



Nectar composition in moth-pollinated *Platanthera bifolia* and *P. chlorantha* and its importance for reproductive success

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Abstract

Main conclusion Sugars (glucose, fructose and sucrose), as well as proteogenic and non-proteogenic amino acids, are present in the nectar of *Platanthera bifolia* and *P. chlorantha*.

Abstract Nectar quantity and quality are floral traits that are subjected to pollinator-mediated selection. Nectar sugar and amino acid (AA) composition in two sister species, *P. bifolia* and *P. chlorantha*, was analysed and the interspecies differences in nectar and the importance of these nectar characteristics for reproductive success were investigated. Nectar was collected from four *P. bifolia* and three *P. chlorantha* populations that exist in different habitats in three regions of NE Poland. Nectar from about 30 flowers (from each population) was sampled and analysed using high-performance liquid chromatography. We found the same primary sugars and AA components in the nectar of both species, although their content varied between the populations according to habitat properties. The nectar of *P. bifolia* and *P. chlorantha* both had low sugar concentrations (9.04–20.68%) and were dominated by hexoses, with sucrose:hexoses ratios between 0.03 and 0.31 across the different populations (the average for the *P. bifolia* populations was 0.17 and the average for the *P. chlorantha* populations was –0.05). Total sugar content did not influence reproductive success and we found positive selection on fructose content. In general, 23 different AAs were detected in both *Platanthera* species. Cysteine and γ -aminobutyric acid were present in only one population of *P. chlorantha*. Sarcosine dominated among the non-proteogenic AAs. To our knowledge, this is the first report that characterizes the sugar and AA profiles in the nectar of *P. bifolia* and *P. chlorantha* in natural populations in the context of effectiveness of reproduction. Total AAs negatively influenced male reproductive success ($r = -0.79$). Pollinators of the investigated species were found to be sensitive to the AAs' taste, from taste classes I and IV. Correlation between male reproductive success and the content of AAs from these groups was 0.79 in both cases. In this manuscript, we investigated the characteristics of *P. bifolia* and *P. chlorantha* nectar, and compared these characteristics to the available data in the context of their adaptations to the requirements of pollinators and with regard to the importance of nectar quality for reproductive success of the studied species.

Keywords Amino acids · NE Poland · Sugars · Pollination success

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Introduction

The wide varieties of flower types that have evolved in plants are adapted in different ways to their pollinators. The structure of the flower and other traits that include colour or scent are often subjected to pollinator-mediated selection (Maad 2000; Leiss and Klinkhamer 2005; Nepi et al. 2018; Parachnowitsch et al. 2019). One of these traits being the evolutionary answer for pollinators' preferences is nectar. Many studies document that nectar concentration and composition are often linked with pollinator types (Baker and Baker 1983; Nicolson and Thornburg 2007; Petanidou 2007; Witt

et al. 2013; Fowler et al. 2016; Tiedge and Lohaus 2017). Pollinators have different requirements with respect to nectar content, both for sugar and amino acid (AA) concentrations, composition and other nectar components such as nectarins, vitamins, phenolics, alkaloids, terpenoids, lipids, metal ions and phytohormones (Baker and Baker 1983; Adler 2000; Petanidou 2007; Gijbels et al. 2014, 2015a, b; Roy et al. 2017; Tiedge and Lohaus 2017).

Most studies report three main sugar components in nectar: sucrose, glucose and fructose, which occur in different proportions, although sucrose is most often considered to be the dominant ingredient (Nicolson and Thornburg 2007; Fowler et al. 2016; Parachnowitsch et al. 2019). Nevertheless, plants that produce nectar that is dominated by hexoses are not so rare (Nocentini et al. 2013). Other carbohydrates, e.g. mannose, xylose and maltose are present in floral nectars less frequently (Nicolson and Thornburg 2007). Some authors suggest that the sugar ratio [sucrose/(glucose + fructose)] is linked to the different types of pollinators (Baker and Baker 1990; Nicolson and Thornburg 2007; Witt et al. 2013). The preferences of pollinators are partially caused by the adaptation of their mouthparts to use nectar with a given level of sugar concentration that is dependent on different viscosities and flux. Heyneman (1983), on the basis of data reported by Pyke and Waser (1981) and Baker and Baker (1982), reported that the highest concentration of sugars in nectar is characteristic of bee-pollinated flowers (on average 35%), while the lowest is typical for those pollinated by bats (17%) or hawk moths (19%). Further studies have expanded this information and verified the concept. For example, Willmer (2011) documented that the optimal feeding rates of lepidopteran occur with higher sugar concentrations (35–45%) than earlier authors reported. Simultaneously, Willmer (2011) noted that nectar with a sugar concentration above 30–40% is difficult to suck up for most lepidopterans because of their long tongues, which require dilute nonviscous nectar. Pollinators' preferences also result from the energetic costs connected with their behaviour. For example, hawk moths feed primarily while hovering and mainly at night (at lower temperatures); thus, this type of activity requires more energy (Willmer 2011; Fowler et al. 2016).

The next group of nectar components that play an important role in plant–pollinator interactions is AAs (Nicolson and Thornburg 2007; Roy et al. 2017; Nepi et al. 2018). Despite their concentrations being much lower than sugars, AAs are the basic source of nitrogen for animals that feed on nectar and different AAs may cause different gustatory and stimulatory effects (Gardener and Gillman 2002; Zhang et al. 2006; Fowler et al. 2016). Some studies document that insects feeding on nectars that are rich in AAs are larger, have greater longevity and produce more offspring with higher fitness (Mevi-Schütz and Erhardt 2005; Fowler

et al. 2016). Available studies have also documented the differentiated composition of AAs in nectar from different taxonomic and ecological plant groups and determined the preferences of pollinators for specific AAs (Willmer 2011; Nocentini et al. 2013; Antoñ et al. 2017; Tiedge and Lohaus 2017).

