can lead to changes in the gene expression, modify ion channel activity, and affect protein function. Compounds that can activate or inhibit G proteins can affect pollinator behavior both in a short- and long-term way (Mustard 2020).

In fact, even before their discovery in floral nectar, a number of studies provided evidence of the several effects that they can play on insects, demonstrating for example that their consumption modulates locomotion, phototaxis, reward-seeking, learning, memory and social communication (Barron et al. 2007, Peng et al. 2020, Finetti et al. 2021, Muth et al. 2022, and reference therein). In most cases, though, these studies involved concentrations of biogenic amines hundreds or even thousands of times greater than those naturally occurring in floral nectar (Muth et al. 2022).

In this study, we investigated the effects of both tyramine and octopamine at concentrations representing the two natural extreme values reported for nectar octopamine and tyramine in *Echium*

vulgare (0.1 mM and 1 mM). Specifically, we conducted three experiments targeting i) consumption and survival, ii) locomotion, and iii) gustatory responsiveness.

5.2 METHODS AND METHODS

5.2.1 Treatment solutions

We tested three different artificial nectar solutions: a control solution (Control) containing distilled water and sucrose 50% w/v, and the same solution enriched with either octopamine hydrochloride (Octo) or tyramine (Tyra), either at 0.1 mM or 1 mM, for a total of five different treatment diets (namely: Control, Octo 0.1, Octo 1, Tyra 0.1, Tyra 1). These concentrations were elected as extreme values of nectar natural concentrations found in previous investigations of the floral nectar of *Echium vulgare* (Barberis et al., unpublished data). As common in experiments with biogenic amines, ascorbic acid was added at a concentration equal to 1.75 mg mL^{-1} to the solution to minimize oxidation of the biogenic amines (Linn et al 2020, Scheiner et al. 2002). All chemicals were purchased from Sigma-Aldrich (Milan, Italy).

5.2.2 Model species and experimental conditions

Bumblebee colonies (*Bombus terrestris*) were purchased from Bioplanet srl, Cesena (Italy), then maintained at 24 ± 1 °C and 43 ± 5% RH in continuous darkness and fed *ad libitum* with fresh frozen pollen and sugar syrup for three days before the experiment started. Colonies contained around 60 workers, brood in all developmental stages and a laying queen. We excluded very small and very large sized, as well as newly emerged and old individuals (Sgolastra et al. 2017).

5.2.2.1 Consumption and survival (Exp. 1)

The method described by Sgolastra et al. (2017) was adopted. A total of 169 worker bee individuals were collected from four colonies (each colony being a replicate) under red light and transferred individually to Nicot cages (each treatment represented by a minimum number of 33 individual bees) (Table S1). Selected bees were also individually weighted and divided into weight classes that were then evenly distributed in the treatment groups to avoid a bias due to bumblebee size. Worker bees were then acclimatized to the test conditions with a 50% (w/v) sucrose solution provided *ad libitum* in 2.5 mL tipless syringes functioning as feeders. Syringes were then replaced after the acclimatization period with syringes offering the treatment diets. Every day for 21 days, at the same interval in the morning (from 9am to 12am), syringes were weighted to calculate bumblebee consumption, and filled them with fresh solution every other day or upon necessity. Solutions were administered *ad libitum*. Before- and after-consumption weights were noted down to calculate the difference from their initial volume and corrected for evaporation. Similarly, mortality was recorded

daily. Worker bees were maintained in a dark climate room at stable conditions of temperature and relative humidity (24 ± 1 °C and 43 ± 5% RH).

5.2.2.2 Locomotion (Exp. 2)

A total of 75 individuals were collected from three colonies (each colony being a replicate) under red light, marked with different water-based colors for being individually recognizable and transferred in groups of 5 into 15 experimental cages per colony (five cages per colony, provided with one of the five treatments) (Table S2). Cages were plastic net cylinders (length = 25 cm, diam. = 16 cm) mounted horizontally with the ends closed by transparent plastic lids (Fig. S1). They were maintained at ambient temperature with a 14:10 h L:D cycle.

Once a day, we recorded the amount of each solution consumed by weighing the syringes. The amount was divided by the number of live bees in each cage to obtain individual daily consumption. Behaviors were recorded twice a day for nine days. Each individual bee was observed each day for two slots of one minute (one in the morning, one in the afternoon). Behaviors and their duration (walking, feeding, flying and still) were registered by means of a vocal recorder during the one-minute observation slot. The frequency of each behavioral class was then calculated *a posteriori* as well as the time percentage spent performing a dynamic (flying + walking + feeding) or a static behavior (standing still). Consumption, survival, and behavioral measurements were carried out till the end of the experiment (which lasted 9 days).

5.2.2.3 Gustatory responsiveness (Exp. 3)

A total of 60 individuals (12 per treatment diet) were collected individually from three colonies under red light, transferred into a falcon tube to be tested for measuring their fine feeding responses according to the protocol designed by Ma et al. (2016) (Table S3). The solutions were presented in a microcapillary tube (100 µL) to individual bumblebees that have been previously starved for 2-4 hr. Their behavior was captured on digital videos by means of a microscope camera Dino-Lite (37BIM40A) to allow the analysis of the fine structure of the feeding behavior through continuous scoring of the position of the proboscis for two minutes after the first contact with the solution. Video recordings were analyzed using the event logging software Mangold Interact. The three different behavioral classes taken into account were: (1) drinking, whenever distinct proboscis bouts could be recognized, indicating that the worker was actively sucking the solution; (2) tasting, when the bee was exploring around, looking for the solution with the proboscis extracted, with or without contact with the solution; (3) losing interest, whenever the proboscis was stowed under the head and the bee no longer showed interest towards the feeder. Furthermore, the number of proboscis bouts was counted and recorded during each drinking state. The volume of solution consumed was also recorded

by measuring, by means of a digital caliper, the pre- and post-consumption lengths of the solution inside the microcapillary tube.

5.2.3 Statistical analyses

5.2.3.1 Consumption (Exp. 1)

To evaluate differences in feed consumption between bumblebee workers fed different treatment diets we fitted a generalized linear mixed-effect model (GLMM) with a Gamma error structure-inverse-link function. We set consumption as response variable, whilst treatment as explanatory variable. Treatments comprised the control solution and the four sugar solutions enriched with tyramine and octopamine at two different concentrations (0.1 mM or 1 mM). We included the bee identity code (from now on "bee ID") nested within the colony identity code (from now on "colony ID") as random effects to account for individual autocorrelation.

5.2.3.2 Locomotion (Exp. 2)

To evaluate differences in the flight motivation driven by different treatment diets we fitted a two-part mixed effect model for semi-continuous zero-inflated data. The model allowed to assess i) the effects of the treatment diets on the likelihood of bees engaging in flight *vs* non-engaging, and ii) the effects of the treatment diets on the duration of flight, when any flight was performed. The model was built by means of the *mixed_model* function of the *GLMMadaptive* package (Rizopoulos 2022), setting a *hurdle.lognormal* distribution as distribution family. Duration of flight was set as independent variable, treatment was set as fixed effect, whilst bee ID nested within colony ID were set as random effects to account for individual autocorrelation.

To evaluate the influence of the treatment diets on bee dynamism, we created a two-vector variable comprehensive of the proportion of time spent engaging in a static *vs* dynamic behavior. We then built a GLMM model with a binomial error structure-logit-link function where such two-vector variable was set as response variable, whilst treatment was set as explanatory variable. We included once again the bee ID nested within the colony ID as random effects to account for individual autocorrelation.

Individual consumption was calculated by dividing the total daily consumption for the number of bees present in each cage (5), then a GLMM was built with a Gamma error structure-inverse-link function. Again, consumption was set as response variable and treatment as explanatory variable. We included the colony ID as random effect to account for individual colony autocorrelation.

5.2.3.3 Gustatory responsiveness (Exp. 3)

For the analysis of the data on gustatory responses we built either a linear mixed model (LMM) or a generalized linear mixed models (GLMM) with a Gamma error structure-log-link function on the arcsin-transformed percentage of duration of each behavioural state (drinking, tasting, losing interest). For the data on the frequency of each behavioural state and the number of bouts we built generalized linear mixed models (GLMMs) with a Poisson error structure-link function. An additional GLMM with a Gamma error structure-log-link function was built on consumption data as well. In all these models, each variable was set as dependent variable, the treatment diet was included as fixed factor whilst bee ID nested within colony ID were included as random factor to account for individual variability.

All Generalized Linear Mixed Models were built through the *glmmPQL* function of the R package *nlme* (Pinheiro et al. 2022), and all data are presented as mean ± SE and all statistics were performed using RStudio software (version 4.0.2) with the α-error set at 0.05.

5.3 RESULTS

5.3.1 Consumption (*Exp. 1*)

Bees fed with the diet enriched with octopamine at the lower concentration (0.1 mM) consumed more than those fed control (t_{160} = 3.402, p = 0.007), those fed solutions enriched with octopamine at higher concentration (1 mM) (t_{160} = -4.480, p < 0.001), and those fed solutions enriched with tyramine at an equal concentration (0.1 mM) (t160 = -2.901, p = 0.034). Bees fed treatment diets containing tyramine did not differ from those fed control (Figure 1, Table S4).

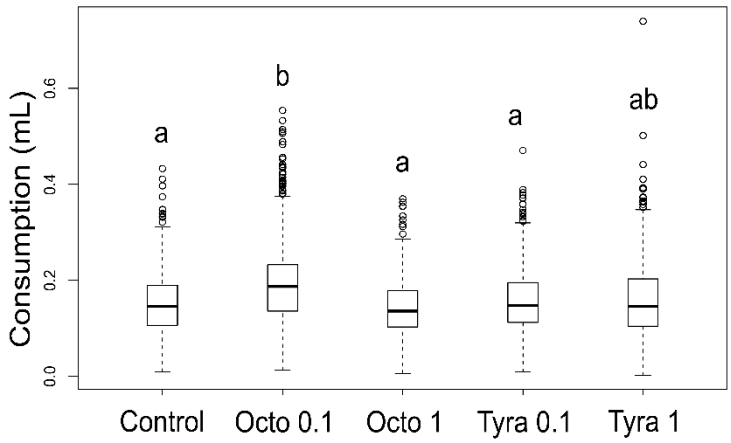

Figure 1. Consumption (mL) of the five treatment diets recorded for worker bumblebees individually caged in Nicot cages (*Exp. 1*). Different letters indicate significant differences among treatments.

5.3.2 Locomotion (*Exp. 2*)

In average, worker bees fed with treatment diets enriched with tyramine at both concentrations (0.1 mM and 1 mM) consumed significantly less solution than those fed control (Control:Tyra 0.1, t_{157} = -2.872, p = 0.037; Control:Tyra 1, t157 = -4.384, p < 0.001). Also, bees fed treatment diets enriched with tyramine at the lower concentration (0.1 mM) consumed significantly less than those fed the diet enriched with octopamine at the same concentration (Octo 0.1:Tyra 0.1, t_{157} = -3.082, p = 0.020). (Figure 2, Table S5).

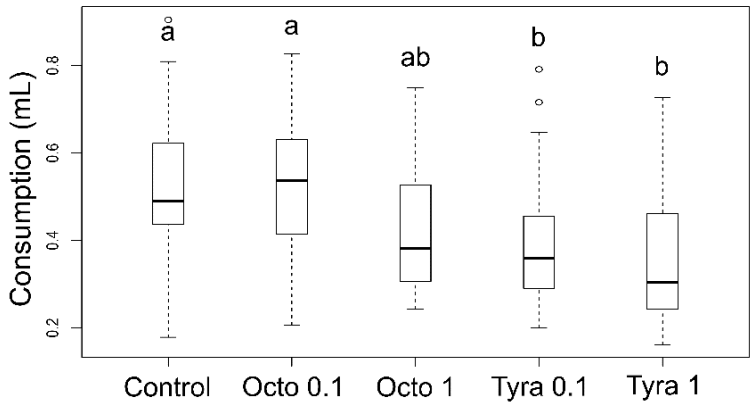

Figure 2. Consumption (mL) of the five treatment diets recorded for worker bumblebees caged by five in small flight cages (*Exp. 2*). Different letters indicate significant differences among treatments.

Bees fed the treatment diet enriched with octopamine at the lower concentration (0.1 mM) appeared significantly less motivated in engaging in flight (Octo 0.1, z = 2.784, p = 0.005) (Figure 3a, Table S6), whilst when engaging in flight, bees fed the treatment diet enriched with octopamine at the higher concentration (1 mM) flew for longer time (Octo 1, z = 2.172, p = 0.030) (Figure 3b, Table S6). No significant difference was highlighted by the following post hoc test.

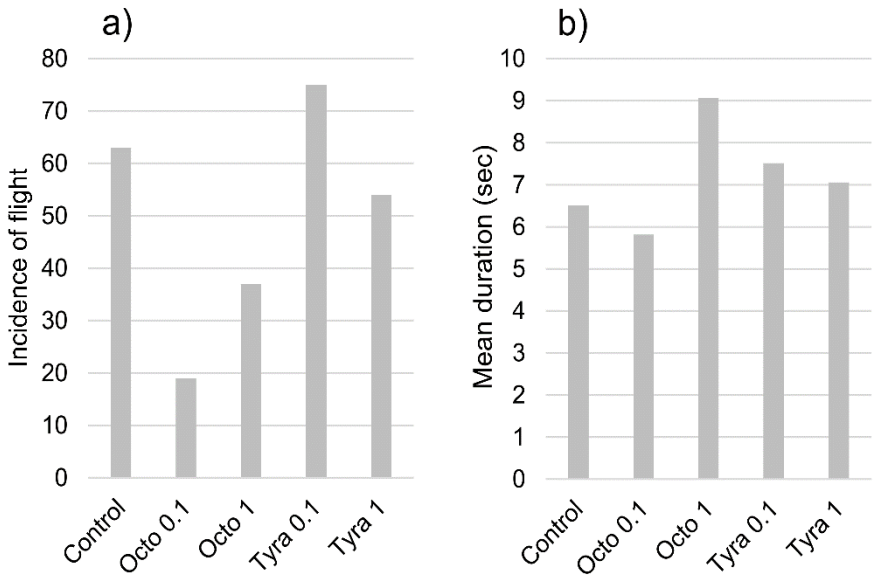

Figure 3. Incidence of flight behavior exhibited (a), and mean duration of flight (b) exhibited by bees fed different treatment diets and grouped by five in small flight cages (*Exp. 2*).

Last, bees fed the treatment diets enriched with octopamine at both concentration exhibited a significant less dynamic behavior than those fed control (Control:Octo 0.1, $t_{68} = 3.498$, $p = 0.007$; Control:Octo 1, $t_{68} = 3.387$, $p = 0.010$) (Figure 4, Table S7).

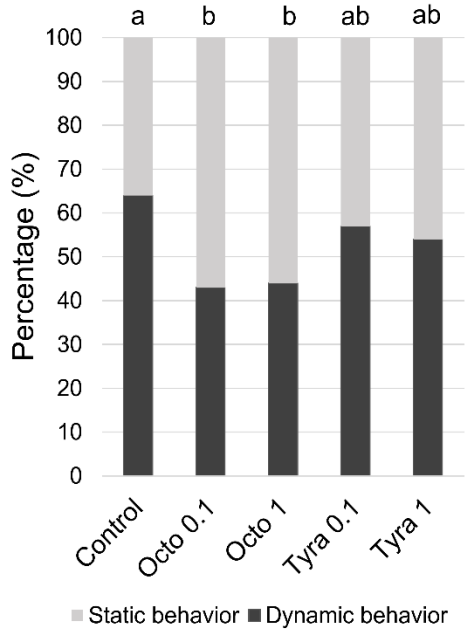

Figure 4. Percentage of dynamic vs static behavior exhibited by bees fed different treatment diets and grouped by five in small flight cages (Exp. 2). Different letters indicate statistically significant differences.

5.3.3 Gustatory responsiveness (*Exp. 3*)

The analysis on the fine feeding behaviour of worker bumblebees showed significant differences for none of the variables considered, except for the frequency of drinking behaviour recorded for bees fed the treatment diet enriched with the higher tyramine concentration (1 mM), which resulted to be the lowest (Tyra 1: $t_{52} = -2.171$, $p = 0.034$; Figure 5, Table S8). Nevertheless, the following pairwise post-hoc test applied did not highlight any significant difference among the different treatments. Similarly, no significant difference was either found for consumption data.

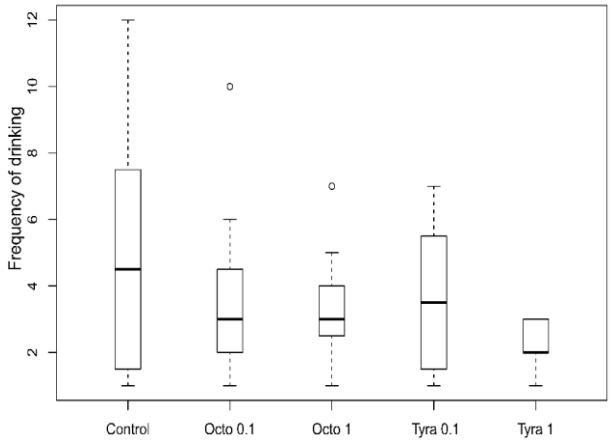

Figure 5. Frequency of drinking behavior showed by worker bumblebees fed different treatment during the essay on gustatory responsiveness (*Exp. 3*).

5.4 DISCUSSION

Previous studies on the effects of ingested biogenic amines on honeybees showed that consumption enhances titers of such compounds in the brain (e.g. Schulz and Robinson 2001), and several other studies showed that their consumption generates important biological effects on insect behavior (e.g. Barron et al. 2007, Agarwal et al. 2011, Arenas et al. 2020). However, most studies on the effects of octopamine and tyramine on insect behavior involve concentrations of biogenic amines hundreds or even thousands of times greater than those naturally occurring in floral nectar (Muth et al. 2022 and reference therein).

Our results confirm that nectar-like concentrations of the biogenic amines octopamine and tyramine provoke relevant biological effects on various bumblebee behaviors which are relevant to plant visitation. Taken altogether, our results on consumption suggest that octopamine rather than tyramine increases feed intake, suggesting a preference for the former over the latter. This result is in line with

what was reported for adult blowflies: when the insects were injected with an octopaminergic drug they became strongly hyperphagic, up to the point of tripling their initial body weights, demonstrating that octopaminergic receptors positively modulate feeding and drinking behavior in this model species (Long and Murdock 1983). Along with these results, other studies have demonstrated how both tyramine and octopamine enhance sucrose responsiveness in bees, by increasing the perceived value of a certain reward and thus stimulating foraging (e.g. Giray et al. 2003, McCabe et al. 2017). However, whether this effect is also exerted by nectar-like concentrations of these compounds remains to be clarified, since different authors have reported contrasting results (Scheiner et al. 2002, Pankiw and Page 2003, Muth et al. 2022).

The general trend revealing a greater consumption of octopamine over tyramine resulted more pronounced when this was observed over an extended period in harnessed bees individually caged, who could not engage in flight nor in extensive walking (such as in *Exp. 1*). These bees consumed more solution when fed with the diet enriched with the lowest octopamine concentration, and this trend – though not significant – was reported also for the consumption recorded for bees grouped by five in small flight cages where they could engage both in short flights and walks (*Exp. 2*). In this case, bees had the opportunity to move and were exposed to a natural dark:light cycle. These second results on consumption stressed that tyramine enriched solutions were consumed less than control rather than highlighting a preference for octopamine enriched diets. This lower feed intake could be easily imputed to a more static behavior. Our results, though, seem to confute this possibility, since bees fed with the treatment diet enriched with tyramine, other than consuming less, showed no differences in their overall dynamism compared to those fed control, somehow strengthening the conclusion that bees like tyramine enriched solutions less than octopamine enriched solutions or that tyramine enriched solutions are less phagostimulant. Moreover, the higher frequency of drinking behavior exhibited by bees during the experiment on gustatory responsiveness (*Exp. 3*) seem to be interpretable in the same direction: bees did not spend significantly less time in drinking when the tyramine enriched solutions were offered to them, nor did they consume less solution during the 2 mins time interval of observation. Nevertheless, they interrupted the feeding behavior more frequently when the higher concentration of such compound was offered.

Finally, effects on consumption and preference were not detectable when the immediate response to the treatment diet was measured in a short-term recording (such as in *Exp. 3*). Given that we used 50% w/v sucrose solutions as base for all the treatment diets, this high sugar percentage may have potentially masked a (putative) preference driven by differences in palatability. Instead, it is possible that the enhancement in feed consumption observed in bees fed octopamine 0.1 mM in *Exp. 1* is rather imputable to some post-ingestion effects, such as phagostimulation.

In our study, bumblebees could not engage in extended bouts of flight in the observation cages, but exhibited short flying hops, so that our data present the limit of not reflecting the real amount of time subjects would have engaged in sustained flight, but they rather bring to light the extent to what bees were motivated to fly. Previous studies revealed that octopamine and tyramine differentially affected flight in honeybees when injected on the thorax, with a general trend revealing that octopamine increased flight, whilst tyramine decreased it (Roeder 2005, Fussnecker et al. 2006). Our results are only partially consistent with these findings: octopamine at the lower natural concentration decreased the motivation of bees to engage in flight. Contrarily, bees fed the treatment diet enriched with octopamine at the higher concentration – even though they did not show a remarkably high frequency of flight engagement – exhibited in average longer flights. Tyramine, on the contrary, seemed to not influence either the frequency or the duration of flight compared to control.

5.5 Conclusions and future perspectives

Considering the concentrations of tyramine and octopamine reported by Muth et al. (2022) our treatment diets at 0.1 mM better represent the average concentrations that these compounds show in the natural nectar of various species. The presence of octopamine in floral nectar at such concentration may encourage insect retention to the flowers, either by stimulating their feeding behavior and by decreasing their motivation to fly, effects which could either result beneficial or detrimental to the plant reproductive success, depending on several aspects such as its degree of self-compatibility. For this reason, every model system should be studied separately to better understand the potential influence that the presence of these compounds exerts on plant reproductive fitness.

Nonetheless, it remains that while making use of their nectar-landscape, insect pollinators encounter and ingest pharmacologically active concentrations of exogenous neurotransmitters, whose consumption does affect a series of behaviors relevant to flower visitation. This evidence strengthens the importance to assess the origin of such compounds in floral nectar, which has been suggested to be of microbial nature. May be nectar biogenic amines byproducts of microbial metabolism, the field of nectar ecology would be driven towards the hypotheses that have started looming in the distance and where a concerted synergy to attract pollinators between plants and nectar-specialist microbes may even result to be a concrete possibility.

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PART 2: REVIEWS

6. SECONDARY METABOLITES IN NECTAR-MEDIATED PLANT-POLLINATOR RELATIONSHIPS

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plants

Review

Secondary Metabolites in Nectar-Mediated Plant-Pollinator Relationships

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Abstract

In recent years our understanding of the complex chemistry of floral nectar and its ecological implications for plant-pollinator relationships have certainly increased. Nectar is no longer considered merely a reward for pollinators but rather a plant interface for complex interactions with insects and other organisms. A particular class of compounds, i.e. nectar secondary compounds (NSCs), has contributed to this new perspective, framing nectar in a more comprehensive ecological context. The aim of this review is to draft an overview of our current knowledge of NSCs, including emerging aspects such as non-protein amino acids and biogenic amines, whose presence in nectar was highlighted quite recently. After considering the implications of the different classes of NSCs in the pollination scenario, we discuss hypotheses regarding the evolution of such complex nectar profiles and provide cues for future research on plant-pollinator relationships.

Keywords

Floral nectar; secondary compounds, plant-pollinator-microbe interactions; pollinator behavior

6.1 INTRODUCTION

Pollination by insects is an ecosystem service that maintains planetary biodiversity and ecosystem functions. It is also fundamental for human food security. About 90% of currently known angiosperm species, totalling just under 300,000 species (Christenhusz and Byng 2016) are pollinated by insects and more than 1500 crops around the world benefit from the same services (Ollerton 2021). Pollen and nectar are the primary alimentary rewards offered by plants to floral visitors, and of the two, nectar is sought by a wider range of animals, mediating the majority of plant-animal relationships (Simpson and Neff 1981). Nectar is a concentrated sugary secretion containing a combination of simple sugars (sucrose, glucose, and fructose) (Nicolson and Thornburg 2007 and reference therein). This ready-to-use energy source powers the flight of feeding insects, birds and other animals (Nicolson and Thornburg 2007, Roy et al. 2017, Nicolson 2022). A co-evolutionary relationship between the relative percentage of sugar in nectar and the food preferences of pollinators was revealed in the early 1980s (Baker and Baker 1983). Although nectar amino acids occur at much lower concentrations than sugars, they are a source of nitrogen for pollinators and contribute to the taste of nectar (Nicolson and Thornburg 2007, Roy et al. 2017, Nicolson 2022). All 20 protein-building amino acids have been detected in nectar (Baker and Baker 1975 and reference therein) and insect preferences for specific amino acids are also known (e.g. Bertazzini et al. 2010, Seo et al. 2019).

