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Nectar chemistry is not only a plant's affair: floral visitors affect nectar sugar and amino acid composition

Gherardo Bogo, Alessandro Fisogni, Joan Rabassa-Juvanteny, Laura Bortolotti, Massimo Nepi, Massimo Guarnieri, Lucia Conte and Marta Galloni

G. Bogo (https://orcid.org/0000-0001-7415-2224) (gherardo1985@hotmail.com), Grupo de Ecología de la Polinización, INIBIOMA (Univ. Nacional del Comahue-CONICET), San Carlos de Bariloche, Argentina. – A. Fisogni (https://orcid.org/0000-0001-6179-2767), Dept of Evolution, Ecology and Organismal Biology, Univ. of California, Riverside, Riverside, CA, USA. – AF, L. Conte (https://orcid.org/0000-0003-0513-0616) and M. Galloni (https://orcid.org/0000-0001-5304-7820), Dept of Biological, Geological and Environmental Sciences, Univ. of Bologna, Bologna, Italy. – J. Rabassa-Juvanteny (https://orcid.org/0000-0002-5554-2304), Faculty of Biology, Univ. of Barcelona, Barcelona, Spain. – L. Bortolotti (https://orcid.org/0000-0001-7372-1579), CREA Research Centre for Agriculture and Environment, Bologna, Italy. – M. Nepi (https://orcid.org/0000-0002-6765-897X) and M. Guarnieri, Dept of Life Sciences, Univ. of Siena, Siena, Italy.

Floral nectar is the primary reward directly consumed by floral visitors and its chemical composition affects their behaviour and fidelity. In turn, floral visitors are expected to alter floral nectar composition directly or indirectly through the introduction of external contaminants, such as pollen grains and microorganisms. To understand the effect of exogenous factors on nectar chemistry, we investigated the modifications of nectar sugar and amino acid composition in relation to floral visitors in the perennial herb *Gentiana lutea*. We quantified nectar contamination by floral visitors through visitor exclusion experiments in the field, estimating pollen and yeast concentration in visited flowers and in uncontaminated nectar at two different flower stages (flower bud and older open flowers). We then assessed changes in the composition and concentration of sugars and both protein and non-protein amino acid in the nectar. We found clear differences in the chemical composition of flower nectar in relation to flower stage and floral visitation. Nectar sugar and amino acid concentrations were significantly higher in older flowers than in flower buds. In addition, nectar sugar concentration was significantly higher in older flowers where visitors were excluded than in visited flowers. On the contrary, amino acid concentration was comparable between visited and non-visited older flowers. We also found that pollen contamination enriched the amino acid profile of the host nectar both quantitatively and qualitatively. Finally, nectar exposed to floral visitors showed a higher number of yeast cells than unvisited flowers although too low to cause the observed changes in the glucose:fructose ratio, suggesting the presence of fructose-related bacteria. Our results show that the chemical composition of floral nectar is altered by several endogenous and exogenous factors in a complex process of ecological relations, and suggest that the modulation of floral visitation through nectar is not solely related to the plant itself.

Keywords: generalist pollination, *Gentiana lutea*, nectar contamination, nectar microorganisms, nectar yeast, plant–pollinator interactions

Introduction

Floral nectar is a complex aqueous solution secreted by plants whose chemical composition is dominated by three simple sugars: the disaccharide sucrose and its monosaccharide constituents, fructose and glucose. These three sugars are derived either from sucrose translocated into phloem sap and/or from the hydrolysis of starch accumulated in the developing nectaries (Pacini and Nepi 2007, Heil 2011). The maintenance of the sugar ratio can be regulated by the enzyme invertase, which hydrolyzes sucrose into glucose and fructose before, during and after nectar secretion (Nicolson and Thornburg 2007). In addition to sugars, other substances are present in smaller quantities, such as proteins, protein and non-protein amino acids, lipids, phenols, alcohols, alkaloids and antioxidants (Nicolson and Thornburg 2007, Nepi 2014).

The chemistry of floral nectar can be shaped by ecological as well as by phylogenetic constraints (Nepi et al. 2010). Among the first, interaction 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). Nectar components have two main functions: to mediate plant interactions with pollinators (attraction) and to protect nectar (protection) from other floral visitors, such as nectar robbers, and from nectar-dwelling microorganisms (Adler 2000, Heil 2011, Chalcoff et al. 2017). Several studies have shown that differences in nectar composition were related to different groups of floral visitors. For example, sucrose-rich nectars are usually preferred by hummingbirds, Megachiroptera, Lepidoptera and long-tongued bees, while hexose-rich nectars are usually preferred by passerine birds, Microchiroptera, short-tongued bees and flies (Baker and Baker 1982, 1983, 1990, Kress 1985, Baker et al. 1998). Moreover, butterflies and flies usually show a preference for nectars with high amino acid concentration (Potter and Bertin 1988, Alm et al. 1990, Erhardt and Rusterholz 1998), honey bees prefer sugar solutions enriched with proline and phenylalanine (Inouye and Waller 1984, Alm et al. 1990, Bertazzini et al. 2010), and bumble bees prefer solutions enriched with β-alanine (Bogo et al. 2019). Recent studies focusing on nectar secondary compounds and their effects on pollinators (Nepi 2014, Stevenson et al. 2017, Mustard 2020) challenge the classical view reported above in which just the alimentary importance of nectar is highlighted. According to the 'manipulation' hypothesis nectar is considered a pollinator manipulator rather than simply an attractant or reward for pollinators (Pyke 2016, Nepi et al. 2018).

Quantity and quality of nectar can vary considerably in relation to several abiotic and biotic factors (Baude et al. 2011). Changes can be induced by water availability, ambient humidity and temperature, soil-related factors, light availability and CO_2 concentration (Chalcoff et al. 2017, Parachnowitsch et al. 2019). At the same time, nectar composition can be affected by flower phenology, interactions with herbivores, nectar robbers or floral visitors, and by the presence of bacteria and yeasts (Aizen and Raffaele 1996, Lasso and Naranjo 2003, Vannette and Fukami 2018, Parachnowitsch et al. 2019) resulting in complex determination of nectar chemical profile.

The concentration of nectar solutes can also change in relation to flower age, independently of external factors (Pacini and Nepi 2007). Once the flowers open, floral visitors can affect nectar composition directly and indirectly in several ways. Visitors can alter nectar sugar composition by feeding on it, often stimulating further secretion. On the other hand, failure to visit can also affect nectar composition, often causing the reabsorbtion of unconsumed sugars (Pacini and Nicolson 2007, Nepi and Stpiczyńska 2008). Floral visitors can also modify nectar chemical composition by adding amino acids present in their saliva or by introducing conspecific or heterospecific pollen into the nectar (Willmer 1980, Gottsberger et al. 1990). Moreover, floral visitors can transfer yeasts directly between flowers or indirectly by transporting yeast-contaminated pollen grains (Herrera et al. 2009, Mittelbach et al. 2015, Pozo et al. 2015). In turn, nectar yeasts can cause important variation in the composition and concentration of nectar sugars (Canto and Herrera 2012, Chappell and Fukami 2018), and can decrease the concentration of certain amino acids (Vannette and Fukami 2018) or increase fatty acid derivatives (Yang et al. 2019).