Many studies have reported the presence of other compounds in nectar. These include non-proteogenic AAs and secondary metabolites, such as alkaloids, phenolics, iridoid glycosides, and glucosinolates cardenolides, which have varying functions (Baker 1977; Adler 2000; Nicolson and Thornburg 2007; Vranová et al. 2010; Roy et al. 2017; Nepi et al. 2018; Parachnowitsch et al. 2019). The majority of the published data suggest that these compounds generally reduce pollinator preferences and simultaneously decrease reproductive success (Adler 2000; Parachnowitsch et al. 2019). For example, experimental studies conducted by Adler and Irwin (2005, 2012) revealed that with higher levels of gelsemine in nectar from *Gelsemium sempervirens*, parameters that quantify reproductive success are decreased. On the other hand, Kessler et al. (2008) found that nicotine in *Nicotiana attenuata* reduces nectar robbing, florivory and nectar consumption, which results in increased reproductive success. Some studies document that pollinators' responses to secondary metabolites can be concentration dependent. At higher concentrations, visitors are most often deterred, while at lower concentrations, nectar may be attractive. This effect translates into higher levels of reproduction (Singaravelan et al. 2005; Manson et al. 2013; Thomson et al. 2015; Baracchi et al. 2017). Moreover, some secondary metabolites (e.g. gelsemine, anabasine or nicotine) may benefit pollinators by increasing their resistance to herbivores, parasites and pathogens (Stevenson et al. 2017; Nepi et al. 2018).

It should be noted that the quantity and quality of nectar depend on pollinators' preferences and also on other biotic and abiotic factors that include the presence of microbes, availability of soil resources, water availability, sun exposure and/or weather conditions (Gardener and Gillman 2001; Nicolson and Thornburg 2007; Farkas et al. 2012; Nocentini et al. 2013; Gijbels et al. 2014, 2015a, b; Nepi et al. 2018; Parachnowitsch et al. 2019).

Because nectar composition is often subjected to strong pollinator-mediated selection (Leiss and Klinkhamer 2005; Gijbels et al. 2015a, b) its quantity and quality greatly affect the reproductive success of nectariferous plants (Tremblay et al. 2005; Duffy and Stout 2011; Willmer 2011; Gijbels et al. 2014, 2015a, b; Pyke 2016). Generally, the majority of the previous studies have reported that greater nectar volume increases pollinators' visitation rate and in effect, increases their reproductive success (Neiland and Wilcock 1998; Tremblay et al. 2005; Nepi et al. 2018), although others have noted results that contrast with this finding or found only a weak (or no) relationship between nectar characteristics and

the level of reproduction (Ackerman et al. 1994; Duffy and Stout 2011). Nectar may not simply function as a reward, increasing visitation rates and furthering reproduction levels, but may also be a ‘manipulator’ of pollinator behaviour, which can also be an important factor shaping reproductive success (Pyke 2016). Despite the undeniable importance of nectar quantity and quality for reproductive success, its presence can have negative effects. It can promote self-pollination and in consequence, increase inbreeding depression, thus reducing the fitness of progeny (Jersáková and Johnson 2006).

Many representatives of the Orchidaceae attract pollinators by offering different rewards, e.g. resins, oil, fragrance or pseudopollen, out of which nectar is most effective (Neiland and Wilcock 1998; Tremblay et al. 2005). Nevertheless, although problems concerning pollinator-mediated selection on floral traits in this group are among the most important and are often examined, there are surprisingly few data regarding the composition of nectar in orchids, especially in comparison to the large number of species that belong to this family. Only a few studies can be found on this topic in orchids, and these are mainly in a restricted range in their reporting of nectar composition (Stpiczyńska and Pielecki 2002; Stpiczyńska 2003a, b; Martins and Johnson 2007; Gijbels et al. 2014, 2015a, b). Some of the most detailed studies were conducted by Stpiczyńska (2001, 2003a) and Stpiczyńska and Pielecki (2002) on *P. chlorantha*. They report the nectar secretion, chemistry and its dynamics during the flowers’ life under laboratory conditions. The recent studies by Lindqvist et al. (2018), which tested a novel technique, are methodical in their character and analysed sugars from only 18 flowers of the two *Platanthera* species. Both species were intensively investigated in the context of pollination biology, spatial variation of reproductive success (focusing on pollinator-mediated selection of floral traits), hybridization and genetic diversity (Nilsson 1983, 1985; Brzosko 2003; Maad and Alexandersson 2004; Maad and Nilsson 2004; Bateman and Sexton 2008; Brzosko et al. 2009; Bateman et al. 2012; Claessens and Kleynen 2011; Steen 2012; Steen and Mundal 2013; Brzosko and Wróblewska 2013; Boberg et al. 2014; Sexton 2014; Esposito et al. 2017; Durka et al. 2017; Duffy and Stout 2011; Esposito et al. 2018; Mötler et al. 2018). Our interest stems from the fact that this species pair is a suitable model system to study co-evolutionary processes. The main differences between the two *Platanthera* species are column structure and connected with this structure’s pollinaria attachment (tongue attachment in *P. bifolia* and eye attachment in *P. chlorantha*) as well as the ethological mechanism, resulting from the differential attraction of pollinators due to differences in floral scent chemistry (Nilsson 1983, 1985; Esposito et al. 2017). These differences result in moths that belong to the noctuids being better adapted to pollinate *P.*

chlorantha, while sphingids are the main pollinators of *P. bifolia*, although both *Platanthera* species may share the main pollinators (Nilsson 1983).

In the context of the above-mentioned problems, it is interesting to examine whether nectar composition is the next trait that differentiates these two sister species. Therefore, the main aims of these experiments were to determine answers for the following questions: (1) What is the composition of nectar produced by *P. bifolia* and *P. chlorantha*? (2) Is nectar chemistry species or habitat dependent? (3) Is reproductive success in populations of both species influenced by nectar content and composition?