For decades nectar chemistry studies concerned analysis of sugars and amino acids, focusing on their basic importance as food rewards in the framework of the mutualistic relationship between plants and pollinators. This classical view of floral nectar was recently challenged by studies focusing on substances present at low concentrations in nectar and not directly related to its food value, i.e. nectar secondary compounds (NSCs) (Stevenson et al. 2017, Mustard 2020). Since several secondary compounds in plants are known to deter herbivores and to have antimicrobial properties (Schoonhoven et al. 2005), NSCs were initially thought to defend against opportunistic nectar-feeding animals and nectar-dwelling microorganisms, protecting plants from exploitation of their nectar (Stevenson et al. 2017 and references therein). The former case was formalized as “nectar forager selection” hypothesys, where opportunistic nectar-feeding animals were identified as scarcely efficient pollinators or nectar thieves/robbers (Nepi 2014). The latter case was instead formalized as “antimicrobial” hypothesis (Adler 2000). A series of studies indeed confirmed the functions suggested in such hypotheses (e.g. Thornburg et al. 2008, Kessler et al. 2008, Barlow et al. 2017), but at the same time other studies clarified that NSCs do not solely play these roles: it became clear, in fact, that they can affect insect foraging behaviour in several additional ways (e.g. Wright et al. 2013, Bogo et al. 2019, Peng et al. 2020), with potential effects on pollination efficiency and plant reproductive success. In this regard it is interesting to note that NSC concentrations are often lower

that those found in plant tissues, where secondary compounds have a clear deterrent effect on herbivorous insects (e.g. Manson et al. 2013). Since the effect of secondary compounds on insects is dose-dependent (e.g. Manson et al. 2013, Liu et al. 2007), it is plausible that NSCs may have functions other than deterrence.

It has since been highlighted that some NSCs affect an array of insect behavioural traits of particular interest in the scenario of foraging activity and pollination of flowers: phagostimulation (e.g. Schoonhoven et al. 2005, Mitchell and Harrison 1984), locomotion (e.g. Bogo et al. 2019, Felicioli et al. 2018), learning and memory (e.g. Wirght et al. 2013, Baracchi et al. 2017, Carlesso et al. 2021), arousal and aggressiveness (e.g. Roeder 2005), olfactory perception (e.g. Gong et al. 2021), phototaxis (e.g. Scheiner et al. 2014), reward-seeking (e.g. Peng et al. 2020) and social communication (e.g. Barron et al. 2007, Tan et al. 2012). According to the recent "manipulation" hypothesis, NSCs can be regarded as tools available to plants for manipulating the behaviour of foraging insects and exploiting their mutualistic interactions: plants rewarding pollinators with "doped" nectar maximize the benefits they obtain, increasing the efficiency of the pollination service (Nepi et al. 2018). Although this hypothesis has some gaps (e.g. lack of experimental evidence directly linking NSCs, pollination efficiency and plant fitness), it opens new ecological and evolutionary scenarios. Here, we bring together the actual knowledge on the plethora of roles played by the most important classes of nectar compounds, with particular focus onto the recently discovered class of biogenic amines, whose presence in floral nectar raises a series of interesting new questions.

6.2 NECTAR PHENOLS

Phenols are organic compounds with one or more six-carbon aromatic rings carrying one or more hydroxy groups (Moss et al. 1995). They are quite common in floral nectar (Baker and Baker 1975, Adler 2000, Baker 1977, Bernardello et al. 1994): indeed, more than 30% of plant species seem to secrete phenolic nectar (Baker 1977). Their ecological role, as well as that of other NSCs, was initially assumed to be a deterrent to scarcely efficient pollinators (Baker and Baker 1975) and nectar thieves such as ants (Janzen 1977). Interestingly, when it was confirmed that phenols in nectar can deter undesirable visitors (Tan et al. 2012, Rhoades and Bergdahl 1981, Verónica et al. 2014, Nicolson et al. 2015), it was simultaneously found that they can attract effective pollinators, reinforcing pollinator fidelity to the plant (Zhang et al. 2018). The study conducted by Gong et al. (2021) provides an interesting example of how nectar polyphenols rule complex interactions beyond the simple deterrence/attraction dichotomy: the results demonstrate that honeybees show a preference for solutions containing polyphenols, and that these compounds are capable of increasing memory retention and affecting sensitivity to bee-alarm odours. These alarm odours are pheromones that

insects can emit while feeding on flowers to alert nest mates to danger (Wen et al. 2017). If polyphenols increase bee sensitivity to such odours, then the visitation rate of bees to flowers marked with such pheromones may decrease. This suggests a negative impact of nectar polyphenols on plant fitness, possibly determining reduced pollination and seed set. Nevertheless, a second scenario is also possible: if there are few sources of danger, the number of flowers marked with alarm odour is low, and increased sensitivity to such signals may reduce visits to flowers that have already been visited, favouring not yet visited flowers.

Nevertheless, we are still discovering actions that phenols seem to exert in floral nectar: for example, they seem to be feeding stimulants for some insects (De Boer and Hanson 1987), while others have antibacterial and antifungal properties (Ataç et al. 2005, Vandeputte et al. 2011, Pimentel et al. 2013). With reference to the latter function, strong antifungal and antibacterial activities of plant tannins have been confirmed (Lattanzio et al. 2006, Montenegro et al. 2013). These tannins are natural water-soluble polyphenols of variable molar mass (Khambabaee and van Ree 2001), often detected in floral nectar (Nepi 2017). Their antimicrobial function is important since it may reduce the proliferation of nectar-dwelling fungi and bacteria, commonly found in nectar, which deplete the food value of nectar by exploiting sugars and amino acids for their own metabolism (Herrera et al. 2009, Pozo et al. 2014). Other nectar phenols are responsible for coloured nectar, that most authors consider to be an honest signal for floral visitors (Hansen et al. 2007, Zhang et al. 2012). The dark colour of some nectar can be due to oxidation of phenolic compounds and the colour is generally lighter in young flowers (Hansen et al. 2007) (e.g. in Fig. 1, personal observation). Coloured nectar can facilitate remote detection of a food source by pollinators, as well as providing an assessment of nectar quantity in individual flowers (e.g. Hansen et al. 2006, Zhang et al. 2012). However there are other possible explanations for coloured nectar, such as a deterrent to nectar-thieves or an anti-microbial effect that preserves the quality of the food resource in long-lasting flowers. Neither explanation is mutually exclusive (Hansen et al. 2007). For instance, the dark purple nectar of *Leucosceptrum canum* is due to the anthocyanidin 5-hydroxyflavylium, the role of which may go beyond that of simple attractant. Birds visiting the flowers of *L. canum* are reported to feed only when the nectar becomes palatable, which coincides with reproductive maturity of the flower and increases pollination efficiency while protecting immature flowers from damage or nectar depletion (Zhang et al. 2012). Such bird behaviour may be driven by the process of oxidation of the compound, known to be highly unstable.

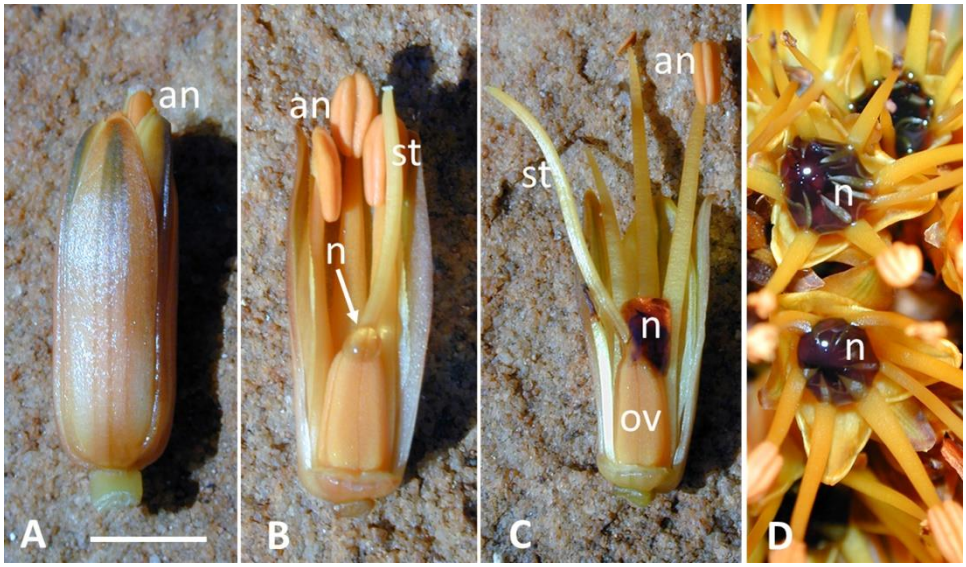

Figure 1. Coloured nectar of *Aloe castanea*. A, B young opening flower containing a small drop of uncoloured nectar (arrow). C, D older flower(s) (2-4 hours after opening) with dark-red coloured nectar. an = anther; n = nectar; ov = ovary; st = style. Bar = 5 mm.

Vividly coloured nectars are found in few plant species and are considered a rare floral trait (Hansen et al. 2007). Some phenols have even fluorescent properties (e.g. Scogin et al. 1979), but our understanding of the phenomenon is still limited. The ecological meaning of fluorescent nectars has been suggested to be guiding pollinators that see in the UVA band towards the flowers, however, not all authors agree on the veracity of this hypothesis (Thorp et al. 1975, Kevan 1976).

Even though the majority of species presents a scentless nectar, another interesting phenomenon worth to be mentioned and involving phenols (and terpenoids, see next section) is that of scented nectars (Raguso 2004). Scented compounds may be dissolved in the aqueous medium of nectar and absorbed passively from the surrounding floral tissues (Nicolson and Thornburg 2007, Weidenhamer et al. 1993). Since floral scents are heterogeneous bouquets of chemicals (Raguso and Pichersky 1999), it is easy to imagine that scented nectars are likewise a complex combination of compounds and not mere attractants. They likely have antimicrobial activity (Knobloch et al. 1989, Lokvam and Braddock 1999), play a role in defence physiology, or act as signals to predators and parasitoids (Pichersky and Gershenzon 2002).

An interesting case concerns plant scents and mate location by pollinators. Mate location often involves species-specific insect pheromones, which have long been considered a major factor for mate-finding success (Thornill and Alcock 1983). However, Xu and Turlings (2018) suggested that plant volatiles play a crucial role as coadjutant in insect reproduction: pollinators are often stimulated to release more pheromones and/or increase mate receptivity by plant volatiles. Although the authors

studied volatiles released from various plant tissues (e.g. leaves, flowers, fruits), it is reasonable to transpose this further ecological role also to nectar scents, which in most cases originate from the volatiles of the surrounding tissues (e.g. Raguso 2004).

The study by Raguso (2004) confirms that in some of the species presenting scented nectar, nectar odours are like those emitted by floral tissues, but intriguingly, the pattern of nectar sharing similar chemical scents with floral tissues is not confirmed for other species, the nectar of which shows a unique bouquet of chemicals.

Along with all the possible functions listed so far, it is also worth mentioning that some plants produce hallucinogenic or narcotic substances that affect pollinator behaviour, disorienting their flight which is often described as sluggish or drunken (Bell 1971). This seems to be determined by phenol derivatives (Jakubska et al. 2005) or alkaloids (Clinch et al. 1972, Manson and Thomson 2009), and appears – at first glance – like a counter-intuitive effect. Whether these substances create addiction or whether floral visitors may find the effect of "getting high" rewarding in itself (things that would both enhance their fidelity) remains to be clarified. In any case, a possible ecological explanation for the presence and maintenance of such compounds in floral nectar could be that sluggish behaviour prolongs the time spent by visitors on the flower, increasing the chance of pollination.

6.3 NECTAR TERPENOIDS

Terpenoids are a large and diverse class of naturally occurring compounds derived from five carbon isoprene units, differentiated from each other by their basic skeleton and functional groups (Moss et al. 1995). They are the main constituents of essential oils and have been detected in the floral nectars of a good number of plant species (Raguso 2004, Juergens 2004, Naef et al. 2004). Although terpenoids are generally thought to be insect attractants (Plepys et al. 2002, Andersson 2003, Tholl et al. 2004), Junker and Blüthgen (2008) confirmed a repellent effect of specific terpenoids commonly found in floral scents, suggesting that their presence in floral nectar may discourage nectar thieves or protect against fungal diseases (Devarenne 2009). Interestingly, many terpenoids also produce satiety in insects (Ozoe et al. 1990).

The case of the nectar terpenoid triptolide, which is found in the floral nectar of *Tripterygium hypoglaucum*, highlights that certain secondary metabolites are tolerated differently by closely related insect taxa. Triptolide is known to impair honeybee foraging responses, dance communication and olfactory learning (Zhang et al. 2022). This specific example supports a coevolution hypothesis, since the sympatric species *A. cerana* shows higher tolerance to the toxin than the introduced species *A. mellifera* (Zhang et al. 2022).

Another important role of nectar terpenoids (and alkaloids, see next section), is to enhance insect immune response to parasites and promote floral-visitor health. The nectar terpenoid abscisic acid, for instance, improves the immune response of worker honeybees and larvae attacked by *Varroa destructor* (Negri et al. 2015), while both classes of chemicals significantly reduce the load of the intestinal parasite *Crithidia bombi* in bumblebee colonies, playing a crucial role in controlling transmission within and between colonies (Richardson et al. 2015). Since a mechanism enhancing plant reproductive success may not only include association of floral traits with nectar taste, but also with the post-ingestive consequences of nectar consumption (Wright et al. 2010), their role in improving floral visitor health may also affect insect fidelity to specific flowers (as may do also other classes of nectar compounds, see the other paragraphs).

6.4 NECTAR ALKALOIDS

Alkaloids are basic nitrogen compounds (mostly heterocyclic) (Moss et al. 1995), whose distribution among living organisms is limited (Pelletier 1983). Most alkaloids have basic properties, are bio-synthesized from amino acids and show a wide variety of chemical structures. Extensive sampling of hundreds of plant species has demonstrated that they are common in the nectar of many plants (e.g. Baker and Baker 1975, Adler and Wink 2001, Palmer-Young et al. 2019).

Again, the occurrence and maintenance of potentially toxic alkaloids in floral nectar has been explained, like in the case of other NSCs, by stating that their presence may be beneficial to the plant by deterring less specialized floral visitors – which would presumably carry a smaller amount of co-specific pollen (Baker and Baker 1975), or nectar thieves and/or robbers (Janzen 1977). The study conducted by Barlow et al. (2017) confirmed that nectar alkaloids in specialized *Aconitum* flowers deter thieving by bumblebees, although they may have co-evolved with specific patterns of nectar secretion aimed at maintaining the benefits of specialized plant-pollinator relationships. On the contrary, though, Haber et al. (1981) found that most floral nectars containing alkaloids were willingly accepted and exploited by ants, indicating that they may not always be an effective barrier against theft of nectar and that their role may be more complex. For example, pyrrolizidine alkaloids have been suggested to represent an adaptation to exclude lepidopterans from exploiting the nectar of several plant families, although some specialized butterflies and moths seem attracted by these compounds (Pliske 1975), collecting volatile derivatives of the alkaloids and using them in predator defence and courtship (Brown 1984, Boppré 1990).

Concentrations of nectar alkaloids sufficiently high to be deterrent may also benefit plants by increasing pollen export (Irwin and Adler 2008) or optimizing the number of flower visitors per

volume of nectar produced, allowing plants to reduce nectar production and energy investment (Kessler and Baldwin 2007).

Another possible ecological meaning attributed to alkaloids is again antibacterial or bacteriostatic and antifungal functions that limit microbial growth (Stevenson et al. 2017, Adler 2000, Nepi 2017). Curiously, the study by Fridman et al. (2012) on the effects of certain nectar alkaloids did not confirm any effect in controlling bacterial growth. Nonetheless, insect pollinators could benefit from the intake of alkaloids. Alkaloids may play a prophylactic or therapeutic role by reducing the pathogen load of insects (e.g. Manson et al. 2010) and honeybees may actively search for alkaloid-enriched nectar to keep pathogens at bay (e.g. Gherman et al. 2014).

What makes nectar alkaloids particularly intriguing is their neuroactive effects on floral visitors (Mustard 2020). Many alkaloids are known to have strong biological activity, explained by their structural relationship with important neurotransmitters (Baracchi et al. 2017). Alkaloids include good examples of compounds that may improve pollination services without benefiting the floral visitors (Stevenson et al. 2017). For instance, nicotine affects learning: at natural doses, bees learn the colour of flowers containing nicotine more efficiently than the colour of flowers offering the same nutritional value but without nicotine (Baracchi et al. 2017). Even more interestingly, after experiencing flowers containing nicotine, bees become faithful to the flowers, even when the reward offered becomes suboptimal compared to other available food resources (Baracchi et al. 2017). Similarly, Wright et al. (2013) found enhanced memory of reward in bees fed solutions containing caffeine. This led them to postulate that memory enhancement can provide an evolutionary advantage to plants through the fidelity of free-flying bees to a caffeine-containing reward. Speculation on the enhancement of plant fitness was somehow confirmed by the subsequent essay of Thompson et al. (2015) on artificial flowers: pollination by bumblebees was higher for flowers containing caffeine. Arnold et al. (2021) also used robotic flowers to provide evidence that inexperienced bumblebees, primed in the nest with caffeine and a target odour, made more initial visits to flowers emitting the target odour than did control bees or those primed with odour alone. Caffeine-primed bees tended to more quickly improve their floral handling time. Although the effects of caffeine were short lived, they showed that the food-locating behaviour of free-flying bumble bees can be enhanced by caffeine provided in the nest.

6.5 NECTAR NON-PROTEIN AMINO ACIDS

Besides amino acids involved in building proteins, non-protein amino acids have also been found in nectar (Nepi 2014, Baker 1977) and may account for up to 30-50% of nectar amino acid composition (Nepi 2014, Nepi et al. 2012, Nocentini et al. 2012). Non-protein amino acids are generally regarded

as secondary metabolites because they are not directly involved in the primary metabolic pathways (Pichersky and Gang 2000), although not all authors consider this classification appropriate (Bell 2003). Classification aside, many different functions have been attributed to nectar non-protein amino acids (Nepi 2014), particularly γ-amino butyric acid (GABA) and β-alanine, which are often the most frequent and abundant in floral nectar (Nepi 2014).

The ecological importance of nectar non-protein amino acids is now well established. As in the case of other secondary metabolites, an early ecological explanation for the presence of nectar non-protein amino acids was again the potential benefit gained by the plant by deterring scarcely efficient or inefficient pollinators (Nepi 2014 and reference therein). Weakening this assumption, more recent findings show that these compounds do not alter nectar palatability (Carlesso et al. 2021) and have low toxicity (Bogo et al. 2019, Felicioli et al. 2018).

Thus more relevant roles of non-protein amino acids in floral nectar may be as neurotransmitters in insect nervous systems (Mustard, 2020), muscle performance promoters (Bogo et al. 2019, Felicioli et al. 2018), or feeding regulators of nectarivorous floral visitors (Passreiter and Isman 1997, Petanidou et al. 2006). A recent study conducted by Carlesso et al. (2021) reported that honeybees were more likely to learn a scent when it signalled a sucrose solution containing β-alanine or GABA, suggesting that the latter enhance learning of determined flower traits, thus favouring pollen transfer among conspecific individuals. Moreover, GABA proved to enhance memory retention. Some non-protein amino acids are suggested to reduce fatigue and sustain muscle activity (Nepi 2014 and references therein). Taurine, for example, is found in the thoracic region of many insects and is associated with fully functional flight muscles (Whitton et al. 1987), whilst the direct involvement of β-alanine in flight metabolism seems confirmed by Bogo et al. (2019): bumblebees fed with solutions enriched in β-alanine at natural concentrations showed the highest flying-index in a behavioural assay. Curiously, Felicioli et al. (2018) reported that GABA- rather than β-alanine-enriched diets enhanced locomotion in *Osmia bicornis*.

GABA is known to stimulate taste chemoreceptors sensitive to sugars and increase feeding activity in caterpillars and adult beetles (Thornburg et al. 2008, Mitchell and Harrison 1984). Indirect evidence of the phagostimulation activity of GABA comes from the finding that satiety in insects is opposed by simultaneous administration of GABA (Passreiter and Isman 1997). Nevertheless, it is speculated that the combination of GABA and NaCl, rather than GABA alone, plays a role in insect phagostimulation (Petanidou et al. 2006). In fact, the absence of effects on the consumption of sucrose solution enriched with GABA alone in the forager honeybees tested by Carlesso et al. (2021) stresses how studying the effects of different NSCs in isolation rather than their combined effects may yield a very different and unrealistic picture of how animal behaviour is influenced.

After all, this is just one of many examples where the effects of GABA coupled with other nectar chemicals help maintain the feeding rate of floral visitors (Nicolson and Thornburg 2007). GABA is also reported to be involved in plant communication with other organisms and accumulates in response to infection by fungi and bacteria (Nepi 2014 and references therein).

6.6 NECTAR BIOGENIC AMINES

Biogenic amines are nitrogenous compounds known to function as neurotransmitters, neurohormones and neuromodulators in invertebrates (Orchard 1982, Roeder 1999, Blenau and Baumann 2001, Scheiner and Baumann 2006, Farooqui 2012). Thus they shape behavioural patterns (Cnaani et al. 2003). Their presence in floral nectar was recently reported for the first time in 15 species belonging to six plant orders (Muth et al. 2022). Tyramine and octopamine are the two biogenic amines so far reported in floral nectar (Table 1). They are the invertebrate counterparts of vertebrate adrenergic transmitters that govern the so-called fight or flight response, namely quick adaptation to energy-demanding situations (Roeder 2005). They are decarboxylation products of the amino acid tyrosine, and though tyramine is the biological precursor of octopamine, the two are considered to act as independent neurotransmitters (Roeder 2005). The highest tyramine and octopamine concentrations so far (averaging about 0.07 mM) have been reported from the species *Citrus x meyeri* (Muth et al. 2022).

Tyramine has not only been found in nectar (Muth et al. 2022), but also in various foods of plant origin. This amine is associated with microbes with aminogenic activity in fermented foods and beverages (Trivedi et al. 2009), but little is known about why it is found, albeit in small amounts, in fruits, flowers, seeds and other parts of plants (Sánchez-Pérez et al. 2018).

Landete et al. (2007) investigated the production of biogenic amines from selected strains of yeast, acidolactic bacteria and acetic bacteria found in wine. Some of the yeast genera identified may also be found in floral nectar of different plant species (Pozo et al. 2012, Pozo et al. 2016). In any case, the ability to produce tyramine and other biogenic amines is correlated more with strain than species (Moreno-Arribas et al. 2003). Yeasts do not appear to be the main producers of the amines found in wine, attributed to lactic-acid bacteria (Landete 2007, Regecová et al. 2022, Garai et al. 2007) that decarboxylate precursor amino acids, tyrosine in the case of tyramine and octopamine.

Besides being produced in nectar by microorganisms that decarboxylate amino acids, tyramine produced by endogenous enzymes such as tyrosine decarboxylase can also be naturally present in various parts of plants or their derivatives (Vazquez y Novo et al. 1989, Preti et al. 2016, Gobbi et al. 2019). According to Servillo et al. (2017), tyramine and its methylated forms, present in *Citrus* plants, are the products of specific pathways involved in response to attack by insects or other herbivores

and pathogens, as they act as neurotransmitters that can modify various behaviours related to flight, feeding and memory (Finetti et al. 2021) and thus herbivore activity.

The enzyme tyrosine decarboxylase appears to be ubiquitous and implicated in various metabolic pathways where tyramine is the first product and in turn the precursor of many other molecules, including dopamine, octopamine and a wide variety of alkaloids (Facchini 2000), implicated in defence against biotic and abiotic stressors (Hagel and Facchini 2005). The production of tyramine and other amines may be induced for defence of the plant itself. Hydroxycinnamic acid amides, including tyramine-derived neutral amides, appear to be directly involved in plant defence against pathogens (Facchini et al. 2002, Knolleberg et al. 2020, Shen et al. 2021, Płonka et al. 2022, Macoy et al. 2015).

Since biogenic amines seem to have such important effects on the invertebrate nervous system, several studies have focused on insects, demonstrating that consumption of these substances modulates behavioural traits such as motivation (Farooqui 2012), reward-seeking (Peng et al. 2020, Schulz and Robinson 2001, Arenas et al. 2020), learning (Mercer and Menzel 1982, Hammer and Menzel 1998, Agarwal et al. 2011) and social communication (Peng et al. 2020, Barron et al. 2007, Finetti et al. 2021, Linn et al. 2020) (Table 1). Octopamine and tyramine both play an essential role in regulating basic motor functions. They differentially affect flight in honeybees when injected in the thorax, octopamine increasing flight and tyramine decreasing it (Roeder 2005, Fussnecker et al. 2006).

Regarding the effects of biogenic amines on food-source communication and exploitation, Barron et al. (2007) showed that octopamine increases the likelihood of dancing by honeybees, and Linn et al. (2020) found that honeybees treated orally with octopamine were less likely to heed social information from waggle dances. This means that even if the food source bees find is poor, they are more likely to retain their personal information than to heed indications of a richer source. This evidence supports the hypothesis that nectar octopamine can increase bee faithfulness to a plant species and may favour its reproductive success. The results of Cnaani et al. (2003) on bumblebees, seem to challenge this view. The authors showed that octopamine-laden solution shortens the time bees need to change their visiting behaviour once they acquire information on changes in food source availability, making them able to direct their visits more promptly to better food sources in a scenario where the pattern of food availability is changing.