In this article we aim to better understand how flower nectar composition is altered by floral visitors and endogenous factors. Specifically, we performed floral visitor exclusion experiments in the field to compare the composition of virgin (i.e. uncontaminated) nectar with that of flower nectar in which visits were allowed, to evaluate the effects of exogenous factors (i.e. insect floral visits and contamination by pollen grains and yeasts) on sugar and amino acid composition. In addition, we analysed the composition of nectar collected from flower buds to evaluate intrinsic changes related to flower age. In this way we can obtain comprehensive information on the alteration of nectar chemistry occurring at different floral stages and after floral visitation. We expected to find differences in sugar and amino acid concentration and in amino acid spectrum richness related to flower age, regardless of exogenous factors. Moreover, based on the potential direct and indirect effects of floral visitors, we also expected an increase in the presence of pollen grains and yeasts in the nectar, which in turn would modify nectar chemistry (Fig. 1).

Material and methods

Model species and study site

Gentiana lutea subsp. *symphyandra* is a long-lived scapose hemicryptophyte. It presents an unbranched stout stem (rarely two), growing up to 2 m tall (Tutin et al. 1972). Flowering occurs between June and July. Flowering stems carry up to ten pseudo-whorls containing about 20 pedicelate flowers each. On average, flower lifespan lasts about three

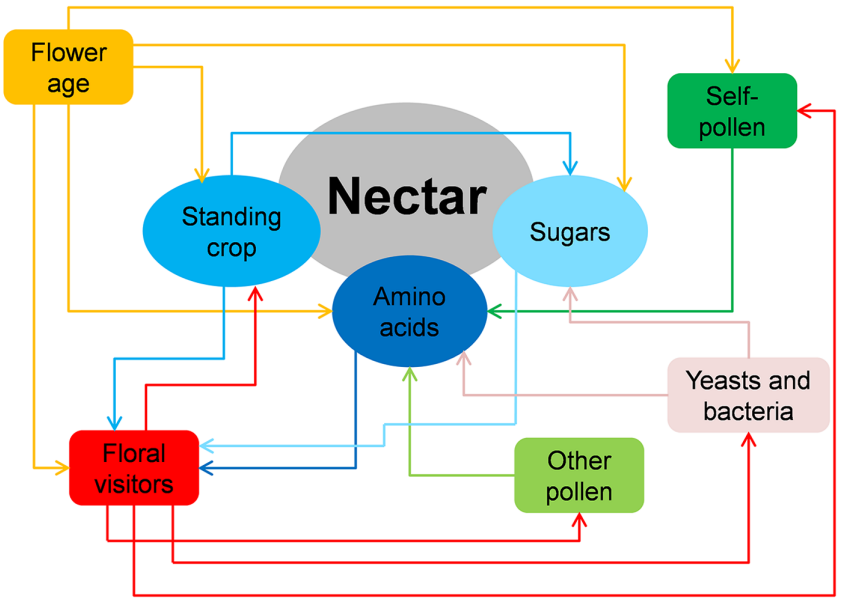

Figure 1. Concept map of the endogenous and exogenous factors that can directly or indirectly influence each other and flower nectar composition.

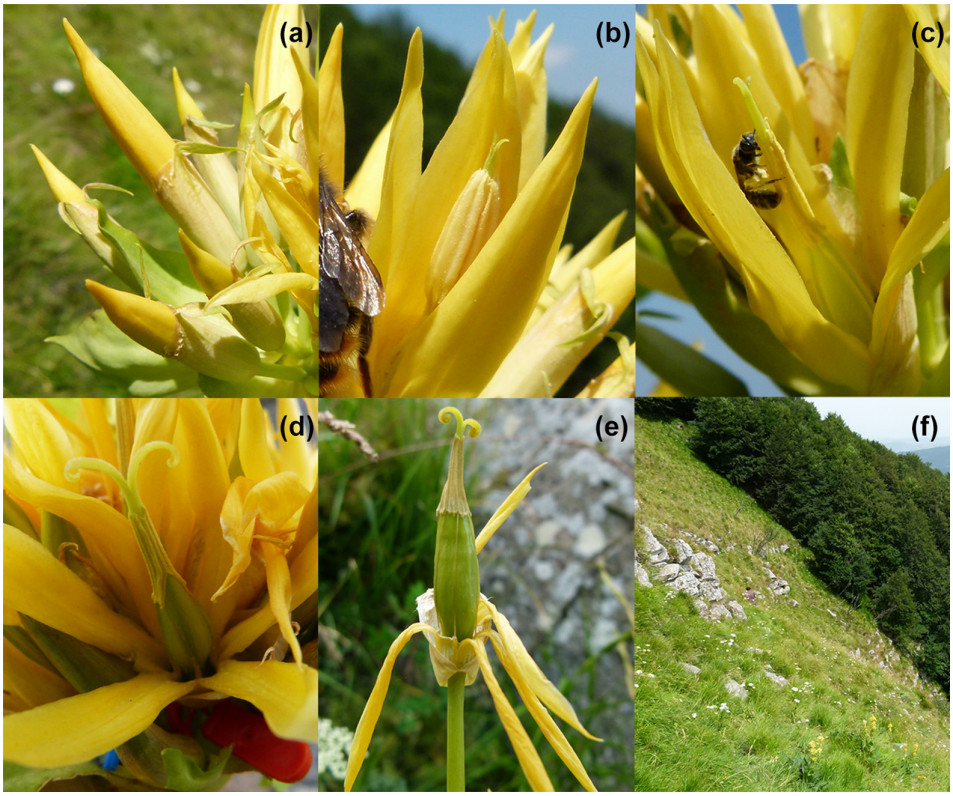

Figure 2. Stages of flower development in *Gentiana lutea* subsp. *symphyandra* (a–e) and study site (f). (a) Floral bud, (b) stage I: perianth open, anthers closed and stigma unreceptive, (c) stage II: perianth open, one to four dehiscent anthers, unreceptive stigma undivided or hardly bilamellate (here a small bee of the family Halictidae is collecting pollen from the anthers), (d) stage III: perianth open, anthers completely dehisced and stigma bilamellate, (e) stage IV: perianth withered.

days and four stages of flower development can be recognized after floral buds (Fig. 2a) have opened (Rossi 2012): 1) perianth open, anthers closed and stigma unreceptive (Fig. 2b), 2) perianth open, one to four dehiscent anthers, unreceptive stigma undivided or hardly bilamellate (Fig. 2c), 3) perianth open, anthers completely dehisced and stigma bilamellate (Fig. 2d) and 4) perianth withered (Fig. 2e). Flowers of *G. lutea* subsp. *symphyandra* are visited by several insect species belonging to the orders Hymenoptera, Diptera, Coleoptera and Lepidoptera, of which wild bees are among the most efficient pollinators (Rossi et al. 2014). Although *G. lutea* is a self-compatible species, partial flower dichogamy reduces within-flower selfing while pollinator-mediated geitonogamy is more likely to occur (Rossi et al. 2014). Seeds developed from self-pollination have lower viability and germination than cross-pollinated seeds (Rossi et al. 2016).