Materials and methods

Studied species and localization

The ‘Butterfly Orchids’ *Platanthera bifolia* L. (Rich.) and *P. chlorantha* (Cust.) Rchb. are a closely related and widely distributed species, which largely occur in sympatry (Hultén and Fries 1986). Both species have overlapping flowering times. The inflorescences of both species develop 10–25 white flowers with a long spur containing nectar. The spur length is differentiated within a geographic range, which is connected with the proboscis length of local pollinators (Nilsson 1985; Maad and Nilsson 2004; Bateman and Sexton 2008; Boberg and Ägren 2009; Claessens and Kleynen 2011). According to Bateman and Sexton (2008), *P. chlorantha* usually possesses longer spurs than *P. bifolia*, although both short- and long-spurred populations of these two species have been reported (Boberg et al. 2014). The two *Platanthera* species differ significantly in flower morphology. In *P. bifolia*, the pollinaria have very short caudicles that are positioned almost parallel to each other, and the distance between viscidia is much smaller than in *P. chlorantha*. Moreover, the column in *P. bifolia* is small with a narrow connective and in *P. chlorantha* the column is wide with a broad connective (Maad and Nilsson 2004). These differences result in the pollinaria of *P. bifolia* being attached to the moths’ proboscis, while in *P. chlorantha* they are attached to the eyes. In effect, *P. bifolia* is predominantly adapted to pollination by hawk moths (Sphingidae) and *P. chlorantha* by noctuids (Noctuidae) (Nilsson 1983). The two *Platanthera* species also differ according to their floral scent chemistry; thus, they attract pollinators in different ways, which is an ethological mechanism of reproductive isolation that reduces hybridization (Nilsson 1983, 1985; Esposito et al. 2018).

This study was performed during 2017 at three locations in NE Poland and included four *P. bifolia* (SMOL, POG, POB1 and BC) and three *P. chlorantha* (BON, LIN, BF) populations. Populations were localized in Białowieża

National Park (two populations), Biebrza National Park (1 population) and Suwałki Landscape Park (four populations) (Table 1). These populations existed in different plant communities: two populations (one of each species, LIN and BC) in multispecies meadows and the remaining in forests or under tree canopy (Table 1).

Reproductive success

To evaluate the reproductive success (RS) in *P. bifolia* and *P. chlorantha* populations, during the peak of flowering, we marked the inflorescences and counted the number of flowers. During late July, female and male reproductive success was quantified. Female reproductive success (FRS) was evaluated (as a percentage) as the proportion of fruits developed to the sum of flowers on the inflorescence. Male reproductive success (MRS) was determined as the percent of pollinaria removal for each plant to the total number of pollinaria present on the inflorescence. The number of plants for which reproductive success was determined is shown in Table 1.

Chemicals

Standards of AAs: L-aspartic acid (Asp), L-glutamic acid (Glu), L-arginine (Arg), L-histidine (His), L-lysine (Lys), glycine (Gly), L-alanine (Ala), L-isoleucine (Ile), L-leucine (Leu), L-methionine (Met), L-phenylalanine (Phe), L-proline (Pro), L-tryptophan (Trp) L-valine (Val), L-citrulline (Cit), L-hydroxyproline (Hyp), L-norvaline (Nva), L-ornithine

(Orn), sarcosine (Sar), taurine (Tau), α -aminobutyric acid (AABA), β -alanine (β -Ala), β -aminobutyric acid (BABA), γ -aminobutyric acid (GABA), L-asparagine (Asn), L-cysteine (Cys), L-glutamine (Gln), L-serine (Ser), L-threonine (Thr), L-tyrosine (Tyr) were purchased from Sigma-Aldrich (St. Louis, MO, USA) in ACS purity. Other chemicals: *o*-phthalaldehyde (OPA) and 9-fluorenylmethyl chloroformate (Fmoc) reagents, borate buffer (0.4 N in water, pH 10.2), NaH_2PO_4 and NaOH, were also purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (ACN), methanol (MeOH) and water were purchased from Merck KGaA (Darmstadt, Germany) in LC–MS grade.

Determination of amino acids and sugars in nectar

The nectar standing crop (nectar column) was measured in the field to the nearest 0.01 mm using an electronic calliper. For all populations, the nectar column was recorded in the same phase of inflorescence development in flowers open to flower visitors. During measurements, the vast majority of them did not have any marks of visitation (pollinaria were present and massules on the column were absent). The measurements were done for the lowest flower on the inflorescence (the first flower from the bottom) for all plants that reproductive success was evaluated. To analyse nectar composition in each of seven populations, nectar from about 30 of the lowest flowers on the inflorescence was sampled. A single analytical sample was nectar from about 30 flowers from about 30 individuals in each population. Nectar from the flowers of each population was placed in a 200- μL

Table 1 Characteristics of *Platanthera bifolia* i *P. chlorantha* populations

Region of NE Poland	Population code	Type of habitat and community	Number of individuals analysed for RS	FRS (%)	MRS (%)	Nectar column length (mm) \pm SE
Suwałki Landscape Park	SMOL	The remnant forest fragment with domination of <i>Betula pendula</i> and <i>Picea abies</i> in tree canopy and poor undergrowth layer	90	73.7	80.1	8.69 \pm 4.50
	POB1	Dense, young, artificial spruce forest with small gaps dominated by grasses	62	26.6	56.9	7.61 \pm 3.53
	LIN	Multispecies meadow surrounded by open areas	96	57.0	95.2	7.87 \pm 3.04
	BON	Broadleaved forests	36	52.5	78.9	9.80 \pm 5.55
Biebrza National Park	POG	The border of alder forest and peat bogs, mainly in open area	46	60.2	85.9	15.94 \pm 5.05
Białowieża National Park	BC	Multispecies meadow surrounded by forests	78	17.8	86.8	5.80 \pm 5.42
	BF	Broadleaved forests	72	73.9	87.3	4.49 \pm 3.10

Eppendorf tube prior to analysis using high-performance liquid chromatography (HPLC). The samples were frozen ($-20\text{ }^{\circ}\text{C}$) until required. Nectar was diluted with water to a volume of $50\text{ }\mu\text{L}$ ($10\text{ }\mu\text{L}$ of nectar and $40\text{ }\mu\text{L}$ of distilled water) for sugar analysis and of $20\text{ }\mu\text{L}$ ($10\text{ }\mu\text{L}$ of nectar and $10\text{ }\mu\text{L}$ of distilled water) for AA analysis. The sample was filtered through a spin column with a $0.4\text{-}\mu\text{m}$ -pore size membrane filter (A&A Biotechnology, Poland) by centrifugation for 2 min at $9000g$ (relative centrifugal force) before injection. The supernatant was loaded into the insert and analysed by HPLC. Analyses were performed using an Agilent Technologies 1260 Infinity series system consisting of a 1260 Infinity Agilent Quaternary pump G1311B, a 1260 Infinity Diode Array Detector (DAD) G1315D, a 1260 Infinity Fluorescence Detector (FLD) G1321B, a 1260 Infinity ALS G1329B Automated Sample Injector, a 1290 Infinity Autosampler Thermostat G1330B, and a thermostated column oven 1290 Infinity TCC G1316C. The system was controlled by Agilent OpenLab ChemStation software.