Besides being described as an enhancer of foraging activity (Peng et al. 2020, Schulz and Robinson 2001, Barron et al. 2002), octopamine has also been demonstrated to be involved in the short-term regulation of forager behaviour in honeybee colonies, regulating the type of food source to which foragers direct their collection activity. Giray et al. (2003) report that higher percentages of foragers

treated with octopamine, but not those treated with tyramine, shifted from pollen-collection to nectar- or water-collection. Nectar-collecting bees treated orally with octopamine also showed a greater likelihood of switching their activity to the collection of water or nectar with lower sugar concentrations. Analysed from a plant perspective, both results suggest a trend directing bees to less valuable resources, and may be explained by the effects of biogenic amines on perception. It is worth mentioning that some studies have provided evidence that both octopamine and tyramine enhance sucrose responsiveness (e.g. Scheiner et al. 2002, Panwik and Page 2003, Mc Cabe et al. 2017). This means that administration of both compounds lowers the sucrose response threshold, i.e. their consumption lowers the sucrose concentration necessary to elicit the proboscis extension reflex (Panwik and Page 2003), enhancing bee perception of the value of a food source. It is worth highlighting, however, that in the above cases, concentrations of biogenic amines hundreds or even thousands of times greater than those occurring naturally in floral nectar were studied in isolation (Table 1). The study by Muth et al. (2022) has the merit of providing the first insights into the effects of administration of nectar-like concentrations of combinations of compounds on bee behaviour. Curiously, the authors found that tyramine and octopamine, given together, did not enhance sucrose responsiveness, but instead seemed to erase the taste aversion for caffeine that bees showed when the alkaloid was tested alone. Similarly, the effect of caffeine on long-term memory was also erased by co-administration of tyramine and octopamine, which did not exert any influence on their own.

Reference	Model species	Chemical	Method	Concentration	Effect
Mercer and Menzel 1982	*Apis mellifera*	octopamine (serotonine and dopamine)	injection into the brain	0.05 mM =	OA enhanced responsiveness to olfactory stimuli
Hammer and Menzel 1998	*Apis mellifera*	octopamine	injection into the brain	0.1 mM =	OA induced associative learning
Schulz and Robinson 2001	*Apis mellifera*	octopamine and tyramine	oral ingestion	2 mg/mL +++	OA increased the number of new foragers, TA did not
Scheiner et al. 2002	*Apis mellifera*	Octopamine and tyramine (and dopamine)	oral ingestion, injection into the thorax	various concentrations, the lowest OA: 1 mM = TA: 0.01 mM =	At nectar-like concentrations, OA and TA didn't affect sucrose responsiveness
Barron et al. 2002	*Apis mellifera*	octopamine	oral ingestion	2 mg/mL +++	OA increased responsiveness to brood pheromone, stimulating foraging
Cnaani et al. 2003	*Bombus impatiens*	octopamine	oral ingestion	various concentrations, the lowest at 2 mg/mL +++	OA shortened the time bees needed to direct their visits to a better food source
Pankiw and Page 2003	*Apis mellifera*	octopamine	Oral ingestion	various concentrations, the lowest at 20 µg/mL =	OA increased sucrose responsiveness (also at the nectar-like concentration)
Fussnecker et al. 2006	*Apis mellifera*	octopamine and tyramine	injection into the haemolymph	various concentrations, the lowest at 0.05 mM =	OA and TA reduced walking and increased grooming and standing, with greater effects at higher concentration.
Giray et al. 2007	*Apis mellifera*	octopamine and tyramine	oral ingestion	various concentrations, the lowest at 125 µg/mL +	OA induced a switch in the type of collected material and affected sucrose responsiveness.
Barron et al. 2007	*Apis mellifera*	octopamine	oral ingestion	10.5 mM +++	OA increased the reporting of source value in dances.
Agarwal et al. 2011	*Apis mellifera*	octopamine (and dopamine)	oral ingestion	various concentrations, the lowest at 0.25 mg/mL ++	OA negatively influenced punishment learning.
McCabe et al. 2017	*Melipona Scutellaris*	octopamine	oral ingestion	various concentrations, the lowest at 10 mM ++	OA increased sucrose responsiveness.
Arenas et al. 2020	*Apis mellifera*	octopamine	oral ingestion	10 mM +++	OA modified the probability that foragers switched the type of collected material.
Peng et al. 2020	*Plebeia droryana*	octopamine	oral ingestion	10 mM +++	OA increased bee feeding and the frequency of individual foraging.
Linn et al. 2020	*Apis mellifera*	octopamine (and dopamine)	oral ingestion	2 mg/mL +++	Bees treated with OA followed fewer dances, increasing the use of private information.
Muth et al. 2022	*Bombus Impatiens*	octopamine and tyramine (coupled)	oral ingestion	OA: 8 µg/mL* TA: 10 µg/mL*	OA+TA interacted with caffeine to alter key aspects of bee behavior.

* Concentrations within the range found in the nectar of *Citrus* x *meyeri*.

Table 1. Studies about the effects of tyramine (TA) and octopamine (OA) on bees. = the concentration used in the study is similar to that naturally occurring in nectar and reported for the first time by Muth et al. 2022; + the concentrations used in the study is higher of one order of magnitude for each +.

6.7 INTRASPECIFIC VARIABILITY OF NECTAR SECONDARY METABOLITES

Within-species variability of NSCs has rarely been investigated. The few studies highlight wide variability at the level of individual plants and patches within a population, as well as between populations (Palmer-Young et al. 2019, Kessler et al. 2012, Egan et al. 2016). Concerning cultivated plants, variability in NSCs has also been demonstrated between cultivars (Palmer-Young et al. 2019). Although the qualitative composition of NSCs seems to overlap somewhat in different populations, quantitative composition differs by orders of magnitude (Palmer-Young et al. 2019, Kessler et al. 2012). Since the effects of NSCs are dose-dependent (Stevenson et al. 2017, Wright et al. 2013, Nepi 2017), this large quantitative variability makes it difficult to predict the effect that a specific compound may exert on a certain type of pollinator in a natural ecological context. It is precisely this high quantitative variability of nectar secondary compounds that may affect pollinator foraging behaviour. For example, nicotine concentration in the flower nectar of *Nicotiana attenuata*, unlike that found in other vegetative tissues, is known to vary unpredictably within and between populations, as well as between flowers of the inflorescence of the same individual (Kessler et al. 2012). This unpredictable variability of nicotine in floral nectar, particularly within an inflorescence, promotes outcrossing, probably because it keeps hummingbirds (the natural pollinators of this species) searching for low-nicotine flowers on a plant, enhancing their movement between flowers (Kessler et al. 2008). It appears clear that for the correct interpretation of the role of NSCs in determining effects on the plant reproductive fitness, the mating system of the species must be kept into consideration. However, the case of nectar nicotine allows a certain degree of generalization: this because the compound is found in some self-compatible species of the genus *Nicotiana* whose reproductive output benefits from cross-pollination provided by animal visitors (e.g. Sime and Baldwin 2003, Issaly et al. 2020).

Nectar-dwelling microorganisms are a possible source of NSC variability. Several traits of the chemical environment of floral nectar, such as high sugar content, specific proteins (Roy et al. 2017) and specific secondary compounds (see previous sections) with known antimicrobial activity, impede the growth of most microorganisms. Nonetheless, specialized yeasts and bacteria that can cope with this "defence arsenal" are common inhabitants of floral nectar (Herrera et al. 2009, Mittelbach et al. 2015, Morris et al. 2020). The presence and proliferation of these microorganisms drastically affect the chemical composition of nectar, generally lowering sugar and amino acid concentrations (Pozo et al. 2014, Canto et al. 2011, de Vega and Herrera 2013). It is also demonstrated that nectar-dwelling microbes may alter levels of secondary compounds. Experiments using synthetic nectars spiked with secondary compounds and an array of inoculated microorganisms highlighted that the bacteria *Erwinia* sp. and *Gluconobacter* sp. and the yeast *Metschnikowia reukaufii* may reduce concentrations

of nicotine and aucubin (an iridoid glycoside) (Vannette and Fukami 2016). Besides lowering the concentrations of nectar secondary compounds, it was recently revived the interest – raised more than a century ago – on that nectar-inhabiting microorganisms themselves can be a source of nectar secondary compounds not secreted by the plant. Biogenic amines, very recently detected in nectar (Muth et al. 2022), may be a class of compounds produced by microorganisms decarboxylating amino acids during fermentation of nectar (Nepi 2017 and references therein).

Since the main vectors transporting nectar-dwelling microorganisms from flower to flower are floral visitors (Belisle et al. 2012, Aizenberg-Gershtein et al. 2013, Bogo et al. 2021), whose foraging activity is not homogeneous among all the flowers of a plant or of a population, nectar-dwelling microorganisms (Belisle et al. 2012) and possible modifications in nectar chemistry (Canto et al. 2011) turn out to be spatially distributed, thus contributing to greater quantitative and possibly also qualitative variability of NSCs.

Another possible source of variability of secondary compounds in floral nectar is the activity of herbivores, which is obviously not homogeneous within or between populations. Leaf herbivory of *Nicotiana tabacum* by *Manduca sexta* increases alkaloid levels in floral nectar, indicating that interactions between species, involving leaf and floral tissues, are connected (Adler and Wink 2006). Besides biotic factors such as the above, abiotic drivers too may affect NSC concentrations. For example, nutrient abundance may affect concentrations of alkaloids in leaves and nectar (Adler and Wink 2006).

6.8 Evolutionary considerations on the origin of nectar secondary compounds

From the above, at least three other general functions can be recognized for nectar beyond food rewards for pollinators: 1) defence against microorganisms; 2) deterrence of exploiters (nectar thieves or robbers (*sensu* Inouye 1980)) and less efficient pollinators by changes in nectar palatability (pre-ingestive effects) or toxic effects; 3) modulation of insect mobility and behavioural traits (post-ingestive effects). Defence against microorganisms is common to all classes of NSCs (Stevenson et al. 2017, Nepi 2014, Adler 2000, Lattanzio et al. 2006, Montenegro et al. 2013, Servillo et al. 2017, Tiburcio et al. 2014, Guimarães et al. 2019). Nectar first appeared in Palaeozoic fern clades (Koptur et al. 2013), when few insects had yet evolved, defence against microorganisms may have been the original function of NSCs. In that era, nectar was not involved in plant interaction with insects. According to the "leaky phloem" hypothesis (De la Barrera and Nobel 2004), nectaries were probably a kind of "sap valve" that exuded excess sugars. These sugary exudations may have been infected with microorganisms, some of which may have been pathogens exploiting nectarostomata to enter plant tissues (Bubán et al. 2003). Thus plants needed protection against microbe proliferation.

Regarding an alternative or concomitant hypothesis on the origin of NSCs, secondary compounds in nectar can be considered a pleiotropic trait, i.e. they occur in other plant organs (leaves, stems), protecting against herbivory, and are transported passively by phloem/xylem during nectar production (Adler 2000, Stevenson et al. 2017). The oldest plant–insect relationship is predation of plants by herbivores. Plants underwent natural selection, evolving chemical defences based on secondary metabolites to cope with herbivory. The first arthropods and insects in the Silurian period may have been herbivorous, driving selection of anti-herbivory secondary compounds in plant tissues, and these compounds presumably flowed passively into the nectar. Anti-herbivory functions are today recognized for all classes of NSCs (see previous sections). These molecules probably interacted with mutualistic insects, namely defenders and pollinators, when they evolved. Most "modern" mutualist insects (Diptera, Lepidoptera and Hymenoptera including ants) radiated 125–90 Mya in the early-middle Cretaceous period, simultaneously with angiosperms (Labandeira 2011). They presumably drove plant selection towards optimal (low) concentrations of secondary metabolites in the secretions they fed on, while plants probably started to "manipulate" insect behaviour pharmacologically by secreting neuroactive compounds into nectar, thus improving their own fitness. In this regard it is noteworthy that true nectar is lacking in gymnosperms but their pollination drops can be considered an ecological analogue of angiosperm floral nectar (Nepi et al. 2009). Interestingly, β-alanine, a non-protein amino acid with neuroactive properties (Mustard 2020), was detected in the pollination drop of ambophilous gymnosperms (i.e. gnetophytes), in which pollination is performed by wind and insects feeding on pollination drops, but not in solely wind-pollinated species (Nepi et al. 2017).

The presence of specific secondary compounds in nectar can also be explained from a microorganism perspective. Most recent hypotheses see nectar as an active interface between flowers and pollinators, in which microorganisms that colonize nectar also play an essential role (Nepi 2017). These, through their metabolism, can affect nectar chemistry, modifying its olfactory attractiveness (Pozo et al. 2014, Rering et al. 2018, Cusumano et al. 2022) and possibly synthesizing secondary compounds or modifying the profile of existing ones, thus changing the behaviour of pollinators. Thus the distribution of microorganisms in a population of flowers is ensured, using flower visitors as vectors (Vannette 2020). In this case evolution of the chemical profile of floral nectar and other floral traits (Rebolleda-Gómez et al. 2019) could be driven by the need of microorganisms to be transferred and to reproduce in other flowers.

It seems likely that multiple drivers, namely plant reproductive fitness, microorganism dispersal and climatic and environmental parameters, were responsible for evolution of the complex chemical profile of the modern floral nectar of angiosperms (Nepi et al. 2021).

6.9 FUTURE RESEARCH PERSPECTIVES

While many studies concern nectar volume and chemical composition in terms of sugars, and to a lesser extent amino acids, comparatively few studies concern the array of nectar secondary compounds (Stevenson et al. 2017). Our knowledge of their distribution at systematic level is therefore limited. Although Palmer-Young et al. (2019) were the first to take a systematic non-targeted metabolomic approach to analysing secondary metabolites, their study only concerns 31 species. The determination of secondary compounds in different systematic contexts is therefore highly recommended for future research.

Another limitation of our knowledge of nectar secondary compounds is that their effects have only been studied in bees, with most of the focus on honeybees and bumblebees Wright et al. 2013, Bogo et al. 2018, Baracchi et al. 2017, Carlesso et al. 2021, Arnold et al. 2021, Muth et al. 2022, Marchi et al. 2021). Future research therefore needs to consider other important taxa of insect pollinators such as flies, butterflies and solitary bees.

More study is also needed on the link between nectar secondary compounds, pollination efficiency and plant fitness in general. Although the "nectar manipulation" hypothesis postulates that NSCs are tools by which plants affect pollinator foraging behaviour, increasing plant reproductive output (Nepi et al. 2018), we have little and inconsistent evidence of this relationship. In *Nicotiana attenuata,* both attractant (benzyl acetone) and repellent (nicotine) compounds are required to maximize pollen export (male function), capsule and seed siring (female function) and flower visitation by native pollinators, whereas nicotine is reported to reduce florivory and nectar theft and/or robbery [17, Kessler and Baldwin 2007). High levels of nectar alkaloids may benefit plants of *Gelsemium sempervirens* via increased male function (pollen export) under a limited set of ecological conditions (abundant efficient pollinators, large floral displays) but have no effect on female function (seed production) (Irwin and Adler 2008). More recent papers dealing with the effect of specific nectar compounds on pollinator behaviour ignore or only partly investigate the possible outcomes for plant reproduction (Wright et al. 2013, Bogo et al. 2019, Felicioli et al. 2018, Baracchi et al. 2017, Carlesso et al. 2021, Muth et al. 2022). Using artificial flowers, it has been demonstrated that caffeine-laced nectar brings more visits by bumblebees and more pollen analogue (dye particles) than nectar without caffeine (Thompson et al. 2015).

The lack of clear evidence of links between NSCs, pollinator behaviour and plant reproductive output is important, since such links are pivotal for considering NSCs to be adaptive and therefore subject to selection. In the absence of data for many species, we cannot exclude the possibility that nectar secondary compounds are non-adaptive and just a pleiotropic trait (see before) (Adler 2000, Stevenson et al. 2017). It should in any case be highlighted that the identity and concentration of

specific secondary compounds may vary between nectar and leaves, suggesting that the production or allocation of secondary compounds may be independently regulated in each plant part (Manson et al. 2012), in turn indicating possible selection pressure by different drivers. The presence of secondary compounds in nectar is probably the result of adaptive and non-adaptive factors, as suggested by Manson et al. (2012).

One more point that needs further attention is the possible interactive effect exerted by a mix of NSCs. In most cases the effects of NCSs on insects have been studied experimentally in isolation (Wright et al. 2013, Bogo et al. 2019, Felicioli et al. 2018, Baracchi et al. 2017, Carlesso et al. 2021, Thompson et al. 2015). This is different from the natural ecological context where nectar-feeding insects experience a complex phytochemical nectar environment characterized by a mixture of substances. Very recent papers underline the importance of studying the effect of mixtures of NSCs and of finding interactive effects with pairs of compounds. Muth et al. (2022) revealed that a combination of tyramine and octopamine, in a range of concentrations occurring naturally in nectar, had no effect on insect behaviour, whereas when combined with caffeine they alter key traits of bumblebee (*Bombus impatiens*) behaviour, such as sucrose responsiveness, long-term memory and floral preferences. Artificial feeding experiments by Marchi et al. (2021) found that single compounds such as arginine and caffeine increased honeybee learning performance but that insect memory retention only increased significantly when feeding treatments offered a combination of the two compounds. These findings highlight that studying the effects of NSCs as single molecules is too simplistic and that it is necessary to test mixtures of NSCs, at concentrations occurring naturally in nectar, also combined with other substances.

A further element of complexity is that nectar chemistry (including NSCs) may affect pollinator behaviour through other floral traits such as colour. For example, bumblebees (*Bombus impatiens*) that had experience with blue flowers preferred blue regardless of nectar chemistry. In contrast, bees having prior experience with white flowers only preferred white in the case of control treatment, whereas bees exposed to caffeine and ethanol showed no preference (Jones and Agarwal 2022).

Another aspect that needs to be considered is the possibility that certain contaminants may alter the effects of NSCs on insect behaviour and other traits. It was demonstrated that the common neonicotinoid imidacloprid attenuated the positive effects of certain NSCs, while an NSC-enriched diet increased the negative effects of pesticide exposure (Richman et al. 2022).

Finally, a further consideration worthy of attention is the link between certain classes of NSCs and abiotic stress. Since plants can synthesize a variety of secondary metabolites to cope with stress, levels of these substances are related to environmental changes. GABA, for example, is involved in drought and heat stress resistance in plants Abdel Razik et al. 2021, Hasan et al. 2021). Higher

temperatures, drought and heat waves are expected to increase significantly in the near future in certain regions of our planet, according to the current climate change scenario (Cramer et al. 2018). An increase in GABA concentrations is likely in plant tissues to counteract increased stress. If this increase also spills over into the nectar, due to the general correlation between levels of secondary compounds in leaves and nectar (Adler and Wink 2006), then plant-pollinator interactions could change, since the effects of GABA on bees are concentration-dependent (Bogo et al. 2019).

6.10 CONCLUDING REMARKS

Today the ecological functions of nectar are recognized to be far more than a simple food reward for pollinators (Parachnowitsch et al. 2019). The complex chemical composition of floral nectar, especially in terms of primary and above all secondary compounds, reflects additional functions that make nectar a plant interface for complex, multi-faceted biotic interactions involving plants, pollinators, nectar exploiters and nectar-dwelling microorganisms (Parachnowitsch et al. 2019, Nepi 2014b) (Fig. 2). Although the "nectar manipulation" hypothesis (Nepi et al. 2018) still has gaps, it is a good framework for shaping future studies in the field of nectar ecology and evolution, also considering the expected scenarios of climate change. In any case, the manipulation of behavioural traits of pollinators is just one facet of the multi-faceted interactions mediated by floral nectar, which should therefore be considered in a more comprehensive perspective. The role of microorganisms, both yeasts and bacteria, in these multifaceted interactions seems largely overlooked (Vannette 2020, Francis et al. 2021, Cullen et al. 2021), limiting an overall understanding of their role in pollinator behaviour, plant-pollinator interactions and plant fitness.

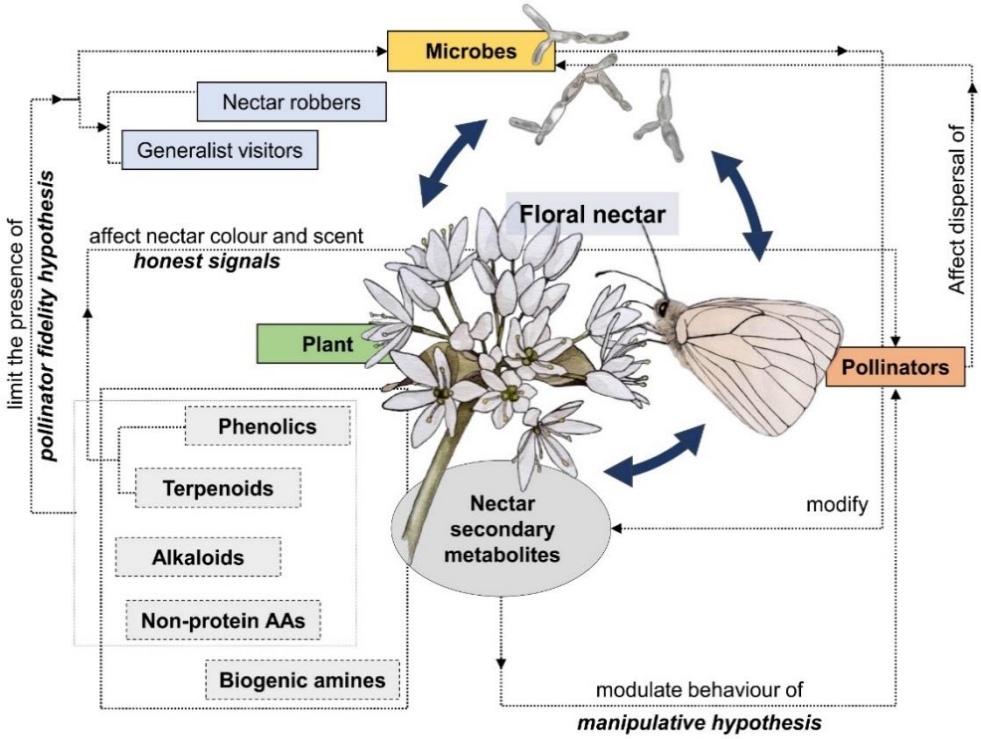

Figure 2. Network of nectar-mediated complex relationships involving plants, microbes and pollinators. Nectar secondary compounds are pivotal in shaping such interactions.

6.11 REFERENCES

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7. FLORAL NECTAR: FIFTY YEARS OF NOVEL ECOLOGICAL PERSPECTIVES BEYOND POLLINATOR REWARD

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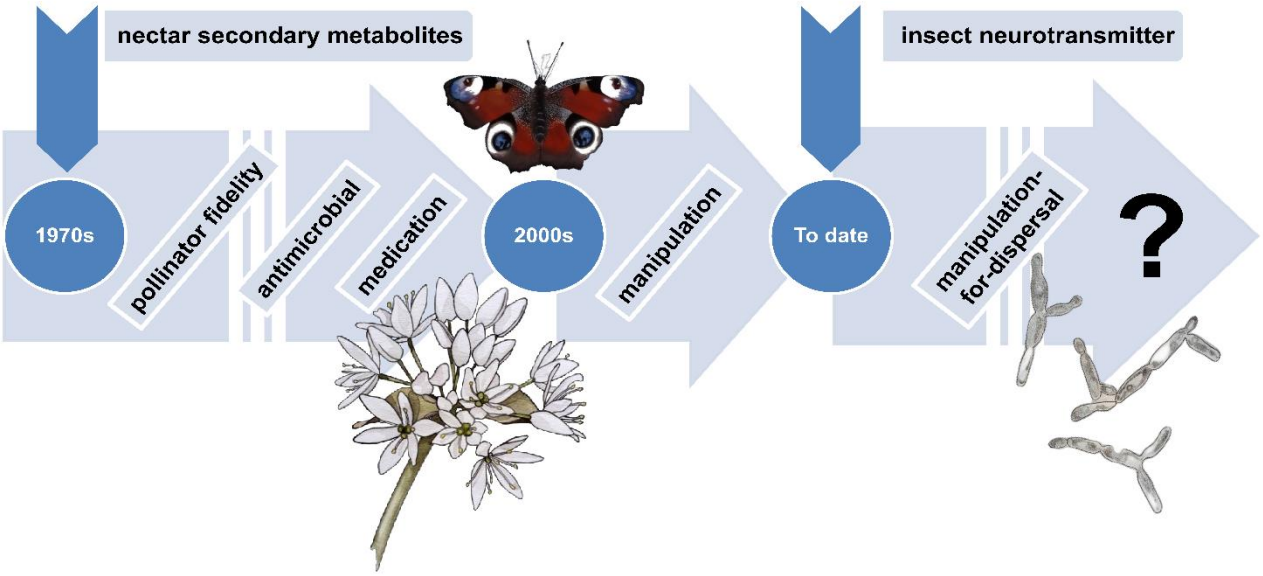
Abstract

Across the past fifty years several ecological significances have been successively attributed to floral nectar in response to the numerous breaking-through findings reported for the field. Here, we review how the historical meaning of floral nectar has been first challenged, then modified and expanded since the first discovery of secondary metabolites in nectar. We then go further in discussing the recent report of the class of biogenic amines, highlighting the importance to assess the origin of such compounds, known to be important insect neurotransmitters, and we conclude the review by pointing out the macro-areas of study which constitute current knowledge gaps in the field of nectar ecology.



Keywords

Nectar biogenic amines, plant-microbe-pollinator interactions, manipulation hypothesis, pollinator fidelity hypothesis, nectar ecology

7.1 BRIEF HISTORICAL OVERVIEW

The scientific definition of nectar, first given by Linnaeus in 1735, sees nectar as the secretion of specific organs, the nectaries. Specifically, the secretion of *floral* nectar is associated with the plant reproductive structures (Nepi 2017), and rewards animals that may perform pollination while visiting the flower (Nepi et al. 2018). Whether pollinator attraction has been the primary driver leading to nectar appearance is a difficult question to answer (e.g. Sprengel 1793, Caspary 1848, Bonnier 1878), nevertheless, its centrality in mediating plant-animal interaction is nowadays undeniable, and it has been somehow recognized as early as in the I century BC, when the poet Virgil, in his Georgics (part IV, 149-227), used the term "nectar" to refer to the substance that honeybees collect from the fields and store in the combs as honey.