Samplings and field observations were performed in a population situated on the northeast face of Mount Grande (Vidiciatico, Bologna – Italy), within the Habitats Directive (92/43/EEC) Site of Community Importance and Special Protection Area IT4050002 'Corno alle Scale' (44°8′57″N, 10°52′10″E, 1380–1460 m a.s.l.). This isolated population (Fig. 2f) occurs in a meadow surrounded by a beech forest and is probably preserved by the steepness of the slope and by the rocky nature of the substrate (Rossi 2012, Rossi et al. 2014). In the studied population plants bloom synchronously with a peak occurring in mid-July, and flowers present copious amounts of nectar (up to 10 µl, Bogo et al unpubl.). Bumblebees are the most abundant flower visitors (about 50% of the total visitors), and present the highest visitation frequency, fidelity and pollinator performance (Rossi et al. 2014).

Test design

We collected nectar samples from four different flower groups (hereafter: flower treatments): 1) flower buds, to investigate the composition of freshly secreted unvisited pure nectar (young virgin nectar, YVN); 2) flowers with open perianth emasculated and bagged from the bud stage, to investigate the composition of unvisited nectar a few days old not contaminated by own or external pollen (old virgin nectar, OVN); 3) non-emasculated flowers with open perianth bagged from the bud stage, to investigate the composition of unvisited nectar contaminated by pollen from the same flower (self-contaminated nectar, SCN); 4) non-emasculated flowers with open perianth, bagged only three to four hours before sampling to allow floral visits but to avoid nectar depletion (naturally contaminated nectar, NCN) (Table 1). Flowers allocated to treatments OVN, SCN and NCN were bagged individually with nonwoven fabric (30 g m^{-2}, white, permeable to air, water and light) to exclude floral visitors. We also sampled nectar from flower buds to test for the presence of invertase activity (INV). Finally, we collected pollen from dehiscent anthers to investigate the effect of pollen contamination on the nectar amino acid profile under controlled conditions. When possible, we applied all treatments to different flowers on the same plant. If the four flower developmental stages required for treatments YVN, OVN, SCN and NCN were not present together, we sampled flowers from different plants (Table 1). The number of treatments allocated per plant ranged between three and six.

Nectar sampling

Nectar was collected from a total of nine plants and 90 flowers of *G. lutea* (Table 1) throughout the study population during two non-consecutive days (July 14 and 17) in 2015. We sampled nectar using Drummond Microcaps of different volumes, depending on its amount in the flower (3, 5 and 10 µl). For each sample, we measured the nectar level in the microcap using a Vernier calliper and then calculated its exact volume as a proportion of the total microcap volume. Nectar samples were then transferred into separate eppendorf tubes filled with 100 µl ethanol. All samples were transported to the laboratory in thermal insulated ice containers on the same day of field sampling, and stored at −20°C until analyses.

Nectar chemical analyses

Just prior to analyses nectar samples were thawed and air-dried in a Speedvac centrifuge to eliminate the ethanol and diluted 1:50 with distilled water. We analysed nectar sugar composition in all samples from the four flower treatments (n = 78 flowers belonging to 4–6 different plants per treatment, Table 1) by isocratic HPLC, using a Waters Sugar-Pak I ion-exchange column (6.5 × 300 mm) maintained at 90°C and a Waters 2410 refractive index detector. Water (MilliQ, pH 7) was used as mobile phase at a flow rate of 0.6 ml min^{-1}. The sample and standard solutions containing glucose, fructose and sucrose (20 µl) were injected. The concentration of each single sugar was calculated by comparing the area under

Table 1. Summary of the experimental treatments and sample size (n) used in this study.

Treatment	Abbr.	Flower stage	Bagged	Emasculated	n plants	n flowers
Young virgin nectar	YVN	bud	No	No	6	19
Old virgin nectar	OVN	III–IV	Yes	Yes	4	19
Self-contaminated nectar	SCN	III–IV	Yes	No	5	20
Naturally contaminated nectar	NCN	III–IV	No	Yes	6	20
Invertase activity	INV	bud	No	No	7	12
Pollen suspension	–	II–III	No	No	3	5

Abbr.: treatment abbreviation. Flower stage II: perianth open, one to four dehiscent anthers, unreceptive stigma undivided or hardly bilamellate. Flower stage III: perianth open, anthers completely dehisced and stigma bilamellate, Flower stage IV: perianth withered.

the chromatogram peaks with standards using the software Clarity (DataApex).

We analysed amino acid composition in a subset of eight randomly chosen samples per flower treatment (n = 32 flowers belonging to 3–5 plants per treatment) by gradient HPLC, using an ion exchange Novapak C18 (15 × 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, the selected volume (20 µl) of each reconstituted sample was amino acid derivatized (Cohen and Michaud 1993) with AQC fluorescent reagent and 0.02 M borate buffer (pH 8.6). In addition to all the protein amino acids (PAA), standards of the non-protein amino acids (NPAA) β-alanine, citrulline, l-homoserine, α-aminobutyric acid (AABA), γ-aminobutyric acid (GABA), hydroxyproline, ornithine and taurine were also used (Nocentini et al. 2012). Tryptophan was not detectable with this procedure. The concentration of each individual amino acid was calculated by comparing the area under the chromatogram peaks with standards using the software Clarity (DataApex).

Pollen and yeast detection

Each of the 78 nectar samples was topped up to 200 µl with ethanol. We than determined pollen and yeast content by counting the number of pollen grains and yeast cells present in aliquots of 10 µl in ethanol. These aliquots were placed on a microscope slide with a glass cover slip and observed under an optical microscope for counting the number of pollen grains and yeast cells present on the slide. We calculated the total number of pollen grains and yeast cells in each sample using the expression $N = (X \times 20)/V$ where X is the aliquot count and V is the nectar sample volume without ethanol. Moreover, we discriminated between pollen of *G. lutea* and pollen of other species based on pollen grain morphology.

Invertase test

Since the floral nectar of *G. lutea* is almost devoid of sucrose, we checked for the presence of invertase activity in the secretion. For this purpose we collected 100 µl of nectar from 12 flower buds (Table 1, INV), with a micropipette K7501 2–20 µl. The nectar sample was diluted 1:20 with a 4.6 mg ml^{-1} sucrose solution. The sugar profile at time zero was determined soon after the dilution. Subsequently, we incubated the solution at 30°C and repeated the sugar determination after 1, 2, 3, 4 and 24 h to quantify the potential hydrolysis of sucrose into glucose and fructose. The determination of sugars was carried out according to the procedure described above.

Pollen suspension test

We suspended 1 mg of *G. lutea* pollen collected from five flowers on three plants (Table 1) in 100 mg of a solution mimicking the average sugar composition of the young virgin nectar (glucose 27.65 mg ml^{-1}, fructose 26.59 mg ml^{-1}, Fig. 4a–b). We sampled 10 µl of the suspension after a few minutes and after 1, 4 and 24 h from the pollen suspension, and measured the amino acid content by gradient HPLC (same methodology explained above) to evaluate if the amino acid profile had changed.

Data analysis

We performed a principal component analysis (PCA) on the subset of eight samples for each of the four flower treatments to explore similarities in nectar composition, considering sugar and amino acid concentration and concentrations of pollen grains and yeast cells (component variables are listed in Supporting information), and a linear discriminant analysis (LDA) with jackknife leave-one-out cross validation to generate posterior probability scores for each treatment based on principal components. Data were scaled and centred around the mean, and analyses were performed using the functions 'dudi.pca' and 'lda' in the R-packages *ade4* and *MASS*, respectively (Venables and Ripley 2002, Dray and Dufour 2007).