A ZORBAX Carbohydrate Analysis Column ($4.6\text{ mm}\times 250\text{ mm}$, $5\text{ }\mu\text{m}$) was used for sugar separation and analysis. A $10\text{ }\mu\text{L}$ aliquot sample or standard solution was injected. The separation was conducted at $30\text{ }^{\circ}\text{C}$ with the mobile phase comprising ACN:water (70:30, v/v) at a flow rate of 1.4 mL min^{-1} . The analytical data were integrated using the Agilent OpenLab CDS ChemStation software for liquid chromatography systems. Identification of sugars was performed by comparing retention times of individual sugars in the reference vs. test solution. The content of glucose, fructose, sucrose, maltose, and lactose was assayed based on comparisons of peak areas obtained for the samples investigated with those of the reference solutions.

Amino acid analysis was performed with gradient HPLC using an Agilent Zorbax Eclipse Plus C_{18} ($4.6\times 150\text{ mm}$, $5\text{ }\mu\text{m}$) column with a guard, i.e. Agilent Zorbax Eclipse Plus C_{18} ($4.6\times 12.5\text{ mm}$, $5\text{ }\mu\text{m}$). The extracts containing primary and secondary AAs were pre-column derivatized using an OPA and FMOC reagent. An injector program was used for derivatization. After the derivatization step, a mixture of each sample was injected into a pre-equilibrated column operated at $40\text{ }^{\circ}\text{C}$. The primary AAs (OPA-derivatized) were monitored at 388 nm by DAD and the secondary AAs (FMOC-derivatized) were monitored by FLD at an excitation wavelength of 266 nm and an emission wavelength of 305 nm . Mobile phase A was $40\text{ mM NaH}_2\text{PO}_4$ (pH 7.8 adjusted using 10 M NaOH solution), while mobile phase B was ACN:MeOH:water (45:45:10, v/v/v). Gradient profile was the following: 0–5 min, 0% B to 10% B; 5–25 min, 10% B to 40.5% B; 25–30 min, 40.5% B to 63% B; 30–35 min, 63% B to 82% B; 35–37 min, 82% B to 100% B; 37–39 min, 100% B; 39–40 min, 100% B to 0% B; 40–43 min, 0% B. A flow rate of 1 mL min^{-1} was used. The total analysis time was 43 min.

Statistics

All statistical analyses were performed using R software (R Core Team 2018). Nectar column length in spurs, FRS and MRS for each population of *P. bifolia* and *P. chlorantha* were further assessed for normality and homogeneity of variances using the Shapiro–Wilk test [‘stats’ package (R Core Team 2018)] and Levene’s test [‘car’ package (Fox and Weisberg 2011)], respectively. The one-way ANOVA (‘stats’ package) was used to determine whether any statistically significant differences exist between parameters. Pearson’s correlation [‘rcorr’ function from ‘Hmisc’ package (Harrell Jr 2018)] was also calculated between these parameters. Due to the small number of populations, we pooled data for the two species when assessing correlations between the parameters analysed. Such an approach is legitimate because the sugars and AAs were the same in both species.

Prior to more sophisticated statistical analysis of AAs and sugars, a set of descriptive statistics (n , mean, standard deviation, minimum, maximum, 95% mean confidence interval) was calculated for 21 AAs (two compounds were omitted due to single detection in seven populations; seven AAs were also omitted due to their absence in all populations) and three sugars with and without grouping by two plant species, i.e. *P. bifolia*, *P. chlorantha*. The data were further assessed for normality using the Shapiro–Wilk test (‘stats’ package). Data were considered to be normally distributed if $p\geq 0.05$ (Table 1S).

To check if a monotonic relationship exists between sugars, Spearman’s rank correlations were calculated using the ‘rcorr’ function from the ‘Hmisc’ package and visualized as a matrix of coefficients and significance levels. Spearman’s correlation was used because only one sugar (glucose) was considered to be normally distributed (Table 1S). To check for the existence of any monotonic relationships between the AAs, Spearman’s correlations were also calculated using ‘rcorr’ function and visualized as a heatmap using the ‘corrplot’ package (Wei and Simko 2017). Spearman’s correlation was used due to the fact that a few AAs were considered not normally distributed (Table 1S). All correlations were considered significant for $p < 0.05$.

To detect the structure and general regularities in the relationships between AAs and to verify detected connections, principal component analysis (PCA) was used. A function named ‘is.singular.matrix’ from the package ‘matrixcalc’ (Novomestky 2015) was used to check if the correlation matrix of all AAs is singular: at least one of its rows (or one of the columns) is a linear combination of the other rows (or columns) of the matrix. Performing the Gauss Jordan elimination method on the Spearman correlation matrix (‘rref’ function from package ‘pracma’) (Borchers 2018) indicated that up to six AAs can be selected to produce a non-singular correlation matrix. An

iterative algorithm was used to retrieve a combination of six AAs that has the highest Kaiser–Meyer–Olkin (KMO) test result value, i.e. measure of sampling adequacy (MSA), because its value close to 1 indicates that the sum of partial correlations is not large relative to the sum of the correlations and so factor analysis should yield distinct and reliable factors (Hair Jr et al. 2014). The following AAs: Asn, His, Cit, Orn, Lys and Sar were selected; their MSA was 0.8769 (Table 2S). To calculate KMO test, ‘psych’ package was used (Revelle 2018). According to Kaiser (1974), the received MSA value is meritorious; therefore, PCA could be performed. A function ‘PCA’ from the ‘FactoMineR’ package was used for this purpose (Lê et al. 2008). Values were normalized (scale.unit = TRUE) and the remaining 15 AAs were assigned as supplementary variables (quanti.sup). A scree plot (Fig. 1S) was created using the ‘fviz_eig’ function from the ‘factoextra’ package (Kassambara and Mundt 2017). A decision was made that two factors, which explain 89.45% of the variance, are sufficient (Fig. 1S, Table 3S). A plot with the quality of the representation of AAs on the new dimensions (factors), created by PCA, (Fig. 2S) was prepared using the ‘corrplot’ package. Furthermore, PCA variables factor map and biplot were created using ‘fviz_pca_var’ and ‘fviz_pca_biplot’ functions, respectively (in the ‘factoextra’ package).