By virtue of its carbohydrate and amino acid content, nectar has been considered as an easily absorbable, cost-effective, alimentary reward offered by plants in exchange for the pollination service mediated by animals (Nicolson 2007, González-Teuber and Heil 2009, Heil 2011). Up to 90% of its dry weight is represented by sugars (Lüttge 1977), whilst the other 10% consists of a plethora of compounds of which amino acids are the most abundant ones (e.g. Lüttge 1961, Mostowska 1965). An historical listing of European scientists who reported the presence of amino acids in nectar between the '50s and the '70s is given by Baker and Baker (1975).

In the '70s, though, the discovery of nectar chemicals not used in primary metabolic pathways (Baker and Baker 1977, 1986) – and thus addressed as secondary metabolites (Pichersky and Gang 2000) – challenged this traditional view. At the time, the majority of the advancements conquered in the field of nectar chemistry was made possible by the pioneering work conducted by Baker and Baker, who were also the first who speculated, in 1975, on the function of these so-called "unfavorable substances". They anticipated the theory nowadays known as "pollinator fidelity" hypothesis, which states that the presence of secondary metabolites in nectar discourages flower-inconstant insects to visit the flowers, whilst favoring specialist visitors. This hypothesis lies on the assumption that specialists are more effective pollinators than generalists and deliver more intraspecific pollen (Rhoades and Bergdahl 1981, Adler 2000 and reference therein). Therefore, starting from the '70s, the main traditional alimentary function of floral nectar started to be flanked by a second crucial role suddenly recognized: that of discouraging those nectar consumers whose contribution to the pollination service is scarce or null. This hypothesis was later expanded by Janzen (1977) and Baker (1978), who proposed that nectar secondary metabolites might deter nectar robbers such as ants. This further interpretation, which many researchers agree to consider as an extension of the "pollinator fidelity" hypothesis, has later been addressed as "nectar robber" hypothesis (Adler 2000). However, studies conducted at the time to confirm such hypothesis revealed that nectar secondary metabolites

could occasionally deter ant robbery, but also showed that in most cases the deterrent effect is conferred by mechanical adaptations (Feinsinger and Swarm 1978, Schubart and Anderson 1978, Guerrant and Fiedler 1981).

In line with the idea that secondary metabolites likely prevent nectar exploitation by the side of inefficient floral visitors, it was then hypothesized how they may prevent nectar wastage by microbes (e.g. Hagler and Buchmann 1993, Verpoorte and Schripsema 1994). By virtue of its sugar and amino acid composition, in fact, nectar has been early recognized as a potentially rich medium for microbial growth (e.g. Boutroux 1884, Schuster and Úlehla 1913, Grüss 1917, Schoellhorn 1919). Curiously, nectar proteins – compounds that are nowadays known to protect the nectar against microorganism proliferation and the plant tissues against infections by pathogens (Nepi 2017 and reference therein) – were discovered more than 90 years ago (Buxbaum 1927), but were initially thought to play a nutritive function, by supplying nectar consumers with organic nitrogen (Lüttge 1961, Heil 2011). This means that the so-called "antimicrobial" hypothesis (Adler 2000) received more support some years later, when more experiments conducted in this direction and proved it to be correct (e.g. Carter et al. 2007, Gonzáles-Teuber et al. 2009, Hillwig et al. 2010).

At first referred as "unfavorable" (Baker and Baker 1975), nectar secondary metabolites have later been often addressed as toxic. In reference to nectar non-protein amino acids, Baker and Baker were again possibly the first authors suggesting that these compounds were likely to be toxic to certain kind of flower-visitors (1977). In the same decade, a series of studies demonstrated the potential toxicity of these secondary metabolites on animal consumers in plant-herbivore interactions. Such toxicity was described as deleterious post-ingestive effects on growth (e.g. Blau et al. 1978, Isman and Duffey 1982), organ functioning (e.g. Berenbaum 1988), and nutrients uptake (e.g. Slansky 1992). These findings led to reconsider the early-adopted assumption stating that the benefits of nectar secondary metabolites must outweigh the costs, and the idea of direct selection for nectar toxins was momentarily obscured by the conjecture that their presence is due to previous selection pressures or pleiotropic constraints (Adler 2000). However, since such studies had been conducted with concentrations equal to those found in vegetative tissues, which are generally greater than those found in nectar (e.g. Adler et al. 2006, Wiese et al. 2018), this led to the intuition that such concentrations could result toxic for insect pathogens but not for their hosts, which may not be as susceptible. From a series of pioneering studies conducted in this direction, raised the conviction that their ingestion by nectar consumers may confer them an improvement in health and life expectancy (e.g. Price et al. 1980, Berenbaum 1988). This new perspective attributing curative benefits to secondary metabolite

ingestion revives the concept that the presence of these compounds in nectar must outweigh the costs associated with their consumption.

In support to this latter view, it is nowadays well established that the putative toxicity exerted by some of these chemicals often depends on the sensitivity of the nectar consumer (e.g. Tiedeken et al. 2016), or it is often reported for introduced species which do not represent the native pollinators of the plants containing such compounds (e.g. Zhang et al. 2022). Moreover, as stated above, the concentrations of such compounds are generally lower in nectar compared to other plant tissues (e.g. Cook et al. 2013, Palmer-Young et al. 2019). In general, pollinators may therefore benefit from the consumption of nectars rich in these metabolites as this may reduce their pathogen loads, enhance their immune response, or even enrich their gut microbiota (Gunasekaran et al. 2020, Baracchi et al. 2022), in line with what may be referred as "medication" hypothesis. A growing number of recent studies supports this view. For example, nectar alkaloids such as gelsemine, anabasine, and nicotine benefit pollinators by increasing their resistance to parasites and pathogens (Manson et al. 2010, Richardson et al. 2015, Thornburn et al. 2015), and the idea that bees may actively search for alkaloid-enriched nectar to keep pathogens at bay (Gherman et al. 2014) has become popular. Such behavior of active search has been explained, at least until recent years, through the homeostasis mechanism, for that an impulse of search for a certain compound appears in an animal when the levels of such compound lower in its body (Samorini 2013). Nevertheless, the consumption of a potentially curative source does not produce an immediate healing, an aspect that Samorini (2013) considers sufficient to reject the idea that the ingestion of curative substances is exclusively ruled by homeostasis mechanisms, rather suggesting some degree of "awareness" or "intentionality".

Nowadays, the existence of self-medication both in vertebrates and invertebrates has finally been established, and a growing number of studies has provided evidence of this (Hutchings et al. 2003, de Roode et al. 2013, Abbott 2014, and reference therein). Self-medication implies that the exposure to secondary metabolites by healthy animals comports a cost, compensated by its beneficial effects in reducing symptoms or clearing infections in parasitized animals (Clayton and Wolfe 1993, Lozano 1998, Singer et al. 2009, Abbott 2014). Additionally, to be in line with the key criteria defining self-medication, an animal must modify its diet preferences, addressing its foraging towards a source containing "nonnutritive" antimicrobial compounds when parasitized (Karban e English-Loeb 1997).

Some of such compounds share structural similarity with important neurotransmitters (Verpoorte 2005), this observation supports the hypothesis that their presence in nectar outweighs the potential costs associated with their consumption. As early as in the '70s, the idea that compounds such as alkaloids, glycosides, and phenolic substances could have a significant effect upon the central nervous

system of flower visitors was already suggested (Baker and Baker 1975, 1977). If a certain chemical can modulate elements of neuronal signal transduction, the concentrations of neurotransmitters, the activity or expression of their receptors can be changed, and this can lead to severe changes in animal behavior (Wink 2018). Occasionally, when the ingestion of nectar secondary metabolites brings to the onset of pharmacological effects on the brain of nectar consumers, these have been addressed as drugs (e.g. Wright et al. 2013). However, the proper definition of a certain substance as a drug is a rather complex task. Historically, drugs have been referred as "nervous foods" (Mantegazza 1871), bringing the focus on two aspects: i) they often interfere with animal nervous system at various levels, and ii) drawing a distinct line separating food and drugs is very hard. Several criteria, in fact, can potentially be adopted for its definition. For example, the sharpening of specific senses or the onset of addiction (Samorini 2013), with only one aspect commonly shared: leading to changes in animal behavior (Wink 2018). Addiction can manifest in various ways, but generally implies a craving for a chemical whose exposure confers the consumer a strong urge once the level of the addictive chemical drops (Wink 2018). Often it also implies consumption despite adverse consequences and perceptual changes in reward strength (Koob 2015, Fattore and Diana 2016). The key drivers of addiction are reflected in altered expressions of motivation and learning, capacities which emerged early in the Precambrian (Menzel and Benjamin 2013), so that recent views frame addiction as a phenomenon with deep evolutionary roots and widely spread among invertebrates (van Staaden et al. 2018).

Cases where the consumption of nectar secondary metabolites implies a cost for the nectar-feeding animal – in contradiction to the conservational instinct – were initially considered incidental, but the observation of repeated feeding behavior on inebriating sources rose the question onto what extension the ingestion of these compounds is "intentional". The hawkmoth *Manduca quinquemaculata*, for example, feeds on nectar of *Datura meteloides*, a plant belonging to the family Solanaceae, whose nectar probably contains the same hallucinogenic substances present in the other plant tissues (Grant 1983). These compounds intoxicate the insects, making them sluggish and disoriented (Grant 1983). When the daze moths lie on the ground, they are highly exposed to predation (Grant 1983). Similar is the case of bee exposure to hallucinogenic or narcotic substances offered by orchid species such as *Epipactis helleborine* (Jakubska et al. 2005).

A recent study conducted by Galpayage Dona et al. (2022) provided first evidence on that bumblebees may engage in activities not directly related to the urge of satisfying a primary need. Despite the absence of external incentives, in the study, bees repeatedly engaged in rolling wooden balls, suggesting that such activity – fully ascribable to play – is rewarding in itself, an aspect in line with the criteria defining play. This finding, along with a series of other studies, represents a breakthrough in the field of insect behavior, since it provides additional evidence to a list of studies on the existence

of a form of sentience in bumblebees (e.g. Bateson 2014, Held and Špinka 2011, Solvi et al. 2016, Birch 2020). This may rise the question on whether the search for hallucinogenic/inebriating substances could also be rewarding in itself, and if the returning to such nectar sources may be not exclusively dictated by the insurgence of physical dependance.

Beyond the causes of this animal retention, the ecological explanation that has been given to the presence and maintenance of such inebriating compounds in the floral nectar is that of increasing the chance of pollination by inebriating floral visitors.

Other coercive mechanisms not necessarily implying intoxication are known, for instance that of offering nectar containing nicotine: after experiencing such nectar, bees keep returning to the food source even when this becomes suboptimal compared to other available rewards (e.g. Baracchi et al. 2017). In line with what Rhoades and Bergdahl (1981) predicted, in this case pollinator retention may increase the mobilization of conspecific pollen grains, providing a benefit to the plant reproductive fitness.

The examples illustrated above share a common aspect: the emergence of a potentially harmful behavior in floral visitors as a consequence of nectar ingestion. Such cases possibly represent the best exemplification – though not unique – to frame the concept of pollinator manipulation, a term that researchers have started using from the early 2000s (e.g. Biernaskie and Cartar 2004, Bayleis et al. 2007), and which gained full recognition after the formal introduction by Pyke (2016) of the "manipulation" hypothesis. However, to be fair, as early as 1981, Rhoades and Bergdahl wrote, in reference to various nectar secondary metabolites, the following statement: "though at first sight the presence of these toxic substances seems incompatible with the reward function of nectar, they probably represent a mechanism to manipulate pollinator behavior to the advantage of the plant and to exclude nectar thieves". They intuited that a combination of rewarding and defensive chemicals could model the insect patterns of visit in favor of the plant fitness beyond the pollinator fidelity hypothesis alone.

Along with those secondary metabolites that have strong biological activities due to their structural relationship with animal neurotransmitters (Verpoorte 2005), an additional case is represented by those nectar chemicals that represent exogenous invertebrate neurotransmitters in themselves. This is the case of biogenic amines (Roeder 1999, Blenau and Baumann 2001, Scheiner et al. 2006, Farooqui 2012), a class of compounds which has been reported for the first time in floral nectar only recently (Muth et al. 2022, Barberis et al. unpublished data). More specifically, the two biogenic amines reported in floral nectar to date are represented by tyramine and octopamine, the invertebrate counterparts of the vertebrate adrenergic transmitters, ruling the so-called fight or flight response, which is to say the quick adaptation to energy-demanding situations (Roeder 2005). They are products

of the decarboxylation of the amino acid tyrosine and even though tyramine represents the biological precursor of octopamine, they are considered to act as independent neurotransmitters (Roeder 2005). Their consumption modulates behavioral traits such as motivation (e.g. Farooqui 2012), reward-seeking (e.g. Schulz and Robinson 2001, Peng et al. 2020), locomotion (e.g. Fussnecker et al. 2006, Hardie et al. 2007), learning (e.g. Mercer and Menzel 1982, Hammer and Menzel 1998) and social communication (e.g. Barron et al. 2007, Linn et al. 2020).

7.2 A STEP BACK

So far, nectar-mediated interactions have been described as a bipartite phenomenon between plants and floral visitors, and the way secondary metabolites wind up in nectar has been neglected. This aspect is in fact still largely unclear (Heil 2011 and reference therein). Along with the discovery of nectar secondary metabolites, back in the '70s, this question revived, and one of the main hypotheses explaining the presence of secondary metabolites in floral nectar stated that nectaries secrete almost unmodified substances, coming directly or indirectly through the vascular tissues, in a phenomenon of rather passive diffusion (Lüttge 1977, Fahn 1988). This view suggests that nectar chemistry was originally determined by co-evolutionary processes with herbivores, while adaptive functions rose after chemical defense (Stevenson et al. 2017).

Despite many secondary compounds are indeed transported between plant tissues via the phloem (e.g. Gowan et al. 1995, Merritt 1996), the idea that the occurrence of secondary metabolites may not be exclusively due to phloem transportation was also revived (Adler 2000).

Nowadays, it is well established that the chemical composition of floral nectar can be shaped not only by phylogenetic constraints but can be also shaped by ecological drivers (e.g. Nepi et al. 2010, Bogo et al. 2021). Among these, for example, it is worth mentioning the interactions with specific guilds of pollinators which may drive selection towards convergent nectar chemistry in unrelated taxa (Pozo et al. 2015). In addition, chemical composition can extensively be affected by processes of post-secretion modification, which can be induced by the interaction with abiotic and biotic variables, such as the influence of meteorological conditions (e.g. Corbet et al. 1979, Plowright 1981, Chalcoff et al. 2017, Parachnowitsch et al. 2019) and the interaction with floral visitors (e.g. Bogo et al. 2021). Nowadays, animal visitors are recognized as the principal vectors of bacteria, fungi and other microorganisms to and among flowers (e.g. Herrera et al. 2010, Belisle et al. 2012). However, not even when they are freshly opened, flowers can be considered sterile. Even before the bud opening, in fact, floral nectar often contains bacteria and fungi (e.g. Shade et al. 2013, von Arx et al. 2019), whose abundance increases over time in individual flowers (e.g. Pusey et al. 2009, von Arx et al. 2019, Morris et al. 2020). Besides bacteria and fungi commonly found in air, soil and other habitats

– generally the first to be detected once the flower opens (e.g. Brysch-Herzberg 2004, Morris et al. 2020) – another group of microbes commonly found is that of flower specialists, which exhibit a range of traits that may be adaptations to the nectar environments (e.g. Dhami et al. 2016, Herrera et al. 2010, Pozo and Jacquemyn 2019). Moreover, regardless of continent or habitat type, microbial colonization has been recently demonstrated to occur much more frequently than previously believed (e.g. for yeasts: Herrera et al. 2009).

Upon colonization, microbes can then modify plant-provisioned nectar chemicals or impart their own through secretion of metabolic by-products into the nectar (e.g. Canto and Herrera 2012, Vannette and Fukami 2018, Yang et al. 2019, Vannette and Fukami 2016, Rering et al. 2020). During sugar fermentation, for instance, different volatile organic compounds (VOCs) are released, and additional compounds can be added to the floral olfactory bouquet (Rering et al. 2018). Even in the case mentioned above, that of *Epipactis helleborine*, one of the potentially hallucinogenic/narcotic compounds offered in its floral nectar is ethanol (Løjtnant 1974, Müller 1988), which is believed to be most likely of microbial origin (Ehlers and Olsen 1997, Kevan et al. 1998).

The influence of microbes on floral nectar is mainly pointed as detrimental for its quality (e.g. Eisikowitch et al. 1990, Herrera et al. 2008, Vannette et al. 2013), weakening or negatively interfering with the plant-pollinator mutualism. For example, some studies demonstrated how yeasts reduce the food value of floral nectar by causing a decrease in sugar (Canto et al. 2011, de Vega and Herrera 2013) and amino acid concentrations (Pozo et al. 2014). In general, though, floral microbes are believed to rarely benefit plants (Vannette 2020). Flower pathogen and some nectar bacteria can reduce plant fitness, either directly or by decreasing pollinator visitation (e.g. Vannette et al. 2013). Other studies, however, have demonstrated how in some other cases microorganisms may enhance pollination by producing volatiles that play a role in attracting pollinators, indirectly influencing the plant fitness (e.g. Pozo et al. 2009, Herrera and Pozo 2010, Cullen et al. 2021). Even in the cases where nectar yeasts can increase pollinator visitation, though, this does not necessarily benefit the plant fitness. For example, Herrera et al. (2013b) reported reduced seed set of yeast-colonized plants despite increased pollinator attraction in *Helleborus foetidus*.

In addition, not only microbial presence in nectar can alter pollinator attraction and visitation through volatile emission or chemical modification (Raguso 2004, Herrera et al. 2013a, Rering et al. 2018, 2020), but the presence in itself seems to drive a preference for yeast-containing flowers in pollinators such as bumblebees, who were therefore shown to be able to detect them in the nectar (Herrera et al. 2013b, Schaeffer et al. 2014, Schaeffer et al. 2017). In this regard, nectar yeast cells have been suggested to supplement insects for important nutritional elements such as vitamins, sterols, and minerals (Vega and Dowd 2005, Stefanini 2018). The first study going in this direction was that

conducted by Dharampal et al. (2019), who provided evidence on the benefits that honeybee larvae gain from the diverse communities of symbiotic microbes that inhabit the surface of pollen grains. If pollen microbes represent a crucial dietary resource for larval development, it is very likely that the microbial inhabitants of floral nectar represent an important nutritional component too (Jacquemyn et al. 2021). This view is line with the emerging evidence suggesting that the nectar microbiome can also influence pollinator health (*sensu* López-Uribe et al. 2020) by modifying their nutritional landscape, altering foraging behaviors, and interacting with their symbionts and pathogens (Martin et al. 2022 and reference therein). For example, both nectar yeasts and bacteria have been demonstrated to lead to a general increase in pollinator fitness through the reduction of the pathogen growth inside the gut of the hosts (Pozo et al. 2020) or by an increasing in the reproductive success and development of the colonies (Pozo et al. 2021).

Since biogenic amines can be generated by microbial decarboxylation of free amino acids, it has been suggested that their presence in floral nectar could be imputable to yeast metabolism rather than being a plant product (Nepi 2017, Nepi et al. 2018). To date, though, evidence supporting this hypothesis is still missing, so that the most conservative explanation still sees them as plant byproducts. Tyramine, for instance, can be found in various plant parts or their derivates thanks to the production of endogenous enzymes (Vazquez y Novo et al. 1989, Preti et al. 2016, Gobbi et al. 2019) appearing to be ubiquitous and implicated in various metabolic pathways of which tyramine – precursor of many other pharmacologically active compounds – is the first product (Facchini et al. 2000). As tyramine can be the product of specific pathways activated in response to attack by various plant enemies (Servillo et al. 2017), the production of biogenic amines may be a general response induced as defense against pathogens or phytophagous (Facchini et al. 2002, Macoy et al. 2015, Knolleberg et al. 2020, Shen et al. 2021, Płonka et al. 2022). In fermented foods and beverages of plant origin, however, its presence is associated with microbes with aminogenic activity (Trivedi et al. 2009). In addition, some microbes found in wine and producing biogenic amines have been also found in floral nectar (Landete et al. 2007, Pozo et al. 2012, Pozo et al. 2016).

7.3 KNOWLEDGE GAPS

Nectar chemical complexity is nowadays established, despite for long its composition has been assumed as a constant trait within a species, assumption which encouraged the search for patterns, whilst justifying pooling nectar samples when volumes were not enough for analyses (Nicolson 2022). This approach has masked for decades the actual variability in nectar, its complex physiology, polygenetic structure, and environmental dependency, which all make its study extremely challenging

(Brandenburg et al. 2009 and reference therein). Explanations on the ecological role of nectar in mediating plant-animal interactions are thus less certain because of the variable chemical expression (Stevenson et al. 2017). More insights into the molecular and genetic mechanisms ruling its secretion and composition are therefore needed.

A second level of complexity is represented by the most recent findings concerning animal cognition. Flower visiting involves perception, memory, expectation, and decision making (Waddington 2001), all tools known to be influenced by emotional states, at least in human beings (e.g. Mathews and MacLeod 1994, Lerner and Keltner 2000). In recent years, the scientific community seems to have recognized the existence of emotions not only in vertebrates such as fish and birds (e.g. Rey et al. 2015, Valance et al. 2008), but also in insects such as bees and flies, which have turned out to fulfill the basic requirements of emotional behavior (Baracchi et al. 2017 and reference therein), as well as showing a form of sentience (Galpayage Dona et al. 2022). Moreover, in recent years, several studies have established that insects possess high levels of cognitive sophistication (e.g. Avarguès-Weber et al. 2011, Collett et al. 2013, Giurfa 2013, Klein et al. 2017). These important breakthroughs challenge the way we have been tackling the subject on how floral visitors make use of the floral nectar-landscape. The rising evidence demonstrating that insects can self-medicate or engage in activities rewarding beyond their primary needs – for pleasure, one would say – possibly represent the main findings encouraging research in this direction.

Moreover, despite the importance of having more information on wild pollinators for their conservation has been acknowledged at overall levels (Pegoraro et al. 2020), the effects of nectar secondary metabolites on the great majority of wild pollinators are largely undervalued. For what concerns wild bees, this is probably a consequence of our limited understanding of how establishing and maintaining their nests in laboratory conditions (Leonard and Harmon-Threatt 2019). So far, most research has been focusing mainly on managed honeybees, bumblebees, and hummingbirds (e.g. Muth et al. and reference therein, Stevenson et al. 2017 and reference therein, Kessler et al. 2012), despite pollinators vary greatly in several characteristics such as life cycle, sociality, and dietary specialization (Muth et al. 2017). For example, even a simple response as that measurable through the elicitation of the proboscis extension reflex under laboratory conditions seems to be deeply influenced by the degree of sociality exhibited by the bee species (Vorel and Pitts-Singer 2010). This stresses the importance to couple (when possible) laboratory essays with investigations performed in natural or semi-natural conditions on wild pollinators, also in the light of recent findings revealing that experiments conducted in controlled conditions may not always yield a realistic picture when it comes to animal behavior (e.g. Mujagic and Erber 2009, Ayestaran et al. 2010).

Even more relevant is filling up the knowledge gap concerning the synergic effects that the complex combinations of chemicals found in nectar may exert on pollinator behavior. Despite foliar chemical ecology has highlighted the relevance of synergistic effects (Richards et al. 2016) and recent studies have demonstrated how these can result in unpredicted behaviors (e.g. Muth et al. 2022), studies on the field of nectar chemistry generally involve the usage of single phytochemical at a time (e.g. Wright et al. 2013, Baracchi et al. 2017, Estravis-Barcala et al. 2021, Hernández et al. 2018, Marchi et al. 2021, Richman et al. 2022, Thorburn et al. 2015).
A better understanding of how nectar-like concentrations of combined co-occurring secondary metabolites affect animal behavior represents a sort of crucial pre-condition for the assessments of how the human-induced dispersion of chemicals in the environment may interfere in plant-pollinator interactions. How nectar secondary metabolites interact with phytochemicals is also still largely unknown, but some first studies have showed that even a single acute exposure to a pesticide can reshape the interactions mediated by nectar secondary metabolites between plants and floral visitors (Richman et al. 2022). This finding highlights the importance of using realistic concentrations of chemicals, comparable to those found in natural nectar.

So far pollinators have been regarded as the main source of selection that leads to the establishment of given concentrations of secondary metabolites in floral nectar (Stevenson et al. 2017), besides current research has outlined how these can also be affected by floral microbes (e.g. McArt et al. 2014, Parachnowitsch et al. 2018, Rebolleda-Gomez et al. 2019, Rivest and Forrest 2020). Other than circumventing the plant defensive mechanisms – such as high concentrations of reactive oxygen species (Thornburg et al. 2003) or proteins with antimicrobial properties (Schmitt et al. 2021 and reference therein) – nectar specialized microbes need to colonize new spaces to maintain their populations (Morris et al. 2020), as the flowers where they live generally present short lifespans (e.g. Primack 1985). To do this, it has been suggested that microbes may affect flower attractiveness to increase their chance for dispersal (Vannette 2020). However, besides few examples like that of the fungal pathogen *Fusarium moniliforme*, which enhances bird visitation for spore dispersal (Lara and Ornelas 2003), there is little evidence showing that microbial species rely on floral visitors for their population maintenance. This means that further investigations are needed to verify what we may address as “manipulation-for-dispersal” hypothesis.