We estimated the effect of flower treatment on sugar and amino acid concentration by fitting linear mixed-effects models (LMMs) using the function 'lmer' in the R-package *lme4* (Bates et al. 2015). For sugars, we fitted four separate models using the log-transformed glucose concentration, fructose concentration, total sugar concentration and glucose:fructose ratio as response variable, respectively. For amino acids, we fitted four separate models using the log-transformed PAA concentration, NPAA concentration, total amino acid concentration and PAA:NPAA ratio as response variable, respectively. In all models we included the four flower treatments (YVN, OVN, SCN, NCN) as explanatory variables, and whorl identity on the plant stem nested within plant identity as random effect to account for intra- and inter-plant variability. We then performed pairwise comparisons between treatments by estimating the marginal means (EMMs) with Tukey-adjusted p-values using the function 'emmeans' in the R-package *emmeans* (Lenth et al. 2020).

We analysed sucrose, glucose and fructose concentrations among the different time intervals in the test 'invertase' using a Pearson's chi-squared test.

We measured the diversity of amino acids based on their concentration using Hill numbers of order 1, corresponding to the exponential of Shannon entropy (Jost 2006). We estimated the effect of flower treatment on amino acid diversity by fitting linear models (LMs) using Hill numbers as response variable and the four flower treatments (YVN, OVN, SCN, NCN) as explanatory variables. Flower treatments were then compared between them using EMMs with Tukey-adjusted p-values.

We estimated the effect of flower treatment on pollen and yeast concentration by fitting two separate LMMs including $\log(x+1)$-transformed pollen and yeast concentrations as response variables, respectively, and the four flower treatments (YVN, OVN, SCN, NCN) as explanatory variables. Whorl identity nested within plant identity was included

as random effect to account for inter-flower and inter-individual variability. Flower treatments were then compared by estimating EMMs with Tukey-adjusted p-values. Figures were drawn using the R-package *ggplot2* (Wickham 2016), and all analyses were performed in R ver. 4.0.2 (<www.r-project.org>).

Results

Nectar analyses

The PCA showed a clear separation of the flower treatments with regard to nectar composition, with the first two components explaining 73.0% of the variance (Fig. 3). The first component was positively correlated with the protein amino acid and total amino acid concentrations (PC1 loadings = 0.44 for both). The second component was positively correlated with the concentration of yeast cells and with the glucose:fructose ratio (PC2 loadings = 0.45 and 0.58, respectively; Supporting information). The LDA showed a total correct assignment by cross-validation of 93.75% of samples (Supporting information). The correct assignment was 100% in treatments YVN, OVN and SCN, and 75% in treatment NCN.

Sugar composition

We excluded sucrose from the analysis of nectar sugar composition because its concentration was always lower than 1.5% of the total sugar concentration. We found significant effects of flower treatment on glucose ($F_{3,74} = 376.97$, $p < 2.2e^{-16}$), fructose ($F_{3,74} = 461.38$, $p < 2.2e^{-16}$) and total sugar ($F_{3,74} = 422.05$, $p < 2.2e^{-16}$) concentration in nectar. Sugar concentrations always showed the same pattern, with the lowest value found for YVN, followed by NCN, OVN and the highest value for SCN (Fig. 4a–c, Supporting information).

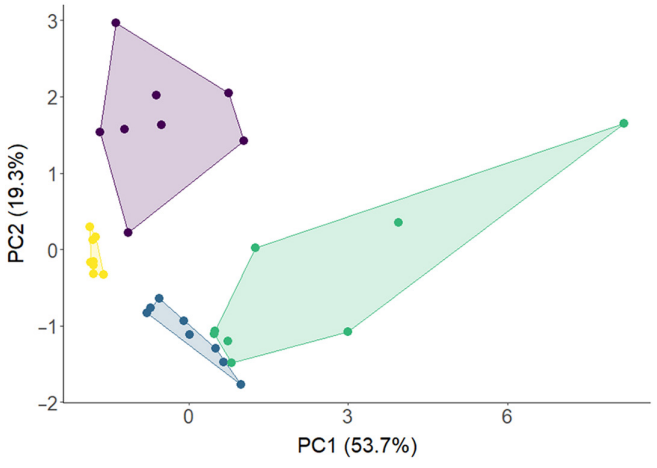

Figure 3. Principal components analysis (PCA) of the nectar composition in eight flowers for each of the four flower treatments, based on sugar and amino acid concentration and on concentrations of pollen grains and yeast cells. Yellow dots: young virgin nectar (YVN); blue dots: old virgin nectar (OVN); green dots: self-contaminated nectar (SCN); purple dots: naturally contaminated nectar (NCN).

Glucose:fructose ratio was significantly higher in the NCN than in the other treatments ($p < 0.0001$ all tests), while no other differences were observed (Fig. 4d, Supporting information).

Amino acids composition

We found significant effects of flower treatment on protein ($F_{3,28} = 17.55$, $p < 7.1 \times 10^{-06}$), non-protein ($F_{3,28} = 28.32$, $p < 2.9 \times 10^{-07}$) and total amino acid ($F_{3,28} = 33.51$, $p < 2.1 \times 10^{-09}$) concentration in nectar. YVN always had the lowest concentration (Fig. 5a–c, Supporting information). Both PAA and total amino acid concentrations were similar among the OVN, SCN and NCN treatments (Fig. 5a–c), while NPAA concentration did not differ significantly between the two contaminated treatments with the highest value observed in the OVN treatment (marginally significant difference between OVN and SCN, $p = 0.09$; Fig. 5b, Supporting information).

The PAA:NPAA ratio was significantly different among treatments ($F_{3,28} = 6.64$, $p = 0.0026$). The YVN and OVN treatments had similar low ratios, while SCN and NCN had significantly higher ratios than OVN and marginally higher ratios than YVN ($p = 0.06$ and $p = 0.08$, respectively; Fig. 5d, Supporting information).

The diversity of amino acids significantly differed among treatments ($F_{3,28} = 10.97$, $p = 6.152 \times 10^{-05}$). Both uncontaminated nectars (YVN = 2.38 ± 0.29, OVN = 1.80 ± 0.09) had a significantly lower diversity ($p \leq 0.01$, Supporting information) than contaminated nectars (SCN = 4.50 ± 0.60, NCN = 4.70 ± 0.66). β-Alanine was the most concentrated amino acid in both the uncontaminated (YVN and OVN) and in the NCN treatments, while it was the second most concentrated single amino acid in the SCN treatment (Supporting information, Fig. 6). Proline was the most and the second most concentrated amino acid in the SCN and NCN treatments, respectively. We discarded the arginine/threonine peak in the SCN treatment, since it was not possible to separate the two amino acids in the chromatograms (Supporting information, Fig. 6).

Pollen and yeast detection

The concentration of pollen grains in the nectar samples significantly differed among flower treatments ($F_{3,74} = 26.55$, $p = 6.713 \times 10^{-09}$), and was significantly higher in the SCN and NCN than in the YVN and OVN treatments ($p < 0.0001$ in all significant pairwise comparisons; Fig. 7a, Supporting information).