To analyse the effect of AAs on insect chemoreceptors, all identified and determined AAs were grouped into four classes: I. Asn, Gln, Ala, Cys, Gly, Ser, Thr and Tyr (no effect on the chemoreceptors of fly); II. Arg, Asp, Glu, His and Lys (inhibition of fly chemoreceptors); III. Pro and Hyp (stimulate the salt cell); IV. Ile, Leu, Met, Phe, Trp and Val (ability to stimulate the sugar cell) and presented as a ternary plot (‘ggtern’ package) (Hamilton and Ferry 2018).

Results

Reproductive success

Fruiting shaped at the lowest level in two *P. bifolia* populations (17.8% in BC and 26.6% in POB1), while the highest was in BF *P. chlorantha* and in SMOL *P. bifolia* populations (almost 73.7%, Table 1). The MRS was higher than FRS. The lowest was noted in the POB1 *P. bifolia* population (56.9%); however, in the remaining populations, it ranged between 78.9% and 95.2%. Statistically significant differences are reported for the levels of reproductive success between populations of *P. bifolia* (FRS: $F=60.128$; MRS: $F=9.274$; $p<0.001$) and *P. chlorantha* (FRS: $F=7.624$; MRS: $F=11.215$; $p<0.001$).

Nectar content and chemistry

Nectar column length in spurs was different in populations of both species. The lowest nectar column among four *P. bifolia* populations was observed in BC population (5.79 ± 5.42 mm), which was almost three times lower than that in the POG population with the highest nectar column (15.94 ± 5.05 mm) (Table 1). In the case of *P. chlorantha*, the lowest nectar content was also noted in Białowieża National Park, in the BF population (4.49 ± 3.09 mm), while the highest was in the BON population (9.80 ± 5.55 mm). Significant differences of nectar column were noted between both *P. chlorantha* ($F=17.415$, $p<0.001$) and *P. bifolia* populations ($F=32.292$, $p<0.001$). Nectar column length influenced FRS in *P. bifolia* ($r=0.23$, $p<0.05$) and MRS in *P. chlorantha* populations ($r=0.19$, $p<0.05$).

Nectar in studied populations differed in sugar concentration. The highest (1103.1 mM) was measured in the BF *P. chlorantha* population and the lowest (430.7 mM) was measured in the POB1 *P. bifolia* population (Table 2). On average, nectar of *P. chlorantha* contained more sugars (842.9 mM) than *P. bifolia* (599.5 mM). The amount of sugars was relatively low and ranged between 90.4 and 206.8 mg/mL (Table 3). In both *Platanthera* species, sugar components found in the nectar included glucose, sucrose and fructose (at varying ratios) in each of the population (Tables 2, 3). In the studied populations, hexoses (glucose and fructose) dominated in the nectar, with higher concentrations of glucose being present except for the POG *P. bifolia* populations where the levels of glucose and sucrose were similar. The highest percentages of sucrose were found in two *P. bifolia* populations, POG and POB1, where sucrose/hexoses ratios equalled 0.59 and 0.43, respectively. In the remaining populations, this ratio was in the range of 0.06–0.14 (Table 3). On average, the nectar of *P. chlorantha* contained more glucose and fructose in comparison to *P. bifolia*, while in the nectar of *P. bifolia*, there was about two times more sucrose than in *P. chlorantha*. No statistically significant correlations were found between the total sugar content in nectar and MRS or FRS. Fructose content positively influenced both MRS ($r=0.82$, $p<0.05$) and FRS ($r=0.79$, $p<0.05$). Moreover, no statistically significant correlations was found between glucose, fructose or sucrose (Table 4).

In general, 23 different AAs were detected in seven populations of both species, but the number of detected AAs differed in particular populations. The lowest (9 AAs) was in BC *P. bifolia* population, and the highest (20 AAs) was in POB1 *P. bifolia* and BON *P. chlorantha* populations (Table 5). Cys and GABA were present only in LIN *P. chlorantha*. However, seven AAs (Thr, Trp, Leu, Tyr, β -Ala, Tau, Nva) were detected neither in *P. bifolia* nor in *P. chlorantha*. Ser was a dominant AA in four populations: POG, SMOL, POB1 and BON (about 25%). However, Sar dominated in

Table 2 The concentration of sugars (mM) in *Platanthera bifolia* and *P. chlorantha* nectar with descriptive statistics

	<i>P. bifolia</i>				Mean ^a	SD ^a	Lower quartile ^a	Median ^a	Upper quartile ^a
	POG	BC	SMOL	POB1					
Glucose	283.23	431.39	416.11	175.69	326.61	120.62	256.35	349.67	419.93
Fructose	199.77	198.01	205.21	175.74	194.68	12.99	192.44	198.89	201.13
Sucrose	150.13	46.21	37.43	79.27	78.26	51.19	44.02	62.74	96.99
Average	211.04	225.2	219.58	143.57					
Sum of sugars	633.1	675.6	658.8	430.7					

	<i>P. chlorantha</i>			Mean ^b	SD ^b	Lower quartile ^b	Median ^b	Upper quartile ^b
	BON	BF	LIN					
Glucose	496.43	611.42	394.31	500.72	108.62	445.37	496.43	553.92
Fructose	161.02	441.75	314.17	305.65	140.56	237.59	314.17	377.96
Sucrose	36.93	49.93	22.64	36.50	13.65	29.79	36.93	43.43
Average	231.46	367.7	243.71					
Sum of sugars	694.4	1103.1	731.1					

SD standard deviation

^aCalculated using a sugar value of POG, BC, SMOL, POB1 population

^bCalculated using a sugar value of BON, BF, LIN1 population

Table 3 The amount of sugars (mg/mL) in *Platanthera bifolia* and *P. chlorantha* nectar