Finally, despite floral microbes are believed to rarely benefit plants, to date a few cases are known of plants which exhibit adaptations to promote microbial growth in flowers. The results of the study conducted by Wiens et al. (2008), for example, suggest that the palm *Eugeissona tristis* may

encourage the growth of ethanol-producing yeasts, selecting mammal pollinators adapted to consume fermented nectar whilst discouraging the less specialized ones. Despite being still an untested hypothesis, if floral microbes could enhance the plant fitness through the imparting of specific compounds such as exogenous insect neurotransmitters into the nectar, then further investigations should examine the potential for plant chemical adaptation to facilitate microbe colonization of nectar. In other words: may plants show chemical adaptations of nectar that promote microbial settlement into the flower to guarantee optimal pollinator attraction?

7.4 CONCLUSIVE REMARKS

All actors involved in the plant-microbe-pollinator interactions are under simultaneous and reciprocal selective pressures, all influencing the ecology and evolution of their reciprocal partners (Figure 1). Clearly, accepting nectar-dwelling microorganisms as a third partner in the nectar-mediated plant-animal interactions adds a further level of complexity to the potential ecological functions of floral nectar (Stevenson et al. 2017, Nepi 2017). Plants need to balance the concentrations of nectar compounds to not deter specific pollinators by exceeding their tolerance thresholds (e.g. Manson et al. 2013, Wright et al. 2013) to guarantee their visitation to flowers, whilst flower specialized microbes need to disperse among flowers to maintain the growth of their populations. In this scenario, floral microbes and plants appear to share – at least up to a certain extension – the need to attract floral visitors, even though for the fulfilment of different needs.

In this review, floral nectar has been presented as an aqueous solution, to which have been attributed several ecological meanings across the recent decades in response to the successive breaking-through findings reported for the field. Since the presence of microbial communities in floral nectar is a phenomenon more ubiquitous and abundant than previously believed (e.g. Herrera et al. 2009, Álvarez-Pérez et al. 2012), though, we conclude with a suggestion open to debate on whether nectar should be rather addressed also as a chemically dynamic suspension of living organisms in order to fully recognize the centrality of their role in shaping plant-pollinator-microbe interactions.

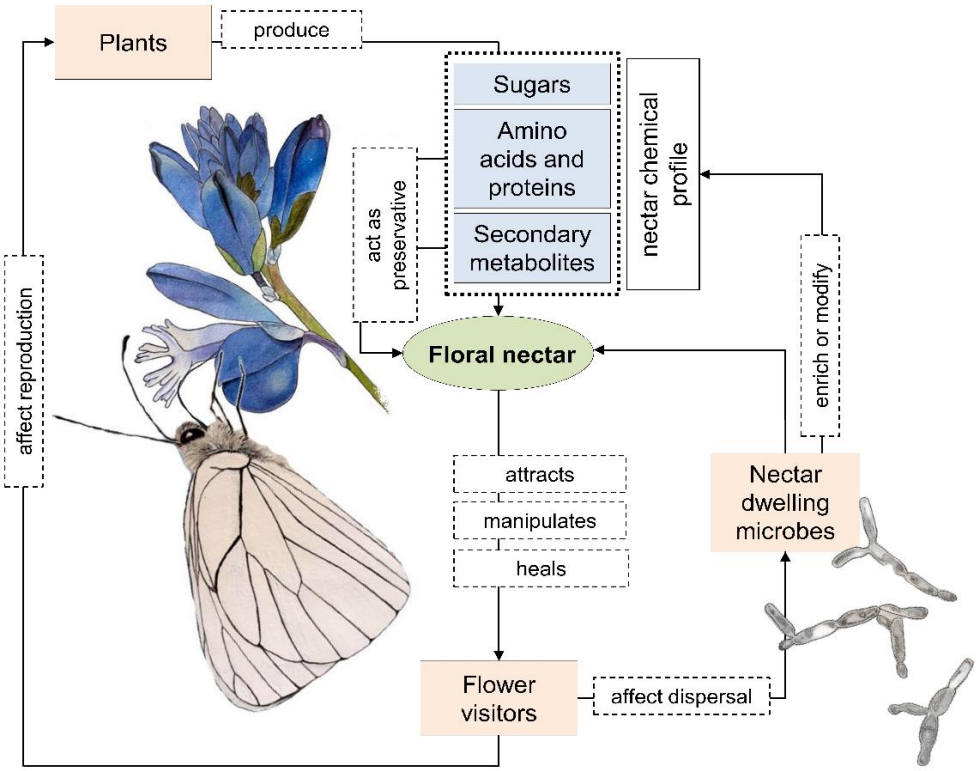


Figure 1. Network of the complex nectar-mediated plant-pollinator-microbe relationships.

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CONCLUSIONS

The work done within the framework of my PhD project contributed to draw two main general conclusions. First, when insects make use of their food-environment, they encounter and ingest low concentrations of several secondary compounds, some of them playing the role of important exogenous neurotransmitters, which can in turn appear coupled together or isolated. This work brings further evidence on how nectar-like concentrations of both amino acids and biogenic amines can influence aspects of floral visitor behavior relevant for the decision-making process implied in flower visitation, and potentially crucial for their pollination performance. The results obtained also confirm that field studies represent good opportunities to set the baselines for laboratory assessments and, sometimes, they can yield more trustable pictures of a given phenomenon, even though the results gained in such way can be difficult to interpret. This brings to light another aspect highlighted by this work: the combination of different approaches is desirable to tackle the study of such complex animal-plant-microbe interactions.

Second, reviewing the existent current and past literature over the field of nectar ecology highlighted what I see as the most urgent question to be answered to step forward in our evolutionary and ecological perspective over the role of nectar. Establishing the origin of nectar biogenic amines is crucial to confirm (or reject) the current multiple speculations on possible evolutive scenarios regarding plant-pollinator-microbe interactions.

APPENDIX STUDIES

APPENDIX 1. DATA PAPER: POLLINATOR-FLOWER INTERACTIONS IN GARDENS DURING THE COVID-19 PANDEMIC LOCKDOWN OF 2020

Noteworthy Data Sets

POLLINATOR-FLOWER INTERACTIONS IN GARDENS DURING THE COVID-19 PANDEMIC LOCKDOWN OF 2020

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Abstract

During the main COVID-19 global pandemic lockdown period of 2020 an impromptu set of pollination ecologists came together via social media and personal contacts to carry out standardised surveys of the flower visits and plants in gardens. The surveys involved 67 rural, suburban and urban gardens, of various sizes, ranging from 61.18° North in Norway to 37.96° South in Australia, resulting in a data set of 25,174 rows, with each row being a unique interaction record for that date/site/plant species, and comprising almost 47,000 visits to flowers, as well as records of flowers that were not visited by pollinators, for over 1,000 species and varieties belonging to more than 460 genera and 96 plant families. The more than 650 species of flower visitors belong to 12 orders of invertebrates and four of vertebrates. In this first publication from the project, we present a brief description of the data and make it freely available for any researchers to use in the future, the only restriction being that they cite this paper in the first instance. The data generated from these global surveys will provide scientific evidence to help us understand the role that private gardens (in urban, rural and suburban areas) can play in conserving insect pollinators and identify management actions to enhance their potential.

Keywords bees, flowers, hummingbirds, insects, pollination, species interactions

INTRODUCTION

Pollinators such as flies, bees, moths, birds, and bats are important components of ecosystems and provide crucial functions and services by facilitating the reproduction of most wild plant species and crop varieties (Klein et al. 2007, Ollerton et al. 2011, Rodger et al. 2021). However, the diversity and abundance of pollinators have declined in some parts of the world, largely driven by land use changes and agricultural intensification, with concomitant effects on seed set (Potts et al. 2010, Ollerton 2017, 2021, Millard et al. 2021). Domestic and public gardens are increasingly recognised as potential synanthropic hotspots of pollinator diversity within the matrix of human-dominated landscapes that characterises many parts of the world, and as areas that deliver multiple ecosystem services, including pollination of fruit and vegetable crops (Matteson et al. 2008, Davies et al. 2009, Owen 2010, Erenler 2013, Norfolk et al. 2013, 2014, Camps-Calvet et al. 2016, Foster et al. 2017, Bendifallah and Ortiz-Sánchez 2018, Baldock et al. 2019, Levé et al. 2019, Marín et al. 2019, Majewska and Altizer 2020, Tew et al. 2021, Prendergast 2021, Prendergast and Ollerton 2021). However, the effectiveness of gardens in supporting pollinators varies according to taxon, locality, garden management, and generalization specialization range of occurring interactions, especially in urban areas (Maruyama et al. 2019, Theodorou et al. 2020, Baldock 2020, Prendergast et al. 2022, Tew et al. 2022). To date, surveys of pollinators and their interactions with garden plants have usually been constrained in their geographical scope. This limits our understanding of the diversity of pollinators associated with gardens and how they vary globally, and our ability to answer questions such as: Do pollinators interact similarly with flowers in different parts of the world? How are different types of garden crop plants integrated within the wider network of plant-pollinator interactions? Does the role of super-generalist species such as honey bees (*Apis* spp.) vary according to region and garden type? What is the relative value of native versus non-native plant species to pollinators and how does this vary geographically? There is thus a clear need for more geographically extensive data on the relationships between pollinators and garden plants to have a better understanding of how this varies globally and to identify plant species in different regions that are important for supporting pollinators, particularly early and late in the season when little else may be in flower other than exotic garden plants. It could also help us to understand the pollinator and plant traits that distinguish garden communities from non-garden communities. Increasing our understanding of garden pollinators will help identify actions that gardeners can take to support these declining insects. During the lockdown precipitated by the COVID-19 pandemic of 2020, which limited the movement of individuals within and between countries, the lead author coordinated an ad hoc network of ecologists to collect standardised data on plant-pollinator interactions from gardens to which they had access. The purpose of this impromptu project was fivefold: (1) To take advantage of a difficult situation that would allow ecologists to focus

more time and effort into understanding the ecology of their own gardens; (2) To generate a standardised data set that could be used by researchers whose field work had been curtailed by the pandemic; (3) To help to improve the physical and mental wellbeing of those field based scientists whose access to nature was severely limited; (4) To build a data set that could be used to address unanswered scientific questions such as how the diversity of pollinators varies with garden size and geographic position, and how ornamental and food plants are used by the pollinators in home gardens; (5) To make the data freely available to give it significant future value beyond the immediate generation of research outputs, e.g. for teaching, informing extension and outreach efforts such as "best plants for pollinators", and so forth. In this initial paper from the project, we provide an overview of the data set and discuss how it may be used in the future, with encouragement for others to do so.

METHODS

While recruitment of participants was on an ad hoc basis, all had previous experience of pollinator surveys and insect and plant identification in their region. Three protocols for garden data collection were used which we refer to as Type A, B and C surveys. Individuals chose to undertake one, two, or all three types depending on their personal circumstances and time availability. Type A surveys involved regular walks at a steady pace around the garden, recording the insects and other flower visitors that were active on particular flowers (representing potential pollinators, hereafter for brevity referred to as "pollinators"). Each walk was timed and the amount of time spent surveying was proportional to the size of the garden and the number of plants in flower present. For example, in the first author's 10 m x 20 m garden he undertook 15-minute walked surveys, always following the same route one way, then returning, pausing to record data. In addition, where possible, the number of inflorescences and flowering area of all plants in bloom were estimated regularly (area in m2 and number of floral units), including both those plants that were visited and those not visited by potential pollinators. The frequency with which this occurred varied by observer but was typically whenever a change for a particular species seemed to be happening, most often weekly, or every 1-2 days during periods of rapid change if monitoring was that regular. "Floral units" varied according to taxa, from individual flowers in the case of species with large, distinct blossoms (e.g., species of Malvaceae), to dense inflorescences in the case of many smaller Lamiaceae, or inflorescences (flower heads) functioning as single blooms in species of Asteraceae. Type B surveys were based on the protocol for the UK Pollinator Monitoring Scheme (PoMS – see: https://ukpoms.org.uk/ and Carvell et al. 2016). This involved 10-minute timed observations focused on a patch of flowers belonging to one species, in an area no larger than 0.5 m x 0.5 m. The observer recorded all flower visiting insects as well as

the number of flowers each pollinator visited and the number of flowers of the target species within the 0.5 m x 0.5 m area. Type C surveys were ad hoc observations of flower visitors made outside the formal periods in which Type A and Type B surveys were undertaken. We include these data as they comprise some rare interactions that were not observed during the formal survey periods, as well as observations by individuals who were not able to complete the Type A and B protocols. Surveyors were asked to prioritise the collection of data via Type A surveys and this constitutes the majority of the data (86.9%), followed by Type B (11.8%) and Type C (1.3%). In all cases, flower visitors and plants were identified to the lowest taxonomic level possible given the observer's skill and ability, most frequently species or genus. Identification advice was provided by local experts where required, using photographs or captured specimens. There were only 17 cases where the plant could not be identified beyond family, and 3,169 where identification was only to genus. These represented just under 13% of the records in the data set. For the flower visitors, almost 70% were identified to species level and only just under 18% could not be identified to at least genus. Two of the authors (JO and JT) have corrected spellings of species names and updated the taxonomy as far as possible, using a wide range of sources for the animals and the International Plant Names Index (IPNI) (www.ipni.org) for the plants. But anyone using the data in the future is advised to check it for accuracy.

The Data set

Formal surveys took place between 16th March (day 76) and 14th October (day 288) 2020, though we also included some earlier *ad hoc* data that had been collected by participants. Data was collected by scientists from 14 countries, in gardens ranging from 61.18° North in Kaupanger, Norway to 37.96° South in Black Rock, Australia (Fig. 1). Metadata for each garden are provided and explained in Table 1 and an explanation of the elements within the data set is given in Table 2. The resulting data set comprises surveys from a total of 67 gardens, ranging in size from c. 5 m2 to 8,000 m2 in extent, and from 2 m.a.s.l to 2,655 m.a.s.l in elevation. Twenty-two of the gardens were in a rural setting, 14 in a suburban locality, and 31 were considered urban. Total observations in the gardens involved over 1,000 species and varieties of plants belonging to more than 460 genera in 96 flowering plant families. Importantly, this includes plants to which visits were not observed during the surveys, which provides important information about the relative importance of plants in different contexts. Almost 47,000 visits to the flowers of these plants were recorded, by more than 650 species of pollinators, belonging to more than 250 genera in 110 families. In total, the data set comprises 25,174 rows of data arranged in columns according to the headings shown in Table 1. In the data set, 1 row = 1 unique interaction record for that date/site/plant species, recording the flower visitor species and number of individuals or visits, or a zero-visit observation.

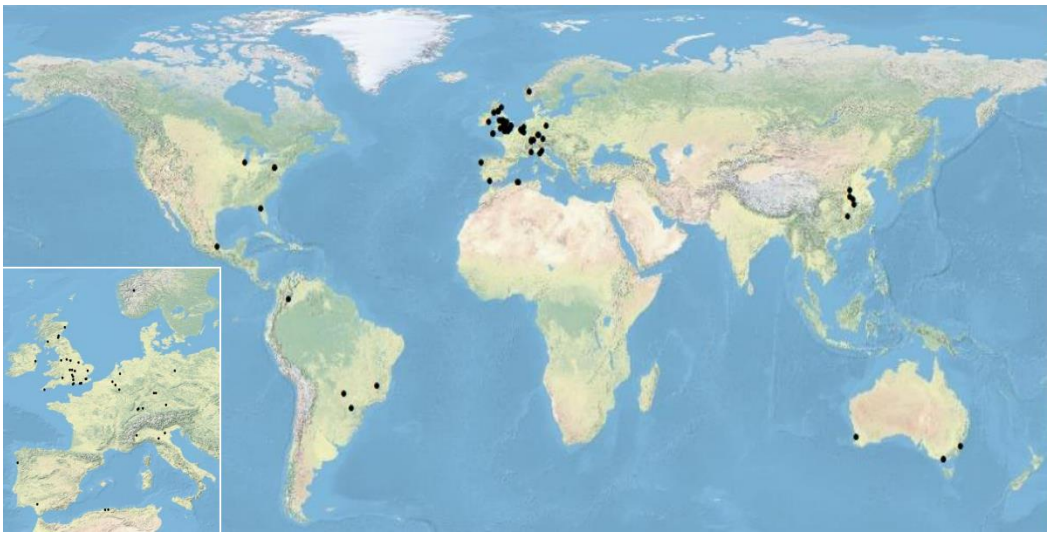

Figure 1. Locations of the gardens surveyed in this study, globally (main map) and within Europe and the Mediterranean (inset map).

Metadata item	Format	Description
Five letter identifier	Text	A code that identifies each garden
Surveyor(s)	Text	The name(s) of the individual(s) who carried out the surveys
E-mail address	Text	The latest email address of the lead individual surveyor
Locality	Text	The town, city or region where the survey took place
Country	Text	The country in which the survey took place
Latitude	Numerical	The decimalised latitude of the garden in which the survey was conducted. Accuracy is limited to two decimal places for reasons of privacy and security
Longitude	Numerical	The decimalised longitude of the garden in which the survey was conducted. Accuracy is limited to two decimal places for reasons of privacy and security
Elevation (m.a.s.l)	Numerical	The approximate elevation of the garden in which the survey was conducted in metres above sea level
Garden size (m^2)	Numerical	The approximate size of the garden in which the survey was conducted in square metres
Type	Text	The locality of the garden in relation to its surroundings. Options are “urban”, “suburban”, “rural”
Trees?	Text	The presence or absence of trees in the garden. Options are “yes” or “no”
Shrubs?	Text	The presence or absence of shrubs in the garden. Options are “yes” or “no”
Lawn?	Text	The presence or absence of a lawn in the garden. Options are “yes” or “no”
Herbaceous perennials?	Text	The presence or absence of herbaceous perennials in the garden. Options are “yes” or “no”
Compost heap(s)	Text	The presence or absence of one or more compost heaps in the garden. Options are “yes” or “no”
Age of property (years)	Numerical	The approximate age of the garden
Other relevant information	Text	Some participants included additional information about their gardens

Table 1. Explanation of the metadata for the data set. Note that where metadata are missing “NA” has been added.

Data item	Format	Description
Five letter identifier	Text	A code that identifies each garden (refer to Metadata)
Survey type	Text	Refer to text. Options are "A", "B", "C"
Date	Text	The date in 2020 on which the survey was carried out. Format is DD/MM/ (day/month/)
Day of the year	Numerical	The day of the year on which the survey was conducted, with 1st January = 1
Start time	Numerical	The time at which the survey commenced, format = 24 hour clock
Duration (min)	Numerical	The length of the survey in minutes
Plant family	Text	The taxonomic family to which the observed plant species belongs
Plant genus	Text	The taxonomic genus to which the observed plant species belongs
Plant species	Text	The taxonomic identify of the plant species observed
Plant species comments	Text	Relevant information about the plant species concerned, e.g. the variety or common name
Total floral cover (m^2)	Numerical/Text	The approximate area of flowers of that species. Values are numerical and in square metres, except for very small areas in which the "< "symbol has been used to qualify the number
Number of floral units	Numerical/Text	The approximate number of flowers or inflorescences present. In some cases this has been qualified with a "+" symbol
Flower visitor order	Text	The taxonomic order to which the observed flower visitor species belongs
Flower visitor family	Text	The taxonomic family to which the observed flower visitor species belongs
Flower visitor genus	Text	The taxonomic genus to which the observed flower visitor species belongs
Flower visitor species	Text/Numerical	The taxonomic identify of the flower visitor species observed. A zero ("0") indicates that no flower visitor was observed
Sex/caste	Text	The sex ("male", "female") or bee caste ("worker", "queen") when noted
Flower visitor species comments	Text	Some participants included additional information about the flower visitor species
Number of individual	Numerical	The number of individual flower visitors observed
Number of flowers visited	Numerical	The number of floral units on which the flower visitor foraged
Photo or specimen taken?	Text	Whether or not a physical record of the flower visitor was preserved

Table 2. Explanation of the data set. Note that for some items, where data are missing "NA" has been added.

The most frequently represented plant species that was visited by pollinators in these gardens was *Taraxacum officinale* agg. (550 records of interactions, that represented 2.5% of the plants observed). The most frequent plant family visited was Asteraceae (2,540 records, 11.6% of the plants) followed by Brassicaceae (1,663 records, 7.6% of the plants) and Boraginaceae (1,214 records, 5.6% of the plants). The pollinator-dependent crop plants within the data set include plums (*Prunus domestica*), apples (*Malus domestica*), soft fruit in the genus Rubus, Brazilian pepper (*Schinus terebinthifolia*),

coriander (*Coriandrum sativum*) and edible Brassicaceae, mainly *Raphanus* and *Brassica* spp. The phylogenetic diversity of the pollinators extended across 12 orders of invertebrates, 10 of them insects, and four orders of vertebrates. The most frequently encountered pollinators belonged to the genus *Bombus* (2,566 records, 19.5% of the pollinators) whilst the single most common species was, unsurprisingly, the ubiquitous Western honeybee (*Apis mellifera*) with a total of 1,536 records (11.7%). Although we have not categorised the plants and flower visitors as native or exotic in the region in which the gardens were surveyed, this could easily be done and would provide important insights into the role of non-native flora in supporting pollinator populations, and the potential for species such as *A. mellifera* to compete with other pollinators.

Data accessibility

The full data set is included as a CSV file with this publication as Supplementary Information 1; the metadata are included as a CSV file as Supplementary Information 2. In addition, the data and metadata are publicly available in Zenodo: https://zenodo.org/record/6342284#.Yikz_O7P2kY

DISCUSSION

This is the largest data set of garden flower visitors ever assembled and is clearly a product of the COVID-19 pandemic; as such we hope that the circumstances under which the data were collected are never repeated. The pandemic, however, provided a unique opportunity for pollinator experts from across the globe to collaborate in the collection of valuable research data. One of the positive aspects of this has been that constraints on field work have resulted in a more local focus on biodiversity that has turned up some surprising results. For example, there is at least one case in our data set of confirmation of a bee species new to a country: *Megachile nigriventris* new to Belgium, discovered by Nicolas Vereecken. Similarly, the scarce UK species *Andrena labiata* was discovered in the first author's garden, its only record in Northamptonshire in decades. Finally, a close focus on her garden in 2020 enabled Ellen Rotheray to describe the puparium and development site of the hoverfly *Rhingia rostrata* for the first time (Rotheray and Rotheray 2021). This highlights the fact that even trained ecologists are sometimes not fully aware of the species present in their immediate vicinity. This paper is the first output from the data set and more will appear in the coming years as members of the team focus on a range of questions. For example: how does garden location and structure affect the patterns that we observe; are there differences between urban versus rural gardens; what influence does garden area and landscape structure (habitat area and connectivity) have on pollinator diversity; which ornamental plant species support pollinators of food plants? Our data should also contribute to discussions about the value of native versus exotic garden plants for

pollinators (Corbet et al. 2001, Pardee and Philpott 2014, Garbuzov et al. 2014, Salisbury et al. 2015, Rollings and Goulson 2019, Giovanetti et al. 2020, Staab et al. 2020, Mata et al. 2021). With additional data gleaned from the literature it should also be possible to address questions such as: Do pollinators prefer plants of similar nutritional quality across the globe? Does the trait-matching between flower and pollinators change in different gardens or continents? There are a number of potential biases within this data set that that must be acknowledged. The first is that the gardens of pollination ecologists may not be representative of those of the wider population. However significant garden heterogeneity has been documented in other studies of garden pollinators and resources (e.g. Prendergast and Ollerton 2021, Tew et al. 2022). There were also a number of surveyors who were isolating with parents or other relatives and therefore not conducting surveys in their own gardens. In addition, a small number of the gardens were actually public spaces. We note also that during the lockdown period there was greater garden use by occupants, plus a decrease in road and air traffic, and other human activities, that might have influenced the patterns of flower visitation observed. There are further geographical biases with respect to where the participants lived. The project began as a UK-based initiative, though soon expanded as word spread, and hence there is a high proportion of data from the UK. As with most ecological studies, there is a lack of data from low-income countries, especially in the Global South, but if opportunities arise for additional surveys these could be added, and we would update the data set in Zenodo. Having said that, it's important to emphasise that the locations of the surveys do cover a wide range of climates and elevations, adjacent to a variety of biomes, in different levels of urbanisation, which makes this standardised data set a unique and valuable contribution to researchers interested in flower visitors and their nectar and pollen sources. In addition to these geographic biases, there will also be a non-random set of plants (and potentially pollinators) included within the surveys because gardeners usually choose plants for their perceived attractiveness and their climatic and edaphic tolerance of where they are planted. These in turn attract flower visitors that are able to exploit those flowers, and which may have a strong association with human settlements. However, rather than being biases per se, we would see these as interesting patterns that could be explored within the data set, for example looking at similarities in the plants and pollinators that widely different types of gardens host. Such phylogenetic patterns are not, of course, independent from geographical biases, nor are they separate from the issue of representativeness. As pollination ecologists, the participants are likely to be more aware than most of the importance of allowing "weeds" to grow that are important for pollinators, such as ragworts, dandelions, and clovers. But again, we see the future potential of comparing such gardens, in which herbicides and pesticides are infrequently or never used, with more typical gardens. The question of the representativeness, or otherwise, of our results is something that could be addressed in the future

by comparing these data with previously published studies or by repeat-surveys of some of these sites. Although we have set up working groups to consider these questions, and others, we wish to make the data set freely available to anyone who wishes to use it in their research, especially those ecologists whose data collection opportunities were curtailed by the pandemic. We ask only that this paper is cited in return. Finally, we dedicate our paper, with our grateful thanks, to all of the front-line workers, health professionals and scientists who worked hard to steer the world through one of the most difficult periods in modern times.

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APPENDIX 2. IDENTIFYING BOTTLENECKS IN THE PLANT LIFECYCLE IN EUROPE: LACK OF KNOWLEDGE HINDERS CONSERVATION ACTIONS

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Abstract

Long term survival of plant populations relies on successful reproductive cycle to obtain generation turnover. Focusing on plant species of conservation concern, we brought together a group of plant conservationists from different countries to assess whether the already available information on plant reproductive biology and autecology is adequate for identifying which phases of single species life cycle might act as bottleneck.