In a few samples of the uncontaminated nectars we found traces of pollen grains that did not belong to *G. lutea*, likely due to contamination during field manipulations. In the SCN treatment 78.5% of the pollen grains detected in nectar samples belonged to *G. lutea* (self-pollen), while in the

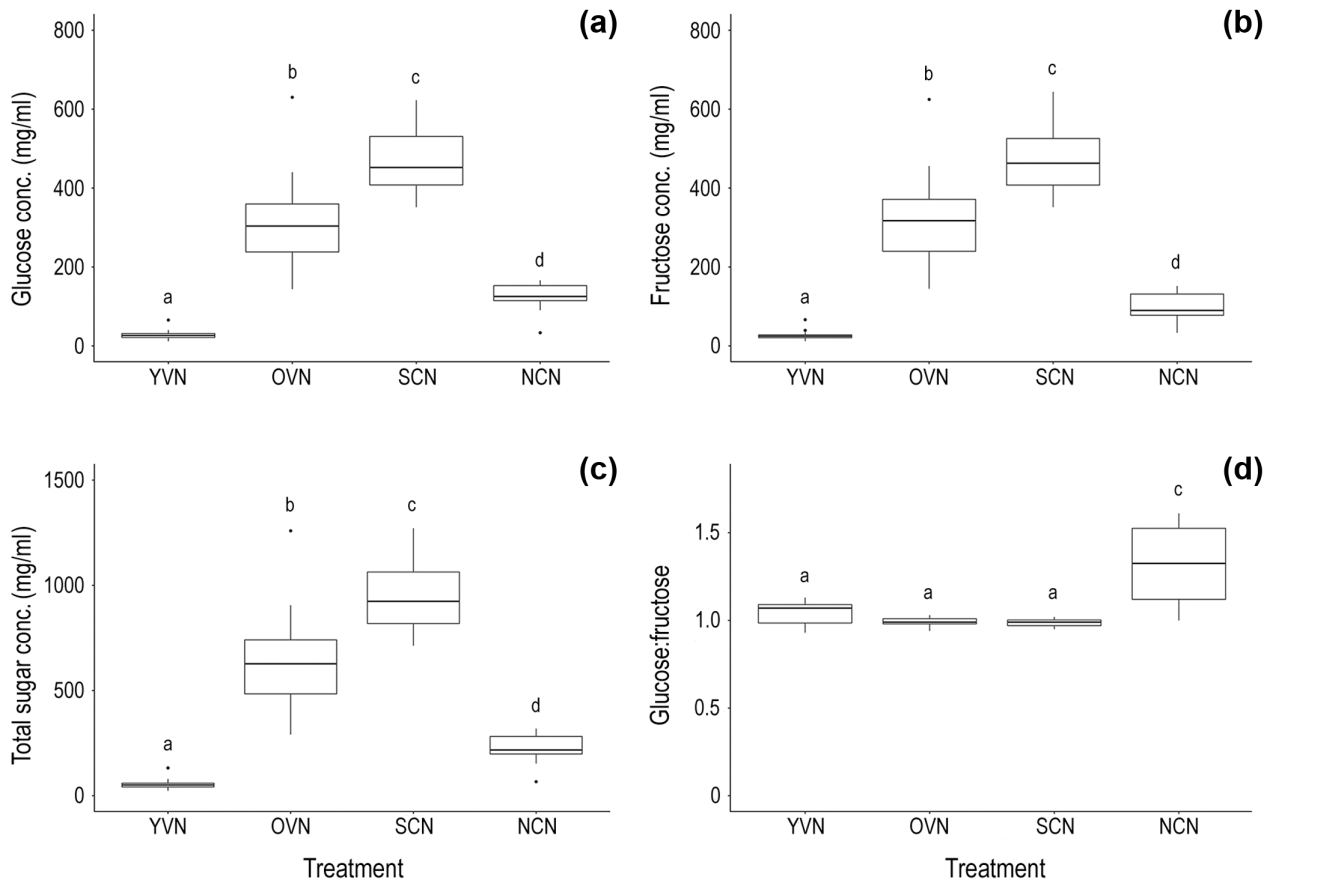

Figure 4. Glucose (a), fructose (b) and total sugar (c) concentration and glucose:fructose ratio (d) detected in the 78 nectar samples of the four flower treatments. Different letters indicate significant differences. YVN: young virgin nectar; OVN: old virgin nectar; SCN: self-contaminated nectar; NCN: naturally contaminated nectar. Note the different scales on the y-axes.

NCN treatment only 31.5% of pollen grains originated from *Gentiana lutea* (self- and conspecific cross-pollen). The concentration of yeast cells in the nectar samples significantly differed among treatments ($F_{3,74} = 33.80$, $p = 3.322 \times 10^{-13}$) and was the highest in the NCN treatment, followed by the SCN, OVN and YVN treatments ($p < 0.01$ in all pairwise comparisons except OVN–YVN; Fig. 7b, Supporting information).

Invertase test

We did not find significant differences among flower treatments and time intervals in the sugar composition ($\chi^2_{10} = 0.093$; $p = 1$). The constant sugar composition through time highlights the absence of the enzyme invertase in the nectar of *G. lutea*, as sucrose was not hydrolysed into glucose and fructose (Supporting information).

Pollen suspension test

The total protein and non-protein amino acid concentrations increased considerably (more than tenfold and more than thirtyfold for PAAs and NPAAs, respectively) after one hour from the addition of pollen grains of *G. lutea* to the sugar solution mimicking the YVN composition. Total amino acid concentration continued to increase after four hours and after 24 h from the pollen suspension, reaching more than 40 times the initial amino acid concentration. The PAA:NPAA ratio was very high at all intervals after the pollen suspension, and increased through time (Fig. 8).

After one hour from pollen suspension, proline was the most concentrated amino acid (3243.6 nmol/100 mg), representing more than 25% of the total amino acid content. After four hours the concentration of proline decreased considerably (382.9 nmol/100 mg), while the overall amino acid spectrum showed minor variations. After 24 h only a few peaks were detectable, and cysteine represented more than 70% of the total amino acid content (Supporting information).

Discussion

We found clear differences in the chemical composition of flower nectar of *Gentiana lutea* subsp. *symphyandra* in relation to flower stage and access by floral visitors. Nectar composition was relatively constant in uncontaminated nectar, especially in floral buds, while it became more variable when nectar was exposed to the external environment and to

