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Nectar tyramine decreases the duration of bumblebee visits on flowers

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Title: 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 solution , suggesting that their behaviour of visit was overall more dynamic. Our results highlight the importance of addressing further investigations on this emerging class of nectarcompounds 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

Conflict of interest

All authors declare that they have no conflict of interest.

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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.

2. Methods

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.

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).

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.

2.2.1 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.

2.2.2 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.

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. Results

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).

3.2 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).

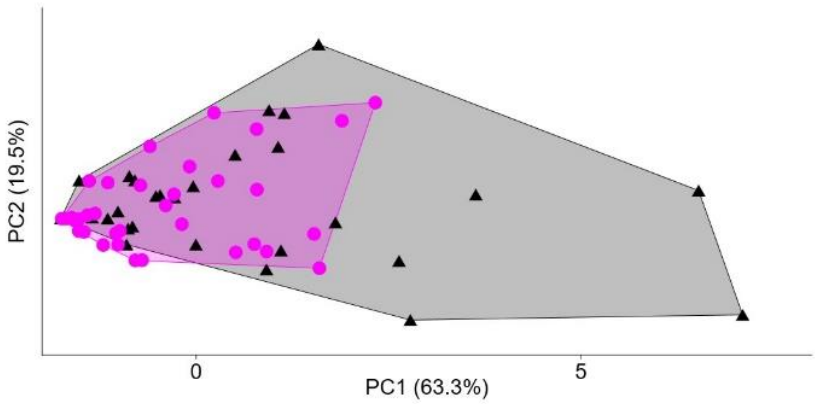

Fig. 1 Principal components analysis (PCA) on the behavioural parameters describing visits performed by free-flying bumblebees. Black triangles represent visits recorded for the treatment C, whilst fuchsia circles represent visits recorded for the treatment T.

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).

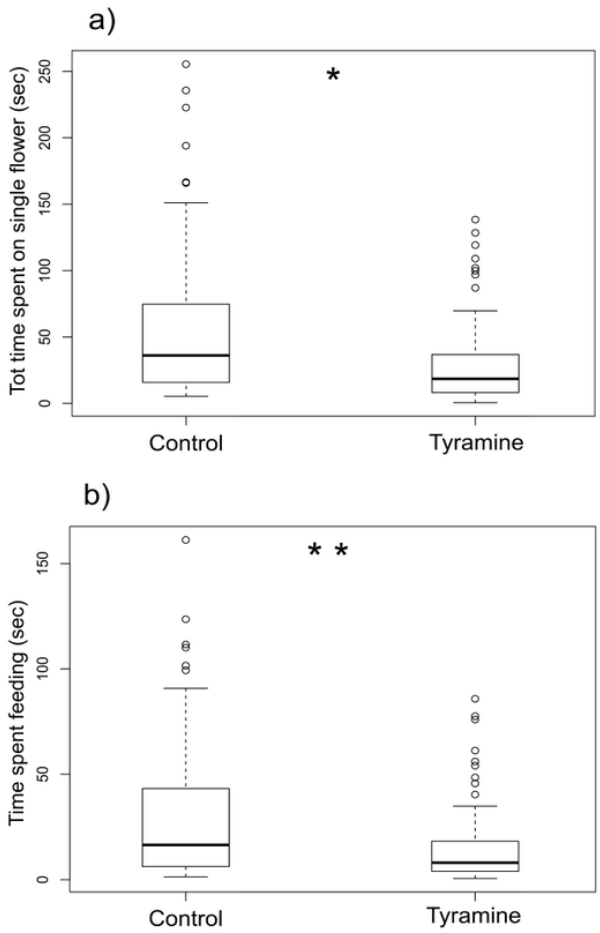

Fig. 2 Total time spent on a single flower (a) and time spent feeding on a single flower (b) out of the 161 flower approaches recorded. Asterisks indicates a significant difference (* = $p < 0.05$ and ** = $p < 0.01$) between treatments according to Generalized Linear Mixed Model where Control treatment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

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.

4.1. 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.

Declarations

- All authors declare that they have no conflict of interest.
- This work was partly supported by the project BeeNet (Italian National Fund under FEASR 2014-2020) from the Italian Ministry of Agriculture, Food Sovereignty and Forestry (MASAF). Marta Barberis was supported by a PhD grant from the University of Bologna.
- All data generated or analysed during this study are included in this published article [and its supplementary information files]

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

Fig. 2 Total time spent on a single flower (a) and time spent foraging on a single flower (b) out of the 161 flower approaches recorded. The asterisk indicates a significant difference between treatments according to Generalized Linear Mixed Model where Control treatment C was set as intercept.