We compiled a list of 80 plant species of conservation concern living on cliffs and rocky slopes, for which biological and autecological information was collected from scientific literature, technical reports, and expert knowledge.

Results have shown that the available information on species reproductive biology and autecology is inadequate to identify bottlenecks in the life cycle of many species and to provide insights for the practical conservation of many more. Available knowledge is mainly referred to the flowering phase, less on seed production and much less on seedling establishment and on cloning. Meanwhile and noteworthy, flowering resulted to be the less critical phase for the fulfilment of the species life cycle.

Overall, with this perspective article we aim to encourage a constructive debate among the scientific community members and policymakers to set up novel concerted strategies for the conservation of plant species of conservation concern. The challenge of the discussion is the implementation of the current approach with new biological and ecological information to be exclusively targeted at identifying the constraints that limit the generation turnover and furnishing specific indications for active management.

Keywords

Conservation management, Plant conservation, Single species biology, Species life cycle.

INTRODUCTION

Modifications of land cover and global climate change are among the greatest human-induced threats to terrestrial biodiversity (IPBES 2019, IPCC 2007, Millennium Ecosystem Assessment, Thuiller et al. 2005, 2005a), with particularly wide consequences on the future of humankind when it comes to plant diversity loss. In addition to playing a fundamental role in sequestering nutrients (including carbon dioxide) in most ecosystems, plants shape habitats worldwide (Giam et al. 2010); their diversity ensures the survival of other living organisms (Huston 1994, Primack and Corlett 2005), guarantees human food security (Kier et al. 2005), and offers essential ecological services (Díaz et al. 2006, Hamilton and Hamilton 2006, Mace et al. 2012, Molina-Venegas et al. 2021, Pereira et al. 2010).

Worldwide, efforts to assess the extinction risk of plant species have undeniably intensified in the last decade (Bachman et al. 2018, Nic Lughadha et al. 2020, Paton and Nic Lughadha 2011); in Europe this occurred mainly as a result of the pursuance of one of the objectives of the Convention on Biological Diversity which called by 2020 for an assessment of the conservation status of all known species, as far as possible, to guide conservation actions (*Convention on Biological Diversity*, 2012). Nevertheless, to date, despite the substantial commitment of the international community to meet the objective, only approximately 10% of the plant species have been globally assessed for extinction risk and listed in the International Union for Conservation of Nature (IUCN) Red List (Nic Lughadha et al. 2020). Additionally, it is estimated that approximately 20% to 39% of plant diversity is currently at risk of extinction (Bachman et al. 2016, Bachman et al. 2018, Brummitt et al. 2015, Nic Lughadha et al. 2012, Nic Lughadha et al. 2020, Sharrock et al. 2014). Thus, it can be argued that the responses adopted by the international community to halt the loss of biodiversity have not been able to keep pace with the rate of increasing threats (Johnson et al. 2017).

So far, Europe has faced huge ecosystem changes driven by past and ongoing human activities, and it is nowadays a mosaic of semi-natural habitats and urbanised areas, with only restricted residual fragments of the original natural habitats. Further on, and as it happens in other geographical areas rich in biodiversity hotspots (Giam et al. 2010), plant species endangerment in Europe increases with habitat loss driven by anthropogenic pressure, lack of extensive traditional management practices and climatic changes (Janssen et al. 2016).

Numerous approaches using varied criteria are proposed for different biodiversity conservation purposes. Among others, prioritization and species-based indicator systems for plant conservation planning are proposed to serve as sources for decision-makers to achieve defensible biodiversity investment decisions (e.g. Arponen 2012, Erdős et al. 2022, Kricsfalusy and Trevisan 2014, Liu et al. 2019). As conservation occurs under time and resource constraints, conservationists consider

impossible to assist all species of conservation concern. However, though being the foundation of many methods for determining factors responsible for species conservation (e.g. Farnsworth 2007, Gabrielová et al. 2013, Kunin and Gaston 1993, Kunin and Shmida 1997, Murray et al. 2002, Pilgrim et al. 2004), the approach based on single species conservation is widely regarded as unaffordable in terms of scientific effort, time, and financial commitment (e.g. Cook et al. 2010, Frankel et al. 1995, Heywood 2015). Nevertheless, in a long-term perspective, biodiversity conservation by means of reproductive success and generation turnover is necessary for the survival of any species (even those with high longevity of single individuals) and the maintenance of any community. Considering that it is not possible to study the biology and ecology of all species, one approach could be that of identifying and addressing the phases in the life cycle of species that limit and/or prevent generation turnover.

In 2018, a group of European plant conservation scientists and other stakeholders established the network entitled *ConservePlants: An integrated approach to conservation of threatened plants for the 21st Century* (COST Action 18201). Considering that the knowledge about the biology of the rarest and most threatened European plant species is limited, this network aimed at improving approaches and methods to protect plant species of conservation concern in Europe from further degradation and extinction (Fišer et al. 2021). Activities in the network were guided on a few key considerations including that the conservation of plant species is based on the conservation of their populations. The number and size of populations influence the probability of extinction of a species. A species with many large populations is less likely to be threatened with extinction than a species with few small populations (Mathies et al. 2004). Plant species of conservation concern, however, are by definition characterised by few small populations that are vulnerable to the combined effects of loss of genetic variability, inbreeding depression, Allee effects, environmental stochasticity and demographic stochasticity (Oostermeijer et al. 2003), which hinder the ability of plant species to successfully undergo generation turnover (Spielman et al. 2004) as bottlenecks occur in their life cycle. A bottleneck in a plant's life cycle can be defined as the inability of individual plants in a population to complete their generation turnover due to constraints at a particular stage in their life cycle (Aronne 2017).

Limited information available about plant species of conservation concern and scarce use of the available data from genetic conservation research were detected as weaknesses for management plans (Salmerón-Sánchez et al. 2021). Inadequate knowledge in biological and/or ecological constraints that prevent generation turnover of species of conservation concern is one of the most important causes of failure in conservation actions (e.g. Kyrkjeeide et al. 2021). One objective of the *ConservePlants* COST Action was therefore to discuss and test possible applications of a species-

based methodological approach to identify bottlenecks in the life cycle of plant species called SHARP (Systematic Hazard Analysis of Rare-Endangered Plants) (Aronne 2017).

The approach of SHARP is based on three phases. A preliminary phase (STEP 0), which consists of collecting all available information on the species reported in scientific articles, technical reports or personal knowledge (Aronne 2017). A first phase of investigation (STEP 1), based on field surveys, aiming to identify which stage in the life cycle of the species presents bottleneck. This will narrow and prioritize further attention on species constraints and is achieved by answering the following questions: (a) Do plants flower? (b) Are seeds produced? (c) Does seedling recruitment occur? (d) Does cloning occur? A final phase (STEP 2), based on laboratory and field experiments, carried out by scientists with ad hoc expertise and aimed at clarifying the causes of the life-cycle bottlenecks and propose possible solutions.

At first sight, information related to bottlenecks in the life cycle of plant species of conservation concern might be considered as already available to any stakeholder involved in species conservation. Indeed, the evaluation of the conservation concerns and further statement of the species conservation status must have been based on some biological/ecological information on the single species of conservation concern. Nevertheless, to the best of our knowledge, it has not yet emerged that available information on the reproductive biology and autecology of plant species of conservation concern is adequate to provide suggestions for executive actions.

We shared the opinion that the current European approach of plant conservation would be much improved by adding a species-based conservation approach aimed at providing information on the life cycle bottlenecks that might constrain generation turnover of the plant species of conservation concern.

During the meetings of the *ConservePlants* COST Action, we have long discussed if this information was already available or not. We realized that most of the statements were based on personal opinions and therefore decided to address the issue using available data from a list of objectively selected species, report the results in this perspective article and expand the discussion within the community of the plant conservationists.

More specifically, we decided to develop the current work within the SHARP framework, and we aimed at verifying if the already available information on European species of conservation concern can be sufficient to identify which phase of the life cycle acts as bottleneck, therefore contributing to species regression. We considered that if this was to occur it could be possible to skip the investigative stage of SHARP and go directly to identify the causes of the life-cycle bottlenecks and elaborate suggestions for conservation actions. To achieve this goal, we focused on a list of species of conservation concern objectively assembled, and analyzed the available information on their

reproductive biology and autecology. The final aim was to discuss whether (and to what extent) the available knowledge can be considered sufficient to identify biological and autecological constraints for the generation turnover and to gain insights into management actions.

MATERIALS AND METHODS

We focused on plants of cliffs and rocky slopes as these habitats host many phylogenetic relicts and rare plant species (Davis 1951, Van der Maarel and van der Maarel-Versluys 1996, Cooper 1997, Soriano et al. 2012, Mifsud 2013, Carta et al. 2019). Indeed, coastal and inland cliffs are described as climatic refugia because they shelter large endemic floras in most unglaciated areas of the world and large relict floras in areas where significant glaciation has occurred (Cooper 1997, Davis 1951, Keppler et al. 2012, Larson et al. 2000). In addition, compared to other habitats (e.g. coastal dunes, semi-natural grasslands, etc.), cliffs and rocky slopes are less affected by human drivers of species extinction (Janssen et al. 2016), which makes them ideal habitats to assess whether species are of conservation concern due to bottlenecks in their life cycle.

The collection of data was made in two consecutive phases: the first aimed at establishing a list of plant species of conservation concern among those living on cliffs and rocky slopes in Europe; the second aimed at building a data matrix on the biological and ecological knowledge that is available and potentially usable to suggest actions for species management. Information was collected in 10 countries (Table 1) spamming all Europe.

Country	Number of species
Croatia	6
Estonia	5
Greece	16
Italy	15
Malta	2
Norway	1
Poland	13
Portugal	18
Serbia	4
Slovenia	6

Table 1 Number of species considered by each country involved in this study.

List of species with conservation concerns living on cliffs and rocky slopes

To compile the list of species of conservation concern living on European cliffs and rocky slopes, we used the official database of Natura 2000 reporting activities for the period 2013-2018 (https://www.eea.europa.eu/data-and-maps/data/article-17-database-habitats-directive-92-43-eec-

2/article-17-2020-dataset/article-17-2020-dataset-microsoft-access-format), hereafter Article 17 Habitats Directive database. Focusing on vascular plants, we applied a query to select all the species with Unfavourable conservation status (U1-unfavourable inadequate or U2-unfauvorable bad, according to Evans and Arvela, 2011) in at least one of the biogeographical regions of the European Union. After removing pteridophytes, the resulting list was exported into a Microsoft Excel worksheet. At the end of this preliminary activity, the spreadsheet encompassed 442 species corresponding to 680 rows because several species occurred in more than one country.

At this point, we examined each species and check marked those living on cliffs and rocky slopes in the geographical area of our expertise. In the cases of countries where the number of species was lower than five, local contributors added to the list species not reported as Unfavourable in the annexes of the Habitats Directive or assessed as threatened with extinction under IUCN protocol at regional level (country).

For each species, the following data from Article 17 Habitats Directive database were reported in separate columns: name of species, ID code, country, annex of Habitats Directive, priority, conclusion assessment. The IUCN threat category was indicated for those species that were not listed in the Habitats Directive. Additionally, we also included information on lifeform, endemic status (according to Melendo et al. 2003, Peruzzi et al. 2014, Petrova and Vladimirov 2010, Piekoś-Mirkowa and Mirek 2003), habitat type (coastal or internal) and type of substrate (calcareous or siliceous).

Matrix of species bottlenecks

The worksheet with the list of species and initial data described above was used as the starting point to build up a matrix containing available information on species reproductive biology and autecology to be subsequently used to identify possible life cycle bottlenecks.

Contributors filled in the worksheet the required information regarding the species of their country. Specifically, four columns were used to report the four main questions as in STEP 1 in the SHARP approach (Aronne 2017): 1) Do plants flower? 2) Are seeds produced? 3) Does seedling recruitment occur? 4) Is cloning highly frequent? Based on the information available for each species, the contributor was allowed to answer the questions with YES/NO/Not Available information. In addition, information on Data Source and Source Reference, was to be given for each of the four questions. Specifically, to compile the columns Data Source, contributors could choose among four different optional Source Types: ST1) Scientific publications on species reproductive biology and autecology and data sheets for the national Red Lists; ST2) Scientific publications on systematic and/or taxonomic revisions of plants, national floras, Master or PhD theses, technical reports (Natura 2000, LIFE projects), other monitoring project reports; ST3) Personal knowledge; ST4) Not

Available information. In the four columns of Source References, the contributors reported details of the citation of the main source of information used to answer the corresponding SHARP question. Finally, an additional column was added to summarize the contributor's opinion on the adequacy of the available information to define the bottleneck in the generation turnover of each species and provide insights for conservation actions. Specifically, the question in the column header was: Is the available information sufficient to determine the critical phase of the species life cycle? To this end, the contributor was allowed to provide a YES/NO answer.

Data analysis

In addition to descriptive results of all information compiled in the matrix, we used two main approaches to analyse the data. First, we investigated if the four questions were associated with response (Yes, No) and if the four questions were associated with data source type (ST1, ST2, ST3, and ST4). We used R (R Core Team 2022) to perform two distinct Chi-square tests of independence with simulated p-value (based on 9999 randomizations) with Bonferroni-adjusted post hoc tests in case of significance of the Chi-square tests (*chisq.posthoc.test* function, *chisq.posthoc.test R package*; Agresti 2007, Beasley and Schumacker 1995). Considering that the Chi-Square test of independence is used to determine whether a significant association exists between two nominal (categorical) variables (McHugh 2013), in the present study, we compared the frequency of each data source type and each response option with the four questions. When addressing the association between data source types and different questions we considered all available information, whereas when considering the association between the response options and the different questions we omitted the cases where no data were available.

Secondly, we wanted to highlight the presence of groups of species sharing the same answers regarding life cycle bottlenecks. To this end, a hierarchical classification was performed. The original nominal variables (life cycle questions) were transformed in a dummy form. In the new raw matrix, each variable (e.g. Are seeds produced?) associated with the three possible values (YES, NOT, Not Available), was split into three final variables (seed produced YES, seed produced NO and seed produced Not Available), each with only two possible answers: 1 = true and 0 = false. The final raw matrix resulted as a matrix of 12 variables containing only presence/absence data.

To evaluate (dis)similarity between records, the qualitative Jaccard index (Jaccard, 1912) was used; the A complete linkage agglomerative method was used in the classification and this was subsequently represented as a dendrogram. For the hierarchical classifications, we used XLSTAT (2017) by Addinsoft.

We used the results from the hierarchical classification to evaluate whether plant species with different levels of conservation concern were associated with different clusters. We defined the level of conservation concern of species by dividing them into 'endemic' and 'non-endemic', as well as 'priority' (as defined by the Habitats Directive) and 'non-priority'. We have considered endemic species as species with relevant conservation concern because of their restricted range, while priority species are those for which the European Union has specific conservation responsibility in view of the proportion of their natural range which falls within the territory (Habitats Directive). In addition, we assessed whether the response option (YES or NO) to the question "Is the available information adequate to determine the bottleneck in each species generation turnover?" was associated with different clusters. For these analyses, three Chi-square tests of independence with simulated p-value (based on 9999 randomizations) were performed (viz. for endemic/non-endemic species, priority/non-priority species, and response options) with Bonferroni-adjusted post hoc tests in case of significance of the Chi-square tests (function *chisq.posthoc.test*, package *chisq.posthoc.test*; Agresti 2007, Beasley and Schumacker 1995).

RESULTS

At the end of the species filtering process, 80 species living on cliffs and rocky slopes were found and included in our data matrix (Appendix A). Among them, 60 are also reported in annexes of the Habitats Directive (46 in Annex II and Annex IV, among which 21 as priority species; nine of Annex IV; five of Annex V). Most of them (56) are species whose conservation status is classified *Unfavourable*, while among the species added by contributors, three are of *Unknown* conservation status and only one is considered as *Favourable* according to Article 17 Habitats Directive database. Nineteen species were added by contributors as included in the national Red Lists of their country, classified as *Threatened with extinction* (seven as CR-*Critically Endangered*, 10 as EN-*Endangered*, two as VU-*Vulnerable*). Finally, the species *Aquilegia iulia* was also included in the list; although not yet processed according to the Red-listing protocol, this species was recently split from *Aquilegia bertolonii* (Nardi, 2011) and not yet been proposed for inclusion in the Annexes of the Habitats Directive. Of the total of 80 species, 64 (80%) are endemic.

Data on the lifeform spectrum highlighted the prevalence of perennial species, including herbaceous plants (hemicryptophytes, 63%; geophytes, 2%; hydrophytes, 1%), bushes (chamaephytes, 26%), and shrubs/trees (phanerophytes, 4%). Only a few species were annuals (therophytes, 4%).

Data on the type of cliffs showed that 52 species (65%) live on internal cliff and rocky slopes, while 23 (28.8%) live on coastal habitats, and only five (6.2%) are not linked to any of the two types.

Among the selected species, 58 (72.5%) are associated with calcareous substrates, 17 plant species (21.3%) with siliceous substrates, and only five (6.2%) with both types.

The total number of 80 study species was not equally distributed among the ten countries and ranged between one and 18 (Table 1). Five species were recorded in more than one country, namely *Arabis scopoliana*, *Cerastium dinaricum*, *Genista holopetala*, *Moehringia tommasinii*, and *Ramonda serbica*. Only the latter species was reported for two countries with different information, specifically on cloning occurrence and source types. Consequently, this species was entered twice in the data matrix to be used for further analyses, thus resulting in 81 records referred to 80 species (Appendix A).

Overall, data analysis on plant life cycle showed that contributors could retrieve information on all plant species for at least one of the four questions. Specifically, of the 324 total questions (4 questions x 81 entries), 219 (68%) got answered, while the rest 105 (32%) remained uninformed. However, the quantity of available information differed among the four questions (Figure 1).

The results showed that flowering did not constitute a bottleneck except for one species (*Athamanta cortiana*). Information on seed production was retrieved for only 69 records (85.2%). Seeds were reported to be produced by all the species with no bottleneck in flowering. Therefore, there is evidence that seed production did not constitute a bottleneck for the species reproduction. Information on seedling recruitment was available in less than half of the records (39; 48.1%) and documented that seedling recruitment did not occur in 6 (15.4%) of the informed cases. The least available information was on clonality (only 37.0% of the records were informed with a positive or a negative answer), and the results showed that cloning occurred in only 36.7% of the informed cases (Figure 1).

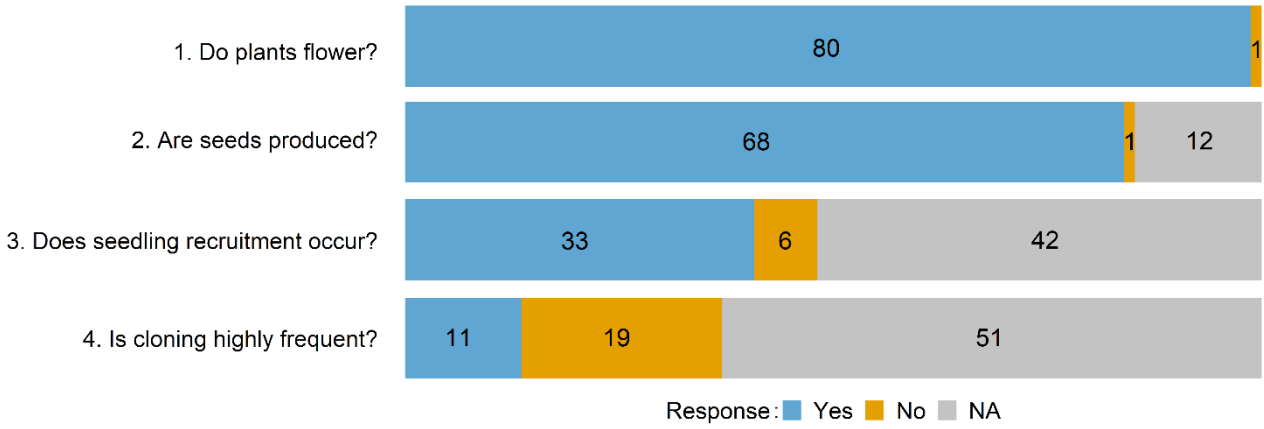

Fig. 1. Distribution of the three possible answers to the four questions regarding the SHARP approach for all the study records. NA: Not Available information.

Further analysis of the data on the plant species for which information was available showed the occurrence of significant associations between the four questions and the YES or NO answers (χ^2 = 89.321, df = 3, $p < 0.001$; n = 219). Specifically, results of the Chi-square tests highlighted that positive answers were associated with questions on flowering and seed production more frequently than expected (Table 2), as were negative answers to the question on cloning (Table 2). Therefore, for species with available information, results revealed that flowering and seed production successfully occur in the majority of plant species. Conversely, cloning is absent in most of the species. No significant association could be observed between response options and the question on seedling recruitment (Table 2). Consequently, it is not possible to deduce whether this phase is a bottleneck in the life cycle of the species.

Question	Yes	No
Do plants flower?	***80*** ***	*1* ***
Are seeds produced?	***68*** **	*1* **
Does seedling recruitment occur?	33 ns	6 ns
Does cloning occur?	*11* ***	***19*** ***

Abbreviations: ns not significant, ** $p < 0.01$, *** $p < 0.001$.

Table 2 Distribution of the records for which information was available to answer the four questions regarding life cycle. Results of the Chi-square tests on the number of plant species according to the YES or NO answers highlight whether there exists a significant association between each of the four questions and the two response options (YES or NO). Associations more than expected are reported in italic and bold, those less than expected in italic.

Results on the types of data sources used to compile information on species life cycle (Appendix B), documented that data were mainly collected from scientific publications and data sheets of the National Red Lists (ST1); this source type informed 151 of the 324 questions (40%). Scientific publications on systematic and/or taxonomic revisions of plants, national floras, Master and PhD theses, technical reports (Natura 2000, LIFE projects), other reports on monitoring projects (ST2), and personal knowledge (ST3) were used to answer 48 and 40 questions (i.e., 15% and 12% of the questions, respectively) (Table 3).

Noteworthy, results of the Chi-square tests highlighted that different source types were significantly associated with different questions (χ^2 = 107.19; df = 9; p < 0.001; n = 324). We found associations of questions on flowering and seed production with scientific publications and data sheets of the National Red Lists (ST1) to be more frequent than expected (Table 3). No associations were detected between questions on seedling recruitment and clonality with scientific publications and data sheets of the National Red Lists (ST1) (Table 3). For questions on seedling recruitment and clonality, absence of data sources occurred more frequently than expected, while questions on flowering and

seed production were associated with the absence of data sources less frequently than expected (Table 3). Therefore, most of the information was available for questions on possible bottlenecks at the phase of flowering or of seed production, and this information was obtained from scientific publications and data sheets of the National Red Lists (ST1), while no significant association was found between the four questions and the other two types of sources (ST2 and ST3) (Table 3). Overall, data highlighted that the scientific publications on plant reproductive biology and ecology refer mainly to flowering and seed production, while studies are less focused on other phases of the life cycle.

Results of the preliminary hierarchical classification performed on variables of the whole set of records (n = 81) highlighted the separation of *Athamanta cortiana* (the only species for which the absence of flowering was indicated as bottleneck) from all other records. According to these results, we considered this species as an outlier and therefore excluded it from subsequent classifications. In addition, we also excluded from further classifications the four variables with no variability in the data matrix (Flowering YES, Flowering NO, Flowering Not Available, Seeds NO).

Question	**ST1**	**ST2**	**ST3**	**ST4**
Do plants flower?	***51*** ***	18 ns	12 ns	*0* ***
Are seeds produced?	***48*** ***	11 ns	10 ns	*12* **
Does seedling recruitment occur?	*18* **	12 ns	9 ns	***42*** ***
Does cloning occur?	*14* ***	7 ns	9 ns	***51*** ***

Abbreviations: ST1- Scientific publications and data sheets for the national Red Lists; ST2- Scientific publications on systematic and/or taxonomic revisions of plants, national floras, Master and PhD theses, technical reports (Natura 2000, LIFE projects), and other monitoring project reports; ST3- Personal knowledge; ST4- Not Available information; ns: not significant, ** $p < 0.01$, *** $p < 0.001$.

Table 3 Types of Reference Sources distributed among the four questions on the life cycle. For each question, the number of records divided by the type of resource used to obtain information is provided. Associations more than expected are reported in italic and bold, those less than expected in italic.

The main hierarchical classification based on a data matrix of 80 records (species) and eight variables (four questions and their data source) considered the records as objects and produced a dendrogram where the species were grouped in two well separated clusters of similar size (Figure 2). The first cluster (Cluster 1) included 41 records (51%) and the second cluster (Cluster 2) included the other 39 (49%).

The subsequent hierarchical classification considered the eight variables as objects and highlighted the main differences between records grouped in the two clusters. The two groups differed mainly in terms of Available/Not Available information (Table 4). In Cluster 1, the total number of records reporting presence of information on species' life cycle was 105 (76.1% of the total matching) while

in Cluster 2 it was 33 (23.9% of the total matching). Conversely, records with Not Available information, were mainly found in Cluster 2 (82.3% compared to 17.6% in Cluster 1).