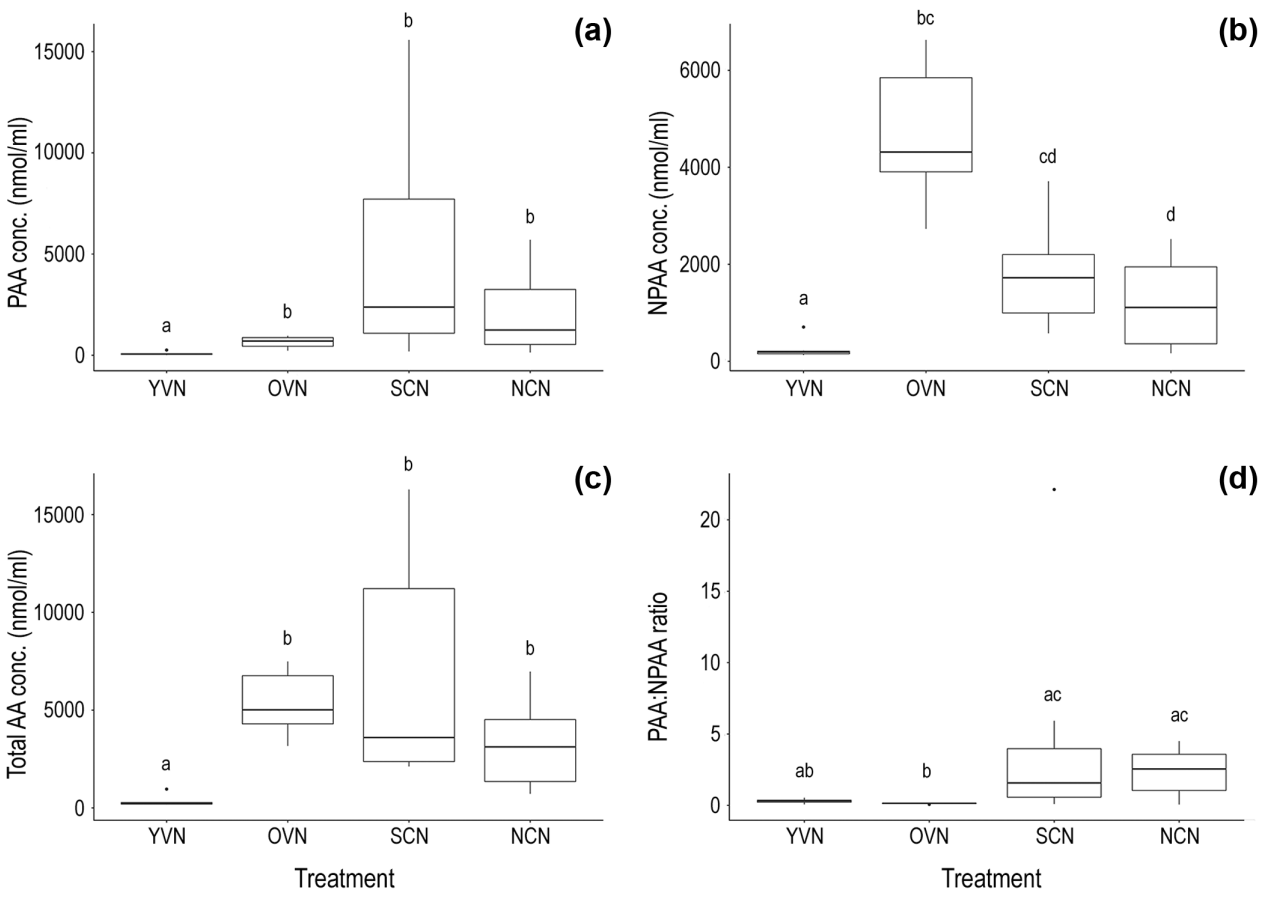

Figure 5. Concentration of (a) protein amino acids (PAA), (b) non-protein amino acids (NPAA), (c) total amino acids and (d) protein:non-protein amino acid ratio in the 32 nectar samples from the four flower treatments. Different letters indicate significant differences. YVN: young virgin nectar; OVN: old virgin nectar; SCN: self-contaminated nectar; NCN: naturally contaminated nectar. Note the different scales on the y-axes.

contamination by pollen grains and yeasts. Nectar sugar and amino acid concentration significantly increased with flower age and as a consequence of contamination by pollen grains. Contamination of nectar by pollen, either autonomously from flowers of the same plant or transported by floral visitors, enhanced nectar amino acid diversity and quantity. Nectar exposed to floral visitors showed a higher number of yeast cells than that of unvisited flowers, although yeasts likely did not significantly alter nectar composition because of their low overall density. These modifications are in turn expected to interact with floral visitors to shape plant-insect interactions.

Nectar sugar composition

The sugar profile of *G. lutea* is dominated by glucose and fructose. The absence of sucrose was not due to the activity of an extracellular invertase but rather to the presence of cell wall-bound invertase such as the one that is required for nectar production in *Arabidopsis* (Ruhlmann et al. 2010) or to other cytoplasmic invertases. Our results showed that the concentrations of glucose, fructose and total sugars followed the same pattern and were significantly different among all treatments. The freshly secreted and uncontaminated nectar presented the lowest sugar concentration, which markedly increased in older flowers. Nectar sugar concentration is often negatively correlated with flower age (Pacini and Nepi 2007), although opposite patterns can be observed (Petanidou et al. 1996, Nicolson and Nepi 2005, Pacini and Nepi 2007). Our results indicated that nectar sugar concentration in *G. lutea* significantly increases with flower age, regardless of external factors. In addition, although pollen only contains low amounts of sugars that could be released into nectar (Nakamura and Suzuki 1981, Pacini 1996, Pacini and Nepi 2007), the higher concentration of sugars found in nectar contaminated by self-pollen (SCN) than in old virgin nectar (OVN) suggests a significant contribution of sugars released by pollen.

The low sugar concentration in nectar of unbagged flowers (NCN) could be related to sugar consumption by yeasts whose presence was highest in this type of nectar. Lowering the sugar concentration is one of the most common impacts of yeasts on nectar quality (Pozo et al. 2015). However, the yeast cell concentration was very low, ranging between 0 and 481 yeast cells μl^{-1}, and only small changes in nectar chemistry have been detected when yeast concentration was lower