	<i>P. bifolia</i>				<i>P. chlorantha</i>		
	POG	BC	SMOL	POB1	BON	BF	LIN
Glucose	51.03	77.72	74.96	31.65	89.44	110.15	71.04
Fructose	35.99	35.67	36.97	31.66	29.01	79.58	56.60
Sucrose	51.39	15.82	12.81	27.14	12.64	17.09	7.75
Sum of sugars	138.40	129.21	124.75	90.45	131.08	206.83	135.39
Sugar content in nectar (w/v) (%)	13.84	12.92	12.47	9.04	13.11	20.68	13.54
Ratio fructose/glucose	0.71	0.46	0.49	1.00	0.32	0.72	0.80
Sucrose/(fructose + glucose)	0.59	0.14	0.11	0.43	0.11	0.09	0.06

Table 4 Correlation matrix for sugars ($n = 7$)

	Glucose	Fructose	Sucrose
Glucose	1	0.21 $p = 0.6445$	-0.36 $p = 0.4316$
Fructose	0.21 $p = 0.6445$	1	-0.07 $p = 0.8790$
Sucrose	-0.36 $p = 0.4316$	-0.07 $p = 0.8790$	1

Values indicate the Spearman’s rank correlation coefficient with appropriate p values

the remaining three populations: BC, BF and LIN. Total AA concentrations negatively influenced pollinaria removal ($r = -0.79, p < 0.05$).

Among the AAs detected in the nectar of the *Platanthera* species, only Ile influenced reproductive success. A statistically significant negative selection of Ile on MRS and FRS ($r = -0.89$ and $r = -0.83, p < 0.05$, respectively) was observed.

Strong to ‘perfect’ positive rank correlations ($r_s = 0.77-1.00, p < 0.05$) were found between the same or different type (polar, acidic and polar, basic and polar, non-polar and non-proteinogenic) AAs, e.g. Ser vs. Asp $r_s = 1$, Lys vs. Met $r_s = 1$, Arg vs. Orn $r_s = 0.83$ and Gly vs. Orn $r_s = 0.77$ (Fig. 1).

Negative scores for the first principal component (Dim1) indicate higher values of Hyp, Pro and AABA than mean values for all the studied populations although these values are positive for other AAs. Moreover, positive scores for the second principal component (Dim2) indicate values for Pro, Hyp, Gln, Phe, BABA, Asn, Ile, Orn and Val that are higher

Table 5 The concentration of amino acids (μM) in *Platanthera bifolia* and *P. chlorantha* nectar with descriptive statistics

	<i>P. bifolia</i>				Mean ^a	SD ^a	Lower quartile ^a	Median ^a	Upper quartile ^a
	POG	BC	SMOL	POB1					
Proteogenic amino acids									
Asp	10.93	6.46	52.07	41.06	27.63	22.40	9.81	26	43.81
Glu	17.24	27.56	20.61	12.85	19.57	6.21	16.14	18.93	22.35
Asn	5.12	0	5.19	5.59	3.98	2.66	3.84	5.16	5.29
Ser	35.82	7.57	121.37	114.04	69.70	56.70	28.76	74.93	115.87
Gln	31.00	0	11.71	21.88	16.15	13.34	8.78	16.8	24.16
His	8.86	6.95	33.35	33.22	20.60	14.67	8.38	21.04	33.25
Gly	11.06	0	24.25	19.65	13.74	10.67	8.30	15.36	20.80
Arg	2.57	0	14.04	7.40	6.00	6.17	1.93	4.99	9.06
Ala	19.54	4.04	45.43	44.04	28.26	20.05	15.66	31.79	44.39
Val	11.34	0	19.95	17.13	12.11	8.83	8.51	14.24	17.84
Met	0	0	5.78	5.47	2.81	3.25	0	2.74	5.55
Phe	9.63	0	5.33	4.21	4.79	3.96	3.16	4.77	6.41
Ile	7.47	0	7.39	8.60	5.87	3.95	5.54	7.43	7.75
Lys	0	0	5.00	3.56	2.14	2.54	0	1.78	3.92
Pro	24.60	30.32	0	16.59	17.88	13.18	12.44	20.60	26.03
Cys	0	0	0	0					
Non-proteogenic amino acids									
Sar	28.83	50.01	80.65	82.28	60.44	25.78	44.72	65.33	81.06
Orn	5.18	0	6.96	8.45	5.15	3.68	3.88	6.07	7.33
Hyp	4.33	0.86	3.80	0	2.25	2.14	0.65	2.33	3.93
Cit	0	0	13.40	5.01	4.60	6.32	0	2.50	7.11
AABA	3.29	6.76	0	5.54	3.90	2.97	2.47	4.42	5.85
BABA	18.02	0	9.58	11.71	9.83	7.47	7.19	10.65	13.29
GABA	0	0	0	0					
	<i>P. chlorantha</i>			Mean ^b	SD ^b	Lower quartile ^b	Median ^b	Upper quartile ^b	
	BON	BF	LIN						
Proteogenic amino acids									
Asp	66.90	14.12	8.52	29.85	32.21	11.32	14.12	40.51	
Glu	25.01	8.79	9.49	14.43	9.17	9.14	9.49	17.25	
Asn	3.62	0	3.06	2.23	1.95	1.53	3.06	3.34	
Ser	159.04	42.11	17.72	72.96	75.54	29.92	42.11	100.58	
Gln	23.68	16.23	12.90	17.60	5.52	14.57	16.23	19.95	
His	43.32	20.52	10.88	24.91	16.66	15.7	20.52	31.92	
Gly	28.81	12.07	1.73	14.20	13.67	6.90	12.07	20.44	
Arg	13.85	4.65	5.13	7.88	5.18	4.89	5.13	9.49	
Ala	57.58	16.67	18.55	30.93	23.10	17.61	18.55	38.06	
Val	25.03	13.08	14.70	17.60	6.48	13.89	14.70	19.86	
Met	7.25	0	0						
Phe	5.52	4.61	0	3.38	2.96	2.31	4.61	5.07	
Ile	8.18	3.63	4.47	5.43	2.42	4.05	4.47	6.33	
Lys	6.37	0	0						
Pro	15.61	10.39	0	8.67	7.95	5.20	10.39	13.00	
Cys	0	0	4.17						
Non-proteogenic amino acids									
Sar	72.69	47.48	67.40	62.52	13.29	57.44	67.40	70.05	
Orn	10.08	0	6.78	5.62	5.14	3.39	6.78	8.43	