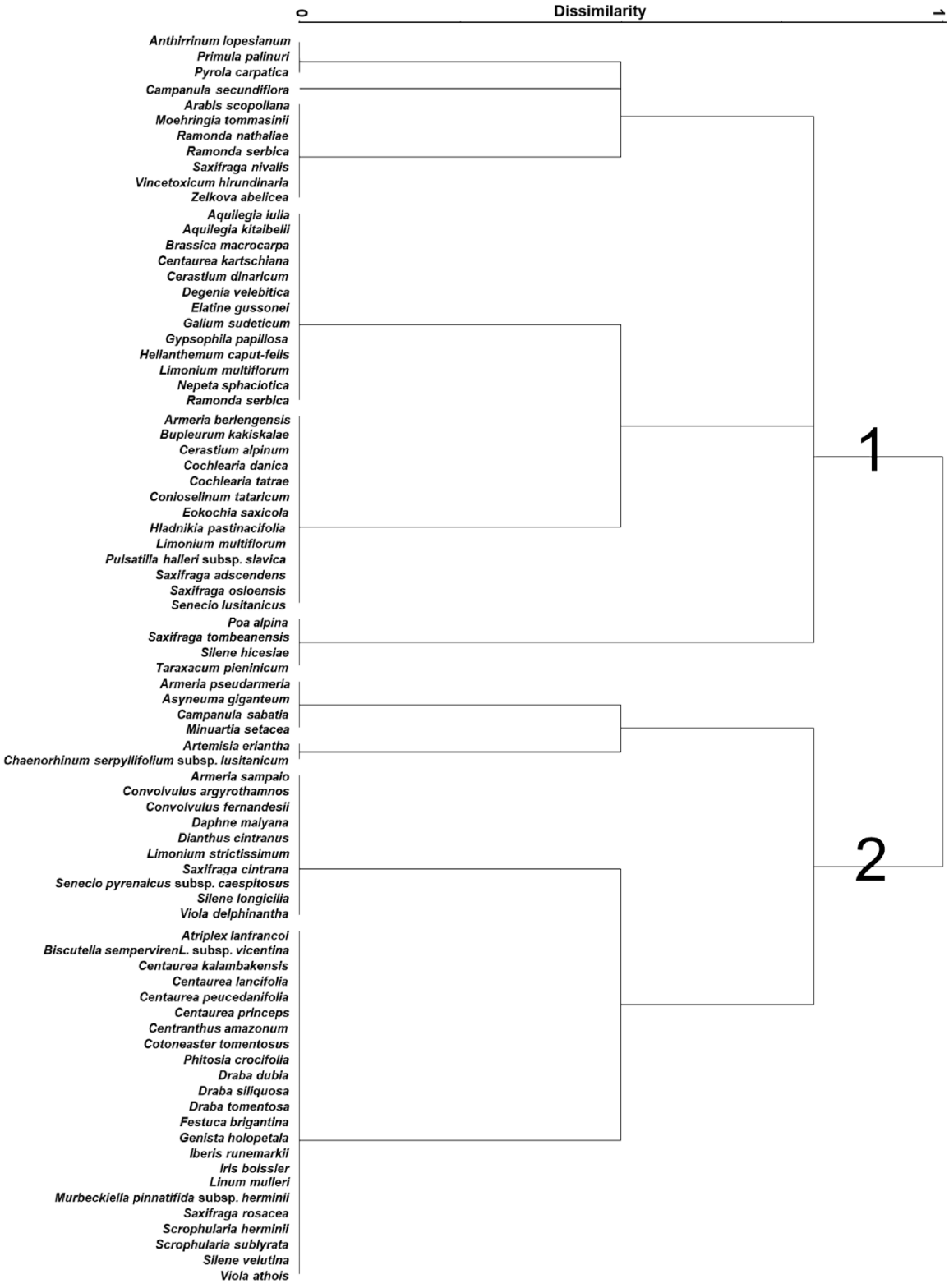

Fig. 2. Dendrogram resulting from hierarchical classification (Jaccard qualitative dissimilarity index and Complete linkage agglomerative method) performed on the raw matrix of 80 records and eight dummy variables.

A more detailed analysis of the cases where information on life cycle was available showed that 100% of records (n = 41) in Cluster 1 consisted of species capable of producing seeds, while this occurred only in 27 records (69.2%) in Cluster 2. Another evident difference between the two clusters regarded the seedling data: in Cluster 1, seedling occurrence was reported for most species (n = 33) and not occurring in only seven. Conversely, for records of Cluster 2, no data were available on occurrence. Concerning clonality, records of Cluster 1 were quite uniformly distributed between presence (n = 11) and absence (n = 13) of cloning occurrence. For Cluster 2 only six records reported absence of clonality, and for the remaining records, no information was available. Overall, results showed that Seed production was the second less critical phase in the species life cycle after Flowering which was reported as normally occurring (and not critical) for all analysed species, except for *Athamanta cortiana*.

Focusing on the records with Not Available information (Table 4), results showed that information on seed production was Not Available for all the cases in Cluster 2 (n = 12), and in none of Cluster 1. Regarding seedlings, 39 records with Not Available information (97.5% of total matching) were found in Cluster 2 and only one in Cluster 1. These results highlighted the occurrence of two critical points in identifying the life cycle bottlenecks: absence of information on seed production and on seedling occurrence for half of the considered species. Regarding clonality, the number of records reporting Not Available information were substantially higher in Cluster 2 (33; 66.0% of total matching) than in Cluster 1 (17; 34.0% of total matching). It is interesting to note that, independently of cluster separation, information on clonality was lacking for 49.0% of the total analysed records, whereas information on seedling recruitment was lacking for 39.1%. These results demonstrate that clonality and seedling occurrence are the life cycle phases less investigated by researchers.

A further analysis of the data aimed at investigating if the species with more available information (Cluster 1) were those with highest levels of conservation concern rejected this hypothesis. No significant relationship was found between endemic and non-endemic species as well as between priority and non-priority species and the two clusters (endemic/non-endemic species; $\chi^2 = 0.04$; df = 1; $p = 0.823$; n = 79; priority/non-priority species; $\chi^2 = 0.33$; df = 1; p = 0.563; n = 79). The endemic and priority species were distributed across both groups (Figure 3) suggesting that knowledge is not disproportionately focused on plant species with different levels of conservation concern.

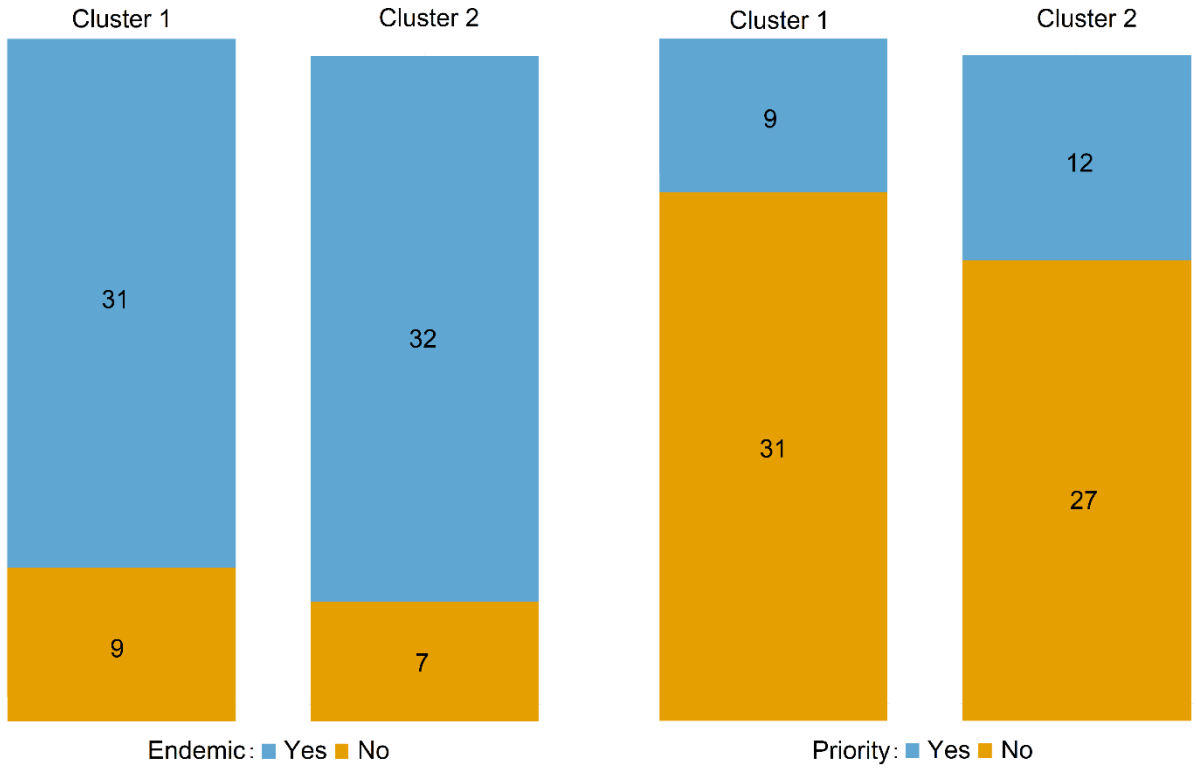

Fig. 3. Number of endemic/non-endemic and priority/non-priority species in Cluster 1 and Cluster 2.

	Available information						Not available information			
	Seeds YES	**Seedlings YES**	**Cloning NO**	**Seedlings NO**	**Cloning YES**	**Total records**	**Seeds NA**	**Seedlings NA**	**Cloning NA**	**Total records**
Cluster 1 (n = 41)	41	33	13	7	11	**105**	0	1	17	**18**
Cluster 2 (n = 39)	27	0	6	0	0	**33**	12	39	33	**84**
Total of matching	68	33	19	7	11	**138**	12	40	50	**102**

Table 4 Number of records and number of matchings resulting for each variable used in the hierarchical classification. Variables are reported following the sequence resulting from the hierarchical classification that used variables as objects. NA: Not Available information.

Finally, we have analysed the replies to the question: Is the available information sufficient to clearly define the bottleneck in the generation turnover of the single species and provide insights for executive actions? Data were considered inadequate to determine the critical phase of the species life cycle for the great majority of the species in the database (n = 67, 83.7%). The frequency of Yes/No answer differed significantly between the clusters (χ^2 = 12.52; df = 1; p < 0.001; n = 80; Figure 4). For the group of the best studied species (Cluster 1), the contributors considered that available information was adequate to define the bottleneck in only one third of the species (13 out of a total

of 41). For species with more lack of information (Cluster 2), the available information was always considered inadequate to allow the identification of the bottleneck in the generation turnover and to provide insights for executive actions.

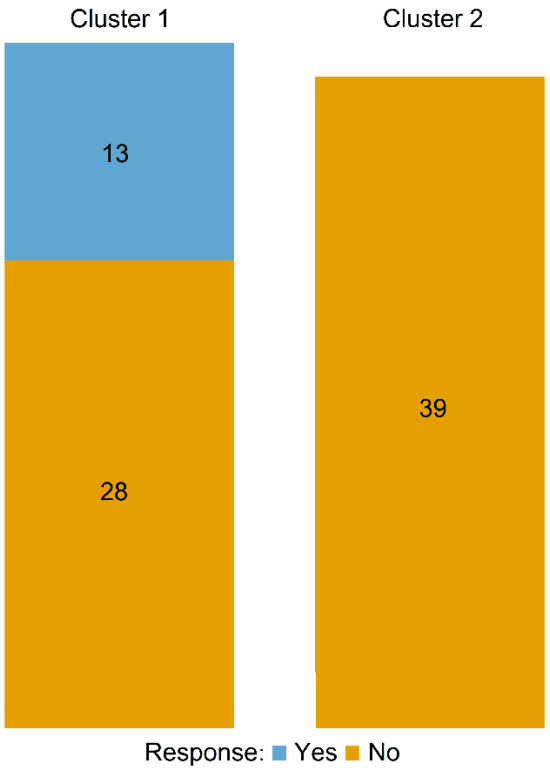

Figure 4. Number of species in Cluster 1 and Cluster 2 whose information on species reproductive biology and autecology was considered adequate/inadequate to determine the critical phase of the species life cycle.

DISCUSSION

In this perspective article we addressed the issue of the lack of knowledge for guiding plant conservation management. Results from our survey on the available information on the reproductive biology and autecology of plant species of conservation concern supported the hypothesis that current knowledge is not sufficient to identify the phase of the life cycle where bottlenecks occur in many species of conservation concern. Moreover, data remarked that even when the critical phase was identified, the available knowledge was not helpful to define management suggestions. Such conclusions may sound as unsurprising to many conservationists but are now based on data of a dedicated survey.

In this study, we used a systematic approach (derived from the SHARP approach) to identify knowledge gaps on species life cycles limiting the implementation of effective management actions.

As expected, part of the information requested to check the successful occurrence of the four phases of the species life cycle according to the SHARP approach (Aronne 2017) resulted to be reachable by reviewing scientific literature or by consulting alternative publications and sources such as, for example, floras or technical reports. For all species in our dataset, it was possible – although to varying degrees – to recover information about their reproductive biology and autecology. Indeed, contributors were able to answer at least one of the four questions for all study species, so that more than half of the questions got answered during the process.

Notwithstanding such results, our investigation has also highlighted a series of critical points. Firstly, available information mainly focused on a few phases of the life cycle, with less documentation of other crucial phases. Our results showed, for instance, that most of the available information focused on flowering. Secondly, the analyses revealed that flowering, although gaining most of the scientists' interest, rarely represents a bottleneck in the fulfilment of the life cycle of the species from cliffs and rocky slopes. Of all the species considered, in fact, only for *Athamanta cortiana* flowering was indicated as a bottleneck, even though the information available was considered inadequate to identify the causes of such a criticality. Thirdly, while proceeding down through the flow of the reproductive process (flowering - seed production - seedling recruitment), the percentage of species with available information decreased. Even though clonality must be considered separately from the other life phases (Aronne 2017), seedling recruitment and clonality resulted the less investigated life cycle phases. However, results highlighted that for the species for which this information was available, local lack of seedling recruitment could be considered as the bottleneck phase for the long-term survival of the species. This could be particularly relevant in the current scenario of global climate changes (e.g. Aronne et al. 2014).

Moreover, when it came to investigating the type of information source, our study revealed that scientific publications and data sheets for the National Red Lists (the best Source Type in terms of information on species reproductive biology and autecology) provided most of such information, but they mainly focused on the flowering and seed production phases. Information on the phases that turned out to be the most critical for the fulfilment of the species life cycle came from other types of publications and/or was based on personal knowledge.

Taken together, our results on the plant species of conservation concern deserving compulsory conservation actions by the European Union, revealed that the already available information on their reproductive biology and autecology paradoxically focuses on the less critical phases of the processes which underpin their long-term survival. Moreover, results also showed that most of the high-quality information is restricted to such phases, whilst missing for the most susceptible ones (for which also low-quality information is missing).

We also considered that, even in the best cases where the information on the reproductive biology and autecology of the species is available and is based on high quality sources, the difficulty to define a bottleneck depends on the fact that those studies were carried out to achieve specific research goals, generally diverging from the identification of the criticalities that might lead to species vulnerability. Remarkably, our results rejected the hypothesis that in a scenario of lack of information on the reproductive biology and autecology of species with conservation concerns, the most (and from the best source type) knowledge was focused mainly on the endemic and priority species. Indeed, we did not find a significantly higher number of endemic and priority species associated with the group characterized by having the most available information. In other words, unless specifically committed, researchers are inclined to choose the study species according to scientific criteria and not to conservation priorities.

What is also worth pointing out is that even in those cases where a bottleneck was identifiable through the already available information from scientific literature or other sources, the causes of the constraint were not necessarily made clear by the achieved knowledge. The final question that contributors were asked on whether they judged the available information as sufficient for the identification of bottlenecks, and for the setting up of specific conservation actions, produced remarkable results. Indeed, for the great majority of the species (83.7%) the answer provided by the experts was negative. Much of the available information, in fact, came from studies which lack a direct management conservation approach. This highlights that many scientific studies aim at advancing with new discoveries and are not necessarily directed to the development of practical strategies to counteract species loss or to develop appropriated conservation measures. In this scenario, to have obtained an answer to the questions on the main phases of the life cycle can be considered, in some way, as fortuitous. Consequently, specific research activities must be planned and commissioned to find out the life cycle bottlenecks with the main goal to develop and suggest feasible solutions that can maintain or restore the populations of a species to a favourable conservation status, as requested by the Habitats Directive. These results altogether are a confirmation that, even after the identification of the bottleneck, more investigations aimed at clarifying the issue and proposing practical action for species conservation are needed. The necessity of such in-depth study reiterates what is reported as the final step of the SHARP approach (Aronne 2017). The Habitats Directive commits each Member State to absorb and implement in their legislation the European indications for the protection of nature by adopting a conservation approach oriented to conserve habitats and thirty years after the Habitats Directive was issued, fundamental knowledge of the life cycle bottlenecks that drive plant vulnerability must be implemented involving much more plant species, as shown by our results.

Our study was based on species with conservation concerns of the European cliffs and rocky slopes. Specific peculiarities of this habitat (including verticality and inaccessibility) might have limited the number of studies and resulting information on the reproductive biology and autecology of the single species; therefore, the scarcity of information might be less critical for species of other habitats in the Habitats Directive (but see e.g. Kyrkjeeide et al. 2021). Nevertheless, the overall knowledge on the life cycle bottlenecks of plant species of conservation concern of cliffs and rocky slopes is alarmingly insufficient to identify the causes of decline and suggest actions for species management. This knowledge is particularly relevant for plant species of cliffs and rocky slopes to predict their long-term survival and possible migration to northern latitudes and higher altitudes, which is expected as an effect of global warming. Most importantly, our work highlighted that even the species-based approach, if intended as any study on the biology and autecology of the species and specifically aimed at overcoming the life cycle bottlenecks, raises the risk of resulting insufficient for the setting up of conservation actions when the focus of the research is not directed to conservation management interventions. Our data on the species of which conservation is required and codified by the Habitats Directive, showed that the already available information on European species of conservation concern is not sufficient to identify the bottlenecks in the life cycle that cause species regression. We claim that the species-based approach is crucial for identifying concrete actions for the conservation of plant species of conservation concern, but the new knowledge on the species must address the bottlenecks in the life cycle that, limiting the generation turnover, might cause species regression. We also remark that future research activities should have an applied focus and that plant conservation would greatly benefit from the adoption of agreed protocols specifically designed for reaching feasible solutions by the side of all possible stakeholders and nature managers.

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SUPPLEMENTARY MATERIAL

SM TO PROLINE AND B-ALANINE INFLUENCE BUMBLEBEE NECTAR CONSUMPTION IN DIFFERENT WAYS WITHOUT AFFECTING SURVIVAL

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Table S1. LMM coefficients for the effect of treatment (i.e., amino acid type), bumblebee weight, and their interaction on the log-transformed solution consumption by bumblebees. Results are given on the log scale. Control solution (i.e., only sugars) is set as intercept.

Variable	**Estimate**	**SE**	**t-value**
(Intercept)	0.616	0.325	1.891
TreatmentB1	−1.706	0.397	−4.293
TreatmentB2	−1.146	0.425	−2.698
TreatmentP1	−1.642	0.441	−3.726
TreatmentP2	−0.605	0.456	−1.327
TreatmentPB1	−1.194	0.435	−2.747
TreatmentPB2	−1.481	0.417	−3.553
log(Weight)	1.335	0.203	6.574
TreatmentB1:log(Weight)	−1.236	0.290	−4.269
TreatmentB2:log(Weight)	−0.810	0.310	−2.615
TreatmentP1:log(Weight)	−1.158	0.315	−3.679
TreatmentP2:log(Weight)	−0.180	0.341	−0.529
TreatmentPB1:log(Weight)	−0.844	0.317	−2.660
TreatmentPB2:log(Weight)	−1.023	0.311	−3.290

B: β-alanine, P: proline, PB: proline and β-alanine, 1: amino acid concentration as found in nectar of *G. lutea* in natural conditions, 2: amino acids at twice the natural concentration.

Table S2. Pairwise contrasts between log-transformed bumblebee solution consumption of different treatments (i.e., amino acid type), based on coefficients estimated by a linear mixed-effects model (see Table S1). Results are given on the response scale.

Treatments	Estimate	SE	t	p-value
S – B1	1.113	0.087	1.376	0.814
S – B2	1.103	0.086	1.256	0.871
S – P1	1.156	0.093	1.800	0.549
S – P2	1.451	0.116	4.664	1.0e−04
S – PB1	1.108	0.088	1.299	0.852
S – PB2	1.171	0.092	2.007	0.413
B1 – B2	0.991	0.075	−0.118	1.000
B1 – P1	1.038	0.081	0.483	0.999
B1 – P2	1.303	0.101	3.427	0.013
B1 – PB1	0.996	0.076	−0.057	1.000
B1 – PB2	1.052	0.080	0.664	0.994
B2 – P1	1.048	0.082	0.594	0.997
B2 – P2	1.315	0.101	3.551	0.008
B2 – PB1	1.005	0.077	0.059	1.000
B2 – PB2	1.061	0.081	0.781	0.987
P1 – P2	1.255	0.100	2.847	0.071
P1 – PB1	0.959	0.076	−0.531	0.998
P1 – PB2	1.013	0.080	0.163	1.000
P2 – PB1	0.764	0.060	−3.440	0.012
P2 – PB2	0.807	0.063	−2.771	0.087
PB1 – PB2	1.056	0.081	0.711	0.992

S: control solution, B: β-alanine, P: proline, PB: proline and β-alanine, 1: amino acid concentration as found in nectar of *G. lutea* in natural conditions, 2: amino acids at twice the natural concentration.

Table S3. Type II ANOVAs to evaluate the contribution of every factor to the model's variance. Each variable was tested against the model without it and without any interactions with other variables. Response variable is bumblebee survival.

Model	Variable	df	χ^2	p-value
a	Treatment	6	5.345	0.500
	Log(Consumption)	1	45.864	1.27e−11
	Log(Weight)	1	0.582	0.446
	Treatment:log(Consumption)	6	11.002	0.088
b	Treatment	6	5.937	0.430
	Log(Consumption)	1	45.587	1.46e−11
	Log(Weight)	1	1.509	0.219
c	Log(Consumption)	1	45.919	1.23e−11
	Log(Weight)	1	1.701	0.192
d	Log(Consumption)	1	46.008	1.18e−11

Table S4. Analysis-of-deviance tables used to test for significance of explanatory variables in bumblebee survival between pairs of nested models.

Comparison	Models compared	Log-likelihood	df	χ_2	p-value$_{adj}$
1	b	−131.29	6	11.758	0.128
	a	−125.41			
2	c	−134.23	6	5.884	0.537
	b	−131.29			
3	d	−135.16	1	1.857	0.290
	c	−134.23			
4	e	−174.10	1	77.879	3.26e−15
	d	−135.16			

Model a: Survival ~ Treatment + log(consumption) + Treatment:log(consumption) + log(Weight) + (1 | Colony/ID)

Model b: Survival ~ Treatment + log(consumption) + log(Weight) + (1 | Colony/ID)

Model c: Survival ~ log(consumption) + log(Weight) + (1 | Colony/ID)

Model d: Survival ~ log(consumption) + (1 | Colony/ID)

Model e: Survival ~ (1 | Colony/ID)

Treatment includes the control solution and the six amino acid (proline, β-alanine, proline and β-alanine) × concentration (natural, twice natural) solutions; Colony indicates colony identity; ID indicates bumblebee identity.

Table S5. Pairwise contrasts of bumblebee survival when bumblebees were fed with different amino acids, based on coefficients estimated by the full model including all amino acid solutions (see Tables S3-S4). Results are given on the log scale.

Treatments	Estimate	SE	z	p-value
S – B1	1.576	1.314	1.199	1.000
S – B2	0.536	0.485	0.634	1.000
S – P1	0.142	0.742	0.192	1.000
S – P2	1.266	0.925	1.368	1.000
S – PB1	−0.386	0.703	−0.549	1.000
S – PB2	0.045	0.780	0.058	1.000
B1 – B2	−1.040	1.386	−0.750	1.000
B1 – P1	−1.434	1.327	−1.081	1.000
B1 – P2	−0.310	1.429	−0.217	1.000
B1 – PB1	−1.962	1.303	−1.505	1.000
B1 – PB2	−1.530	1.343	−1.140	1.000
B2 – P1	−0.394	0.867	−0.454	1.000
B2 – P2	0.730	1.012	0.721	1.000
B2 – PB1	−0.922	0.832	−1.107	1.000
B2 – PB2	−0.490	0.888	−0.552	1.000
P1 – P2	1.123	0.869	1.292	1.000
P1 – PB1	−0.528	0.722	−0.732	1.000
P1 – PB2	−0.097	0.798	−0.121	1.000
P2 – PB1	−1.652	0.882	−1.873	1.000
P2 – PB2	−1.220	0.942	−1.295	1.000
PB1 – PB2	0.431	0.760	0.567	1.000

S: control solution, B: β-alanine, P: proline, PB: proline and β-alanine, 1: amino acid concentration as found in nectar of *G. lutea* in natural conditions, 2: amino acids at twice the natural concentration.

SM TO NECTAR TYRAMINE DECREASES THE PERMANENCE OF BUMBLEBEES (*BOMBUS TERRESTRIS*) ON FLOWERS

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Table S1. Number of visits per treatment, number of different individuals per day and treatment, and number of open flowers available in each compartment of the greenhouse in each day of the experiment. Supernumerary flowers were cut off in order to keep the daily number of visitable flowers equal.

Day of essay	N° visits for control	N° visits for tyramine	N° bees for control	N° bees for tyramine	N° flowers for control	N° flowers for tyramine
1	8	8	4	2	5	5
2	9	8	5	3	3	3
3	6	6	2	2	5	5
4	3	5	2	3	9	9
5	3	6	3	1	11	11
6	2	2	1	1	12	12

Table S2. Retention time (RT) and detection limit (LOD) of the HPLC-DAD method used for biogenic amines detection.