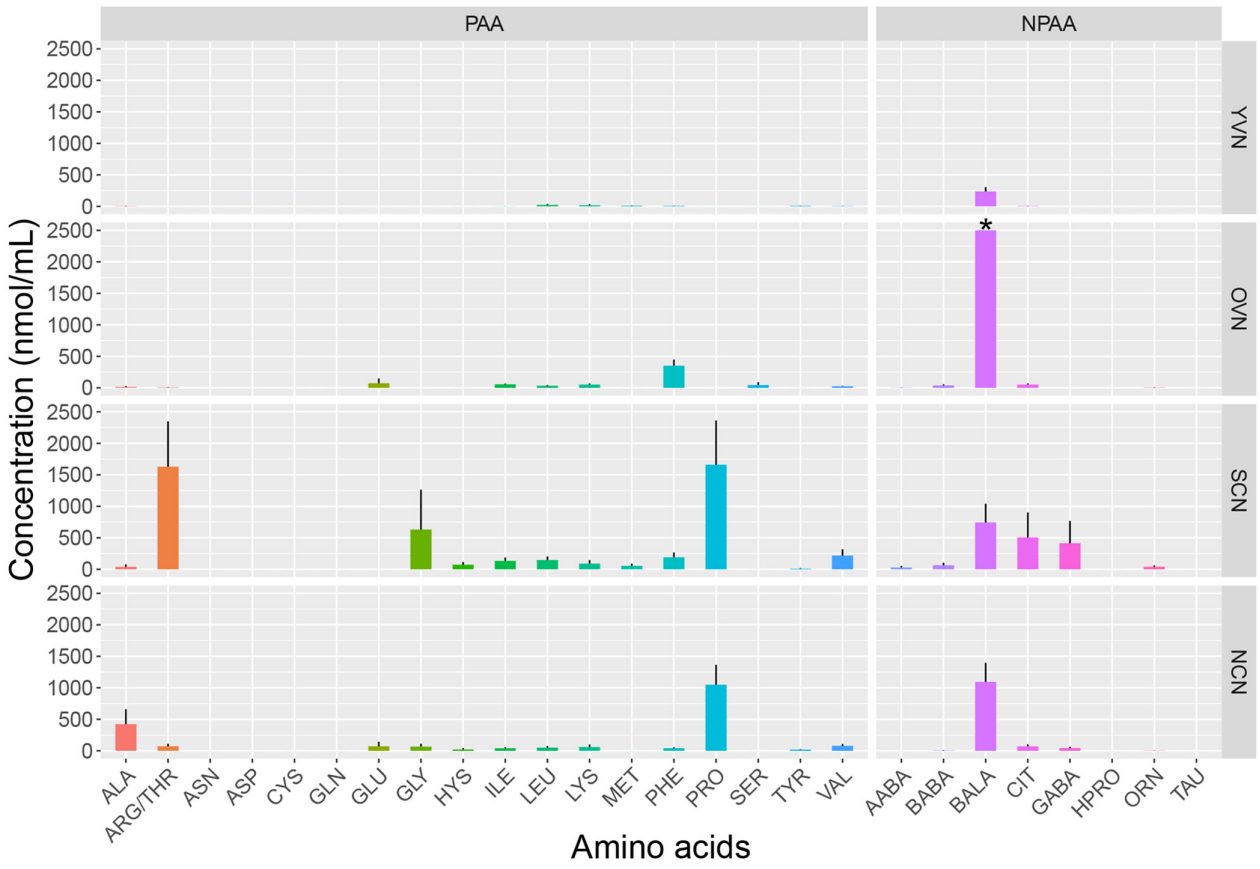

Figure 6. Mean concentration (± SE) of protein (PAA) and non-protein (NPAA) amino acids detected in the 32 nectar samples of the four nectar treatments. YVN: young virgin nectar; OVN: old virgin nectar; SCN: self-contaminated nectar; NCN: naturally contaminated nectar. *: concentration of β-alanine (BALA) in the OVN treatment was 4553 ± 480.9 nmol ml^{-1}. ARG/THR corresponds to the sum of the two amino acid concentrations, because it was not possible to separate them in the chromatograms.

than 10^4 cells μl^{-1} (Hausmann et al. 2017). The low overall concentration of yeast cells can be related to the hexose-dominant nectar of *G. lutea*, as yeasts are more commonly found in sucrose-dominant nectars (Herrera et al. 2008, Pozo et al. 2015). More likely, the low sugar concentration observed can be related to consumption by floral visitors (Pacini and Nicolson 2007), as nectar removal can decrease further sugar secretion by the plant (Galetto and Bernardello 1992, Rivera et al. 1996, Pacini and Nepi 2007).

The glucose:fructose ratio was close to one in all treatments except in the unbagged flowers. The lower concentration of fructose and the subsequent higher glucose:fructose ratio in visited flowers can be explained by the presence of microorganisms. Although yeasts can significantly alter the proportion of sugars in nectar (Canto and Herrera 2012, Pozo et al. 2015, Borghi and Fernie 2017, Schaeffer et al. 2017), they do so by hydrolyzing sucrose into glucose and fructose (Herrera et al. 2008, De Vega and Herrera 2013, Pozo et al. 2015). Because sucrose is very scarce in nectar of *G. lutea*, yeasts are likely not the main cause of the G:F ratio increase observed in visited flowers (NCN treatment). We therefore hypothesise that the low fructose concentration found in visited flowers (NCN treatment) was caused by some fructose-related bacteria, another abundant group of nectar-dwelling microorganisms (Vannette et al. 2013, Pozo et al. 2015).

Yeasts are frequently transported by floral visitors during their foraging bouts, either directly via their proboscis or indirectly through the transportation and involuntary release of pollen grains into nectar (Brysch-Herzberg 2004, Canto et al. 2008, Borghi and Fernie 2017, Hausmann et al. 2017). The lower concentration of conspecific pollen recorded in flowers open to visitors, compared to flowers where visitors were excluded, suggests that under natural conditions most of the pollen produced by *G. lutea* is removed by floral visitors, and that only a fraction contaminates its own nectar (< 31.5%). Although there was no difference in the total pollen content between self- (SCN) and naturally contaminated (NCN) nectar, results clearly indicate that most of the pollen found in the SCN treatment originated from *G. lutea* (> 75%), while almost 70% of the pollen in the NCN treatment belonged to different plant species. This suggests that the higher concentration of yeast cells found in the naturally contaminated nectar was mainly driven by floral visitors, either directly through contact with their proboscis or indirectly through contamination by foreign pollen.

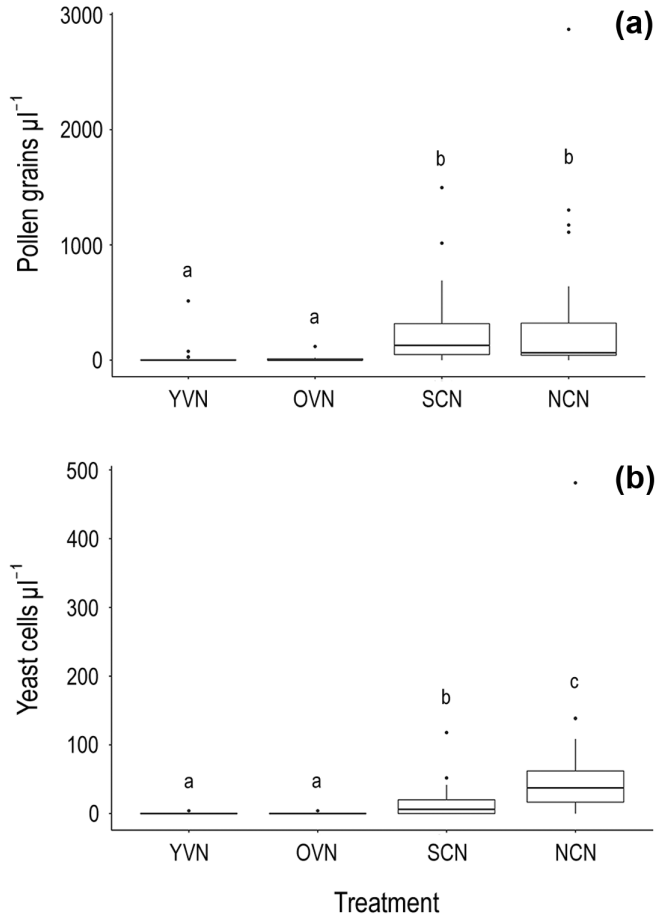

Figure 7. Concentration of (a) pollen grains and (b) yeast cells found in the 72 nectar samples of the four flower treatments. Different letters indicate significant differences. YVN: young virgin nectar; OVN: old virgin nectar; SCN: self-contaminated nectar; NCN: naturally contaminated nectar. Note the different scales on the y-axes.