Table 5 (continued)

	<i>P. chlorantha</i>			Mean ^b	SD ^b	Lower quartile ^b	Median ^b	Upper quartile ^b
	BON	BF	LIN					
Hyp	0	0.86	0					
Cit	11.66	0	2.94	4.87	6.06	1.47	2.94	7.30
AABA	4.93	6.01	3.80	4.91	1.11	4.37	4.93	5.47
BABA	11.94	16.35	0	9.43	8.46	5.97	11.94	14.15
GABA	0	0	2.75					

SD standard deviation

^aCalculated using an amino acid value of POG, BC, SMOL, POB1 population

^bCalculated using an amino acid value of BON, BF, LIN1 population

than mean values for all the populations, and are negative for the other AAs (Fig. 2). The PCA clustered SMOL, POB1 *P. bifolia* and BON *P. chlorantha* populations (all from SLP) together, which are loaded with Val, Ala, Gly, Ser, Met, Cit, Arg, Lys, Asp, Sar and His (the long vectors are associated with high levels of metabolites). These three populations are further characterized by negative loadings (low levels) for Pro and Hyp. The BC *P. bifolia* and BF *P. chlorantha* populations were also clustered because they have the highest number of AAs that are lower than average (positive loadings for all AAs, excluding AABA and Pro). The POG *P. bifolia* population is associated with high levels of Gln, BABA, Phe, Hyp and Pro. However, the LIN *P. chlorantha* population represents the best, having an average value for all AAs (the smallest distance from the origin of the coordinate system). Results of PCA show greater similarities in AA composition between populations from a given region than between populations of the same species.

Amino acids in nectars could present either a source for these compounds in the nutrition of the pollinators or the presence of AAs in nectar may contribute to its taste. The taste AAs are divided into four classes. Possible simulation of insect chemoreceptors by AAs in nectars have AAs from classes II, III and IV (Gardener and Gillman 2002; Nicolson and Thornburg 2007). The results indicate class II AAs dominate (chemoreceptor inhibitors) in both species (Fig. 3, Table 4S). In the LIN *P. chlorantha* population, AAs from taste class III (stimulation of salt cells) and in BC *P. bifolia* population AAs from taste class IV (stimulation of sugar cells) were absent. The participation of AAs from taste class III was on average three times higher in *P. bifolia* than in *P. chlorantha*, whereas participation of AAs from class IV was higher in *P. chlorantha*. Content of AAs from taste groups I and IV negatively influenced MRS in the populations studied (in both cases $r = -0.79$, $p < 0.05$).

Discussion

Many studies have shown that plants have evolved different strategies to increase reproductive success. For example, producing nectar suitable for their potential pollinators, whose life history characters (e.g. growth and fecundity) depend on this food source (Baker and Baker 1983; Nicolson and Thornburg 2007; Petanidou 2007; Witt et al. 2013; Fowler et al. 2016). It is well known that although the concentration and composition of nectar are mainly connected with pollinator types, the constitution of nectar produced by plants of a given species varies in space and time (Pacini and Nepi 2007; Nocentini et al. 2013; Gijbels et al. 2014, 2015a, b).

Similar to other studies, we found differences in the nectar sugars, AA concentrations and compositions across *P. bifolia* and *P. chlorantha* populations (Tables 2, 5). The highest sugar content was found in the nectar from the BF *P. chlorantha* population and the highest AA content in the BON *P. chlorantha* population that exists in broadleaved forest on rich soils. On the other hand, the lowest levels of AAs in nectar were measured in two meadow populations of both species that are known as habitats with low nitrogen availability (Gijbels et al. 2014). Additionally, the low number of AAs (nine compounds) in the BC population may reflect the low levels of nitrogen in the soil. The sugar content of nectar was lowest in the POB1 *P. bifolia* population in a very high density community of young spruces. These results suggest the importance of habitat quality for nectar composition, which is in accordance with other studies. Higher AAs concentrations were previously reported in the nectar of *Agrostemma githago* and *Gymnadenia conopsea* that exist in habitats with more nutrient-rich soils by Gijbels et al. (2014) and Gardener and Gillman (2001), respectively. Also, higher nectar sugar content was reported in *Allium ursinum* by Farkas et al. (2012). The influence of soil composition on nectar AAs content and composition is well documented in experimental studies by Gijbels et al. (2015a). The importance of environmental conditions