Amine	LOD (nmol/mL)	RT (min)
Histamine	0.9	1.97
Epinephrine	0.4	2.38
Norepinephrine	0.5	3.1
Octopamine	0.7	4.3
Dopamine	0.7	5.2
Tyramine	0.6	8.38
Serotonin	0.2	14.98
Tryptamine	0.4	20.2

Table S3. Loading resulted from the PCA on behavior parameters of 66 visits recorded during the experiment.

Parameter	CS1	CS2
Time spent feeding	0.50	-0.13
Time spent walking	0.47	0.16
Total time of visit	0.55	-0.03
Total n° of flowers visited	0.41	-0.50
Total n° of approaches to nectar	0.25	0.84

Table S4. Results of Generalized Linear Mixed Models (GLMMs) on bumblebee behavior exhibited on the single flowers visited. C = Control, T = Tyramine. Values are expressed as mean ± standard error.

Behavioural parameters	C	T	t-value (df = 14)	p-value
N° feeding bouts	1.903 ± 0.390	1.800 ± 0.182	-0.082	0.6928
Time spent feeding (sec)	31.105 ± 3.224	15.181 ± 3.057	-3.456	0.004*
Time spent walking (sec)	25.096 ± 3.611	14.450 ± 3.412	-1.405	0.182
Total time spent (sec)	56.202 ± 6.653	29.631 ± 3.416	-2.308	0.037*

*Indicates significant difference at $\alpha = 0.05$

SM TO FLORAL NECTAR AND INSECT FLOWER HANDLING TIME CHANGE OVER THE FLOWERING SEASON: RESULTS FROM AN EXPLORATORY STUDY

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Figure S1. Geographic location of the two study populations of *Echium vulgare* in northern Italy (red dots). The site named TO is in the Piedmont region (Chiaverano municipality), the site named MO is in the Emilia-Romagna region (Lama Mocogno municipality).

Table S1. Retention time (RT) and limits of detection (LOD) of the HPLC-DAD method used for detection of biogenic amines.

Amine	LOD (nmol/mL)	RT (min)
Histamine	0.9	1.97
Epinephrine	0.4	2.38
Norepinephrine	0.5	3.1
Octopamine	0.7	4.3
Dopamine	0.7	5.2
Tyramine	0.6	8.38
Serotonin	0.2	14.98
Tryptamine	0.4	20.2

Table S2. Taxonomic identification and number of visits (total 319) of insects that visited *Echium vulgare* flowers by period (early and late summer) and population (MO and TO). ND = not determined.

Order	Family	Genus	Species or morphogroup	Early		Late	
				MO	TO	MO	TO
Hymenoptera	Apidae	*Anthophora*	*Anthophora plumipes* Pallas, 1772	9	0	0	0
Hymenoptera	Apidae	*Apis*	*Apis mellifera* Linnaeus, 1758	0	51	28	0
Hymenoptera	Apidae	*Bombus*	*Bombus pascuorum* species group	26	12	18	23
			Bombus sylvarum Linnaeus, 1761	17	0	0	0
			Bombus hortorum species group	5	4	0	0
			Bombus lapidarius (Linnaeus, 1758)	16	0	0	0
			Bombus pratorum (Linnaeus, 1761)	5	0	0	0
			Bombus terrestris group	4	5	0	0
Hymenoptera	Apidae	*Ceratina*	*Ceratina cucurbitina* (Rossi, 1792) *Ceratina chalybea* Chevrier, 1872	2	0	8	5
Hymenoptera	Halictidae	*Halictus*	*Halictus simplex* complex *Halictus* cfr. *scabiosae* (Rossi, 1790)	2	1	0	0
Hymenoptera	Megachilidae	*Anthidium*	*Anthidium florentinum* (Fabricius, 1775)	0	2	0	0
Hymenoptera	Megachilidae	*Hoplitis*	*Hoplitis adunca* (Panzer, 1798) *Hoplitis anthocopoides* (Schenck, 1853)	55	2	0	0
Hymenoptera	Megachilidae	*Osmia*	*Osmia caerulescens* (Linnaeus, 1758)	0	2	0	0
Hymenoptera	Megachilidae	*Megachile*	*Megachile centuncularis* (Linnaeus, 1758)	0	1	0	0
Diptera	Syrphidae	ND	Syrphidae (Latreille, 1802) sp. 1	1	0	0	0
Diptera	Culicidae	ND	Culicidae (Meigen, 1818) sp. 1	0	0	3	0
Lepidoptera	Papilionidae	*Iphiclides*	*Iphiclides podalirius* (Linnaeus, 1758)	0	4	0	0
Lepidoptera	Pieridae	*Pieris*	*Pieris* Schrank, 1801 sp. 1	0	1	0	1
		Gonepteryx	*Gonepteryx rhamni* (Linnaeus, 1758)	0	3	0	0
Lepidoptera	Sphingidae	*Macroglossum*	*Macroglossum stellatarum* (Linnaeus, 1758)	0	2	0	0
		Hemaris	*Hemaris fuciformis* (Linnaeus, 1758)	0	1	0	0

Table S3. Loading resulting from PCA on nectar parameters of the 21 samples collected in the two periods (early and late summer) and populations (TO and MO).

Parameter	PC 1	PC 2
Volume per flower (µL)	0.54	0.11
Total sugar (mg/mL)	-0.46	0.42
Sucrose:Hexose	0.44	0.21
Total amino acids (nmol/mL)	0.02	0.71
Non-protein amino acids (nmol/mL)	0.33	0.43
Octopamine (nmol/mL)	-0.44	0.28

Table S4. LMM coefficients for the effect of period on the different nectar parameters. The early period is set as intercept.

Nectar parameter	Variable	Estimate	SE	t-value	p-value
Volume per flower	(Intercept)	0.589	0.039	15.039	0.000
	PeriodLate	-0.325	0.060	-5.431	< 0.001*
Total sugars	(Intercept)	49.905	7.206	6.926	0.000
	PeriodLate	50.423	11.007	4.581	< 0.001*
Sucrose:hexose ratio	(Intercept)	3.773	0.281	13.439	0.000
	PeriodLate	-1.445	0.429	-3.369	0.003*
Total amino acids	(Intercept)	3.187	0.045	70.96	0.000
	PeriodLate	0.074	0.069	1.075	0.297
Protein:non-protein amino acids	(Intercept)	0.727	0.102	7.118	0.000
	PeriodLate	0.545	0.119	4.562	< 0.001*
Octopamine	(Intercept)	0.333	0.163	2.049	0.055
	PeriodLate	0.956	0.185	5.164	< 0.001*

*Indicates significant difference at $\alpha = 0.05$

Table S5. PCA loadings of nectar composition of 21 samples from both flowering periods (early and late summer), based on amino acid spectra.

Amino acid	PC1	PC2
Aspartic acid	-0.02	0.06
Serine	0.03	0.03
Glutamine	-0.01	0.04
Glycine	-0.01	0.01
Histidine	-0.02	0.03
Arginine	0.02	-0.04
Threonine	0.00	0.01
Alanine	0.03	0.03
Proline	-0.05	0.05
Cysteine	-0.01	-0.05
Tyrosine	0.37	0.09
Valine	0.04	0.04
Methionine	0.00	0.00
Lysine	-0.01	-0.03
Isoleucine	-0.56	0.51
Leucine	0.00	0.06
Phenylalanine	0.71	0.16
Taurine	-0.05	0.09
β-alanine	-0.07	0.03
GABA	-0.01	0.03
Ornithine	-0.19	-0.82

Table S6. LMM coefficients for the effect of period on single amino acid concentrations, showing statistical significance. The early period is set as intercept.

Amino acid	Variable	Estimate	SE	t-value	p-value
TYR	(Intercept)	64.292	37.731	1.704	0.106
	PeriodLate	265.457	43.497	6.103	< 0.001*
VAL	(Intercept)	40.392	10.252	3.940	0.001
	PeriodLate	40.397	15.660	2.580	0.019*
ALA	(Intercept)	37.088	20.019	1.853	0.080
	PeriodLate	38.787	18.130	2.139	0.046*
PHE	(Intercept)	269.183	58.070	4.636	0.000
	PeriodLate	435.850	88.703	4.914	< 0.001*
PRO	(Intercept)	52.951	22.040	2.402	0.027
	PeriodLate	-31.815	13.718	-2.319	0.032*

*Indicates significant difference at $\alpha = 0.05$

Figure S2. Amino acid concentrations detected in the early and late periods (early = light blue (left); late = dark blue (right)) expressed as mean ± SE. The amino acids citrulline and α-aminobutyric acid were not detected in either population or sampling period and are not shown in the histogram. The asterisks denote a statistically significant difference according to a LMM where the early period was set as intercept. NPAA = non-protein amino acids; PAA = protein amino acids.

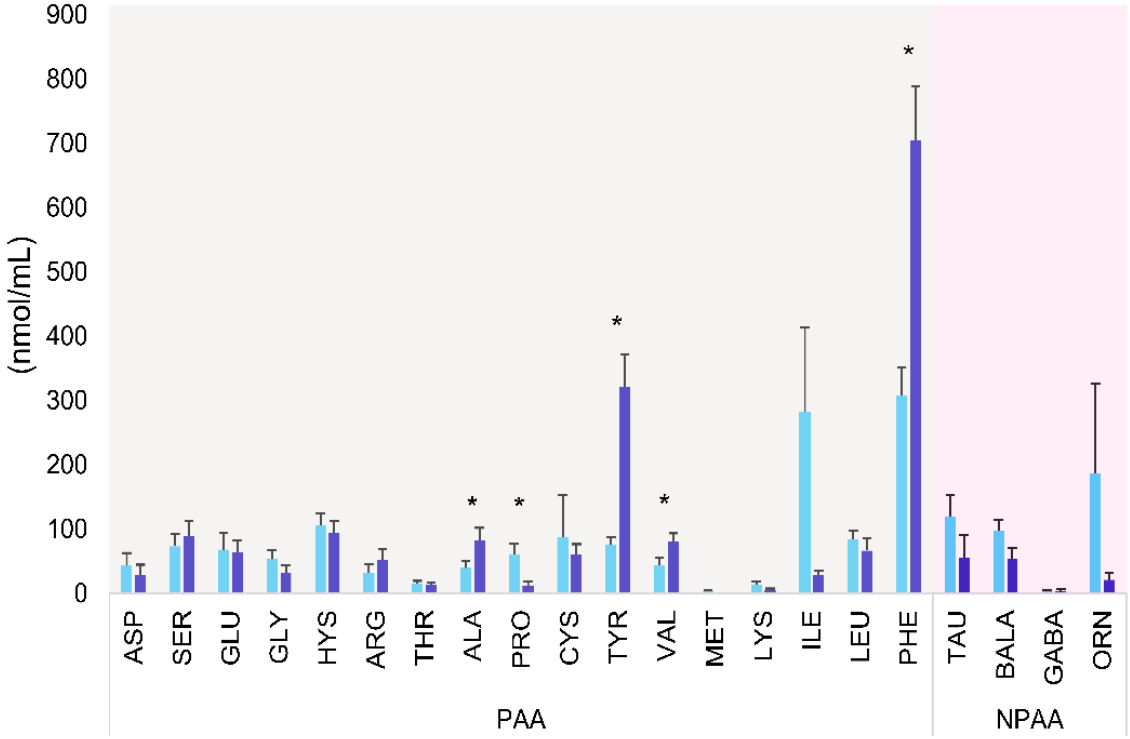

Table S8. GLMM coefficients for the effect of period on the duration of visit to single flowers by bumblebees. The early period is set as intercept.

Parameter	Variable	Estimate	SE	t-value	p-value
Duration of visit (sec)	(Intercept)	0.921	0.157	5.876	0.000
	PeriodLate	0.479	0.147	3.257	0.002

SM TO NECTAR-LIKE CONCENTRATIONS OF EXOGENOUS INSECT NEUROTRANSMITTERS GENERATE RELEVANT EFFECTS IN BEE BEHAVIORS RELATED TO FLOWER VISITATION

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Figure S1. Flight cages used for the experiment on locomotion (*Exp. 2*) consisted of plastic net cylinders (length = 25 cm, diam. = 16 cm) mounted horizontally with the ends closed by transparent plastic lids.

Table S1. Sample size and no. of replicates (different colonies) for the experiment on consumption and survival (*Exp. 1*).

Colony	Treatment	Concentration	No. bees
1	Control	-	9
	Octopamine	0.1 mM	9
		1 mM	9
	Tyramine	0.1 mM	8
		1 mM	9
2	Control	-	9
	Octopamine	0.1 mM	8
		1 mM	8
	Tyramine	0.1 mM	9
		1 mM	9
3	Control	-	7
	Octopamine	0.1 mM	8
		1 mM	8
	Tyramine	0.1 mM	8
		1 mM	6
4	Control	-	9
	Octopamine	0.1 mM	9
		1 mM	8
	Tyramine	0.1 mM	8
		1 mM	10

Table S2. Sample size and no. of replicates (different colonies) for the experiment on locomotion (*Exp. 2*).

Colony	Treatment	Concentration	No. bees
1	Control	-	5
	Octopamine	0.1 mM	5
		1 mM	5
	Tyramine	0.1 mM	5
		1 mM	5
2	Control	-	5
	Octopamine	0.1 mM	5
		1 mM	5
	Tyramine	0.1 mM	5
		1 mM	5
3	Control	-	5
	Octopamine	0.1 mM	5
		1 mM	5
	Tyramine	0.1 mM	5
		1 mM	5

Table S3. Sample size and no. of replicates (different colonies) for the experiment on gustatory responsiveness (*Exp. 3*).

Colony	Treatment	Concentration	No. bees
1	Control	-	2
	Octopamine	0.1 mM	2
		1 mM	2
	Tyramine	0.1 mM	2
		1 mM	1
2	Control	-	2
	Octopamine	0.1 mM	3
		1 mM	3
	Tyramine	0.1 mM	3
		1 mM	2
3	Control	-	2
	Octopamine	0.1 mM	3
		1 mM	4
	Tyramine	0.1 mM	4
		1 mM	6
4	Control	-	6
	Octopamine	0.1 mM	4
		1 mM	3
	Tyramine	0.1 mM	3
		1 mM	3

Table S4. Pairwise contrasts between consumption of different treatment diets of bees individually caged in Nicot cages for the experiment on consumption and survival (*Exp. 1*), based on coefficients estimated by a GLMM.

Contrast	Estimate	SE	df	t.ratio	p.value
Control – Octopamine 0.1 mM	1.478	0.435	160	3.402	0.007
Control – Octopamine 1 mM	-0.487	0.442	160	-1.103	0.805
Control – Tyramine 0.1 mM	0.211	0.440	160	0.479	0.989
Control – Tyramine 1 mM	0.405	0.437	160	0.928	0.886
Octopamine 0.1 mM – Octopamine 1 mM	-1.965	0.439	160	-4.480	< 0.001
Octopamine 0.1 mM – Tyramine 0.1 mM	-1.268	0.437	160	-2.901	0.034
Octopamine 1 mM – Tyramine 1 mM	0.892	0.441	160	2.021	0.261
Tyramine 0.1 mM – Tyramine 1 mM	0.194	0.439	160	0.442	0.992

Table S5. Pairwise contrasts between consumption of different treatment diets of bees grouped by five in small flight cages for the experiment on locomotion (*Exp. 2*), based on coefficients estimated by a GLMM.

Contrast	Estimate	SE	df	t.ratio	p.value
Control – Octopamine 0.1 mM	0.040	0.170	157	0.237	0.999
Control – Octopamine 1 mM	-0.427	0.190	157	-2.241	0.170
Control – Tyramine 0.1 mM	-0.566	0.197	157	-2.872	0.037
Control – Tyramine 1 mM	-0.949	0.216	157	-4.384	< 0.001
Octopamine 0.1 mM – Octopamine 1 mM	-0.467	0.190	157	-2.458	0.106
Octopamine 0.1 mM – Tyramine 0.1 mM	-0.606	0.197	157	-3.082	0.020
Octopamine 1 mM – Tyramine 1 mM	-0.523	0.232	157	-2.249	0.167
Tyramine 0.1 mM – Tyramine 1 mM	-0.383	0.238	157	-1.609	0.494

Table S6. Coefficients calculated on the duration of flight exhibited by bees fed with different treatment diets and grouped by five in small flight cages for the experiment on locomotion (*Exp. 2*), by means of a two-part mixed effect model for semi-continuous and zero-inflated data and divided in fixed effects (a) and zero-part coefficients (b). Control diet was set as intercept.

a)

Fixed effects	Estimate	SE	z-value	p-value
Intercept	1.320	0.159	8.298	< 0.001
Treatment_Octopamine_0.1mM	-0.061	0.265	-0.230	0.818
Treatment_Octopamine_1mM	0.507	0.233	2.172	0.030
Treatment_Tyramine_0.1mM	0.182	0.198	0.919	0.358
Treatment_Tyramine_1mM	0.131	0.210	0.624	0.533

b)

Zero-part coefficients	Estimate	SE	z-value	p-value
Intercept	1.515	0.336	4.507	< 0.001
Treatment_Octopamine_0.1mM	1.426	0.512	2.784	0.005
Treatment_Octopamine_1mM	0.799	0.491	1.627	0.104
Treatment_Tyramine_0.1mM	-0.367	0.463	-0.794	0.427
Treatment_Tyramine_1mM	0.170	0.472	0.359	0.719

Table S7. Pairwise contrasts between a two-vector variable of dynamic *vs* static behavior exhibited by bees fed with different treatment diets and grouped by five in small flight cages for the experiment on locomotion (*Exp. 2*), based on coefficients estimated by a GLMM.

Contrast	Estimate	SE	df	t.ratio	p.value
Control – Octopamine 0.1 mM	0.987	0.282	68	3.498	0.007
Control – Octopamine 1 mM	0.954	0.282	68	3.387	0.010
Control – Tyramine 0.1 mM	0.354	0.282	68	1.257	0.718
Control – Tyramine 1 mM	0.501	0.282	68	1.779	0.394
Octopamine 0.1 mM – Octopamine 1 mM	-0.033	0.278	68	-0.120	1.000
Octopamine 0.1 mM – Tyramine 0.1 mM	-0.633	0.278	68	-2.272	0.167
Octopamine 1 mM – Tyramine 1 mM	-0.453	0.277	68	-1.632	0.483
Tyramine 0.1 mM – Tyramine 1 mM	0.147	0.278	68	0.527	0.984

Table S8. Coefficients calculated on the frequency of drinking behavior exhibited by bees fed different treatment diets during the experiment on gustatory responsiveness (*Exp. 3*), by means of a GLMM. Control diet was set as intercept.

	Value	SE	DF	t-value	p-value
Intercept	1.340	0.182	55	7.374	0.000
Treatment_Octopamine_0.1mM	-0.262	0.257	55	-1.019	0.313
Treatment_Octopamine_1mM	-0263	0.257	55	-1.025	0.310
Treatment_Tyramine_0.1mM	-0.218	0.257	55	-0.848	0.400
Treatment_Tyramine_1mM	-0.558	0.257	55	-2.171	0.034

SM TO EFFECT OF NECTAR AMINO ACID COMPOSITION ON BUMBLEBEES' PREFERENCE: A LABORATORY ASSESSMENT WITH PROLINE AND B-ALANINE

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Table S1. LMM coefficients for the effect of treatment (i.e., amino acid type), bumblebee weight, and their interaction on the log-transformed solution consumption by bumblebees. Results are given on the log scale. Control solution (i.e., only sugars) is set as intercept.

Variable	Estimate	SE	t-value
(Intercept)	0.616	0.325	1.891
TreatmentB1	−1.706	0.397	−4.293
TreatmentB2	−1.146	0.425	−2.698
TreatmentP1	−1.642	0.441	−3.726
TreatmentP2	−0.605	0.456	−1.327
TreatmentPB1	−1.194	0.435	−2.747
TreatmentPB2	−1.481	0.417	−3.553
log(Weight)	1.335	0.203	6.574
TreatmentB1:log(Weight)	−1.236	0.290	−4.269
TreatmentB2:log(Weight)	−0.810	0.310	−2.615
TreatmentP1:log(Weight)	−1.158	0.315	−3.679
TreatmentP2:log(Weight)	−0.180	0.341	−0.529
TreatmentPB1:log(Weight)	−0.844	0.317	−2.660
TreatmentPB2:log(Weight)	−1.023	0.311	−3.290

B: β-alanine, P: proline, PB: proline and β-alanine, 1: natural amino acid concentration, 2: twice the natural amino acid concentration.

Table S2. Pairwise contrasts between log-transformed bumblebee solution consumption of different treatments (i.e., amino acid type), based on coefficients estimated by a linear mixed-effects model (see Table S1). Results are given on the response scale.

Treatments	Estimate	SE	t	p-value
S – B1	1.113	0.087	1.376	0.814
S – B2	1.103	0.086	1.256	0.871
S – P1	1.156	0.093	1.800	0.549
S – P2	1.451	0.116	4.664	1.0e−04
S – PB1	1.108	0.088	1.299	0.852
S – PB2	1.171	0.092	2.007	0.413
B1 – B2	0.991	0.075	−0.118	1.000
B1 – P1	1.038	0.081	0.483	0.999
B1 – P2	1.303	0.101	3.427	0.013
B1 – PB1	0.996	0.076	−0.057	1.000
B1 – PB2	1.052	0.080	0.664	0.994
B2 – P1	1.048	0.082	0.594	0.997
B2 – P2	1.315	0.101	3.551	0.008
B2 – PB1	1.005	0.077	0.059	1.000
B2 – PB2	1.061	0.081	0.781	0.987
P1 – P2	1.255	0.100	2.847	0.071
P1 – PB1	0.959	0.076	−0.531	0.998
P1 – PB2	1.013	0.080	0.163	1.000
P2 – PB1	0.764	0.060	−3.440	0.012
P2 – PB2	0.807	0.063	−2.771	0.087
PB1 – PB2	1.056	0.081	0.711	0.992

S: control solution, B: β-alanine, P: proline, PB: proline and β-alanine, 1: natural amino acid concentration, 2: twice the natural amino acid concentration.

Table S3. Type II ANOVAs to evaluate the contribution of every factor to the model's variance. Each variable was tested against the model without it and without any interactions with other variables. Response variable is bumblebee survival.

Model	Variable	df	χ^2	p-value
a	Treatment	6	5.345	0.500
	Log(Consumption)	1	45.864	1.27e−11
	Log(Weight)	1	0.582	0.446
	Treatment:log(Consumption)	6	11.002	0.088
b	Treatment	6	5.937	0.430
	Log(Consumption)	1	45.587	1.46e−11
	Log(Weight)	1	1.509	0.219
c	Log(Consumption)	1	45.919	1.23e−11
	Log(Weight)	1	1.701	0.192
d	Log(Consumption)	1	46.008	1.18e−11

Table S4. Analysis-of-deviance tables used to test for significance of explanatory variables in bumblebee survival between pairs of nested models.

Comparison	Models compared	Log-likelihood	df	χ_2	p-value$_{adj}$
1	b	−131.29	6	11.758	0.128
	a	−125.41			
2	c	−134.23	6	5.884	0.537
	b	−131.29			
3	d	−135.16	1	1.857	0.290
	c	−134.23			
4	e	−174.10	1	77.879	3.26e−15
	d	−135.16			

Model a: Survival ~ Treatment + log(consumption) + Treatment:log(consumption) + log(Weight) + (1 | Colony/ID)

Model b: Survival ~ Treatment + log(consumption) + log(Weight) + (1 | Colony/ID)

Model c: Survival ~ log(consumption) + log(Weight) + (1 | Colony/ID)

Model d: Survival ~ log(consumption) + (1 | Colony/ID)

Model e: Survival ~ (1 | Colony/ID)

Treatment includes the control solution and the six amino acid (proline, β-alanine, proline and β-alanine) × concentration (natural, twice natural) solutions; Colony indicates colony identity; ID indicates bumblebee identity.

Table S5. Pairwise contrasts of bumblebee survival when bumblebees were fed with different amino acids, based on coefficients estimated by the full model including all amino acid solutions (see Tables S3-S4). Results are given on the log scale.

Treatments	Estimate	SE	z	p-value
S – B1	1.576	1.314	1.199	1.000
S – B2	0.536	0.485	0.634	1.000
S – P1	0.142	0.742	0.192	1.000
S – P2	1.266	0.925	1.368	1.000
S – PB1	−0.386	0.703	−0.549	1.000
S – PB2	0.045	0.780	0.058	1.000
B1 – B2	−1.040	1.386	−0.750	1.000
B1 – P1	−1.434	1.327	−1.081	1.000
B1 – P2	−0.310	1.429	−0.217	1.000
B1 – PB1	−1.962	1.303	−1.505	1.000
B1 – PB2	−1.530	1.343	−1.140	1.000
B2 – P1	−0.394	0.867	−0.454	1.000
B2 – P2	0.730	1.012	0.721	1.000
B2 – PB1	−0.922	0.832	−1.107	1.000
B2 – PB2	−0.490	0.888	−0.552	1.000
P1 – P2	1.123	0.869	1.292	1.000
P1 – PB1	−0.528	0.722	−0.732	1.000
P1 – PB2	−0.097	0.798	−0.121	1.000
P2 – PB1	−1.652	0.882	−1.873	1.000
P2 – PB2	−1.220	0.942	−1.295	1.000
PB1 – PB2	0.431	0.760	0.567	1.000

S: control solution, B: β-alanine, P: proline, PB: proline and β-alanine, 1: natural amino acid concentration, 2: twice the natural amino acid concentration.

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Then, in the months, I lost the file in the guts of my laptop, and now that I really am at the end of my PhD, I have to write it all over again. Not a big deal, though. In fact, since then, several people have joined the list of those I'm grateful to. But anyway, this is very much me: fussing to keep up the pace just to find out that the path in the end has taken a different direction.

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