Nectar amino acid composition

As for sugars, the freshly secreted and uncontaminated nectar had the lowest total amino acid concentration, which significantly increased in older flowers. This difference was mainly driven by a marked increase in non-protein amino acids. Nectar contaminated by self-pollen and by pollen from floral visitors had relatively high and comparable concentrations of amino acids. Moreover, the diversity of amino acids was significantly higher in the contaminated (SCN, NCN) than in the uncontaminated nectar (YVN, OVN). Our results clearly demonstrate a weak effect of yeast contamination and a strong effect of pollen contamination on amino acid diversity and abundance of floral nectar of *G. lutea*, because the total concentration and the profile of amino acids was similar in the SCN (self-pollen contaminated) and NCN (contaminated by pollen and yeasts) treatments. It has also been demonstrated that pollen contamination may increase yeast growth and play an important role in the population dynamics of nectar-dwelling yeasts (Pozo and Jacquemyn 2019).

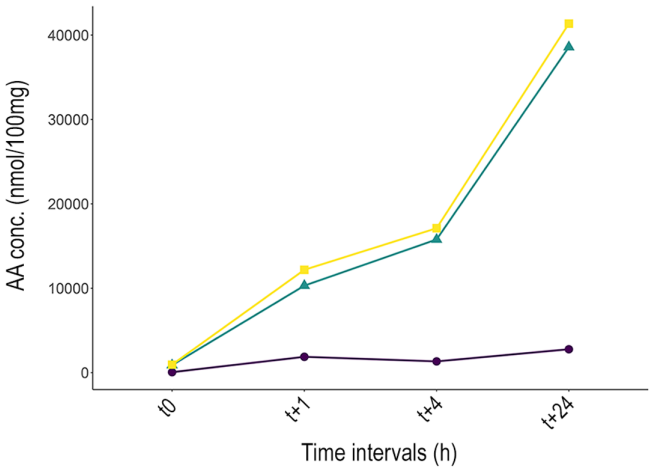

Figure 8. Concentration of total (yellow squares), protein (blue triangles) and non-protein (purple dots) amino acids (AA) at different time intervals after the suspension of pollen grains of *Gentiana lutea* in a solution mimicking nectar sugar composition of young virgin (i.e. uncontaminated) nectar (YVN). t0: after a few minutes, t + 1: after one hour, t + 4: after four hours and t + 24: after 24 h from pollen suspension.

β-alanine was the most abundant non-protein amino acid in all nectar treatments, and one of the most abundant amino acids overall. Since β-alanine was detected at high concentrations in both uncontaminated nectar treatments, we assume that it is produced by the plant. β-alanine is the most common non-protein amino acid in nectar, along with GABA (Nepi 2014), and has important roles in the functioning and regulation of plant physiology and metabolism. It mainly functions as a precursor of Coenzyme A and protects plants from several external stresses (Parthasarathy et al. 2019). Recently, it has been suggested that β-alanine may influence plant–pollinator relationships by affecting insects' nervous systems and stimulating the flight muscle function (Nepi 2014, Felicioli et al. 2018, Bogo et al. 2019). The high concentration of β-alanine produced by the nectar of *G. lutea* could therefore play an important role in the attraction of floral visitors.

The protein:non-protein amino acid ratio was higher in nectars contaminated by self-pollen and by pollen from floral visitors than in uncontaminated nectars. The significant amount of pollen grains observed in contaminated nectars is the most likely source of this increase. Although we only performed a single pollen supplementation test, these preliminary results clearly showed an increase of the total amino acids and in particular of protein amino acids. This was supported by the highest total and protein amino acid concentrations found in the self-contaminated nectar treatment (SCN), while the old uncontaminated nectar (OVN) presented a very low total amino acid concentration and protein amino acids were almost absent. The effect of pollen contamination on nectar amino acid concentration and composition needs further systematic examination, since few direct tests have been performed, and these have yielded contrasting results (Gottsberger et al. 1990, Nicolson 2007).

Both contaminated nectars presented a high concentration of proline, which was absent in uncontaminated nectars. Free proline accumulates in extremely high concentrations in the pollen of several species (Pálfi et al. 1987, Chiang and Dandekar 1995), reaching up to 70% of free amino acids (Hong-qi et al. 1982), and is usually the primary amino acid released in nectar-like solutions (Erhardt and Baker 1990, Gottsberger et al. 1990, O'Brien et al. 2003). It is therefore likely that the increase of proline content resulted from the large amount of pollen grains observed in contaminated nectars, originating either from within-plant pollen transfer or from pollen transported by floral visitors.

Nectar contamination can affect the activity of floral visitors. High proline concentration can increase nectar attractiveness towards floral visitors by stimulating insects' salt cells and consequently enhancing the intensity of feeding behaviour (Hansen et al. 1998, Wacht et al. 2000). Moreover, proline is degraded during the initial stages or the lift phase of insect flight (Micheu et al. 2000), acting as an efficient short-term fuel – more efficient than sugar – that results in quick bursts of energy production (Carter et al. 2006, Teulier et al. 2016).

In a previous study, Rossi et al. (2014) found similar concentrations of proline in different populations and subspecies of *G. lutea*, and hypothesised an endogenous production of proline rather than a contamination by exogenous pollen. However, our results indicate that pollen is the main source of proline in flower nectar of *G. lutea* subsp. *symphyandra*. Since all subspecies of *G. lutea* have generalist pollination and show similar pollinator communities (Rossi et al. 2014), we can expect that the similar patterns observed among nectars of different subspecies are driven by the same mechanism of contamination by floral visitors.

Conclusions

Nectar is the main interface between the plant and its floral visitors, which use it as the main source of energy. Our results show that the floral nectar implicated in this process is not the pure uncontaminated nectar secreted by the plant, but rather a complex intertwining of numerous endogenous and exogenous factors, partially mediated by visitors themselves. We found that nectar sugar and amino acid composition depends on both flower development and on nectar contamination by pollen and microorganisms. While the main impact of microorganism presence is the shift of glucose:fructose ratio, pollen contamination mainly increases the amino acid concentration and diversity. By transferring pollen grains and microorganisms to flowers while foraging, floral visitors appear to be the main indirect cause of major modifications of the nectar chemical composition. In turn, all these modifications are likely to modify the attractiveness of flowers towards floral visitors, in a complex process of ecological relationships.

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Author contributions

Gherardo Bogo and **Alessandro Fisogni** contributed equally to this publication. **Gherardo Bogo**: Formal analysis (equal); Visualization (equal); Writing – original draft (equal); Writing – review and editing (equal). **Alessandro Fisogni**: Formal analysis (equal); Investigation (equal); Visualization (equal); Writing – original draft (equal); Writing – review and editing (equal). **Joan Rabassa-Juvanteny**: Investigation (equal); Writing – review and editing (equal). **Laura Bortolotti**: Investigation (equal); Writing – review and editing (equal). **Massimo Nepi**: Conceptualization (equal); Methodology (equal); Writing – review and editing (equal). **Massimo Guarnieri**: Investigation (equal); Writing – review and editing (equal). **Lucia Conte**: Project administration (equal); Writing – review and editing (equal). **Marta Galloni**: Conceptualization (equal); Investigation (equal); Methodology (equal); Project administration (equal); Supervision (equal); Writing – review and editing (equal).

Data availability statement

Data are available from the Zenodo Digital Repository: <http://doi.org/10.5281/zenodo.4265415>.

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