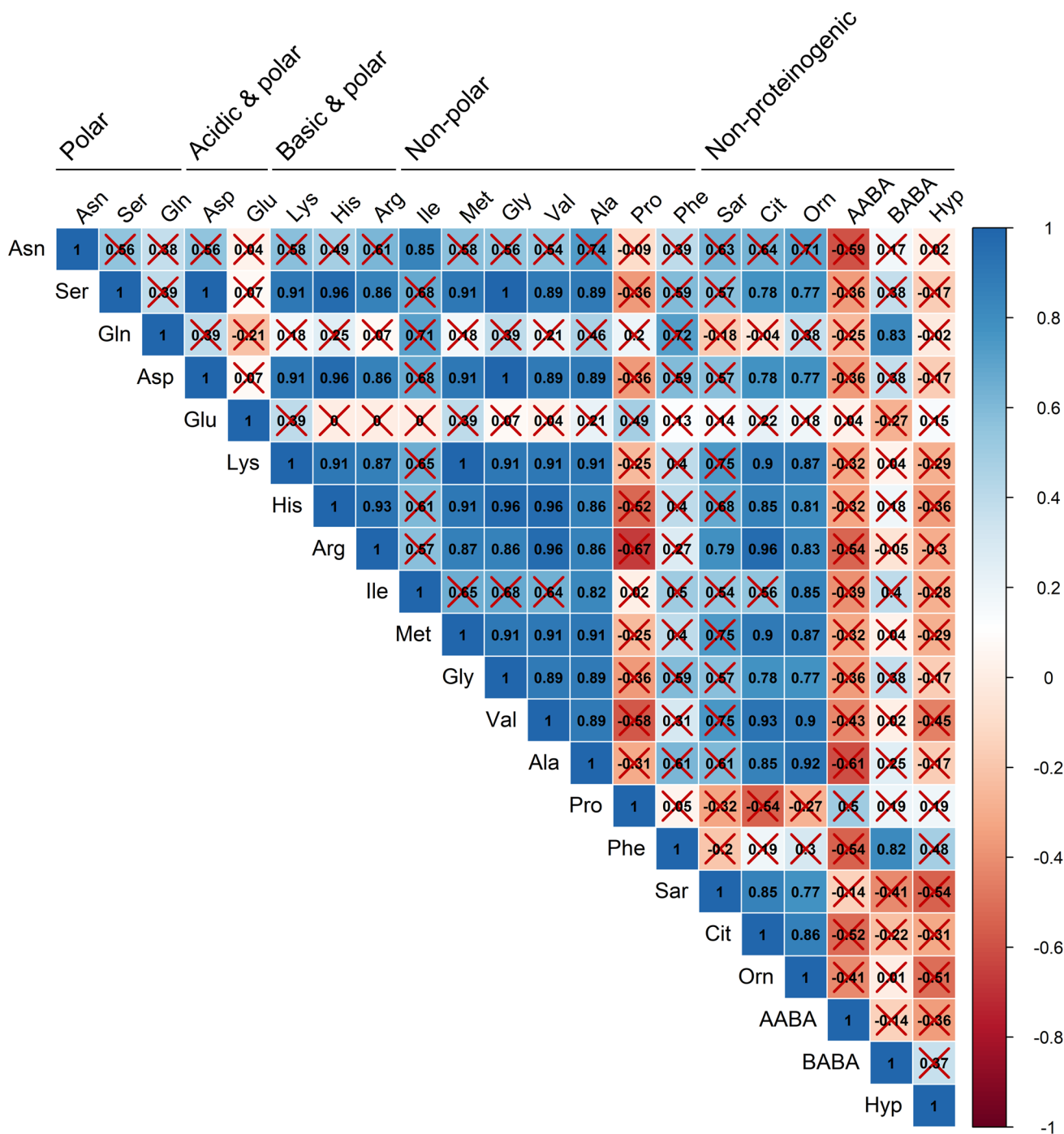


Fig. 1 Correlation heatmap for amino acids ($n=7$). The coloured and labelled scale codes for the value of the Spearman's rank correlation coefficient r_s . Positive correlations are blue while negative correlations are red. However, red crosses show insignificant correlations ($p \geq 0.05$)

also suggests that there are differences in Ser production between populations that exist in open habitats and under a tree canopy. In both meadow populations (one *P. bifolia* and one *P. chlorantha*) that have low levels of nitrogen in the soil, Ser participation in nectar was markedly lower. Gijbels et al. (2014) documented that production of Ser depends on soil nitrogen content. This result is also in accordance with

Nocentini et al. (2013), who found lower production of Ser in sunny places. To determine whether the nectar composition of two *Platantherans* is influenced by soil properties in the populations studied, further studies with soil analyses are needed.

Nectar quantity influenced FRS in *P. bifolia* and MRS in *P. chlorantha* populations. Increased RS could be

Fig. 2 Biplot of amino acid profiles of *P. bifolia* and *P. chlorantha* populations, showing the first two dimensions/factors (Dim1–2) of PCA that together explain 89.45% of variance. Biplot vectors indicate strength and direction of factor loading for the first two factors. Vectors of active variables are in red; however, those of supplementary are in blue. Individuals (populations of orchids: POG, BC, SMOL, POB1, BON, BF, LIN) were colour coded to distinguish between *P. bifolia* and *P. chlorantha* populations

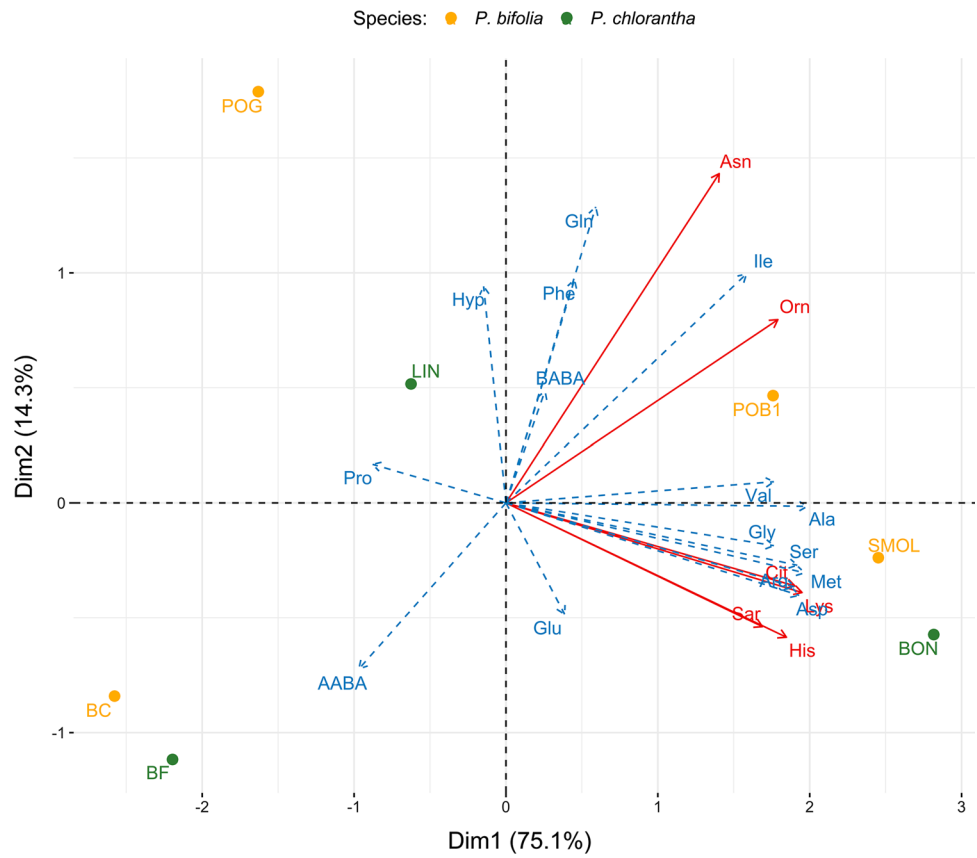
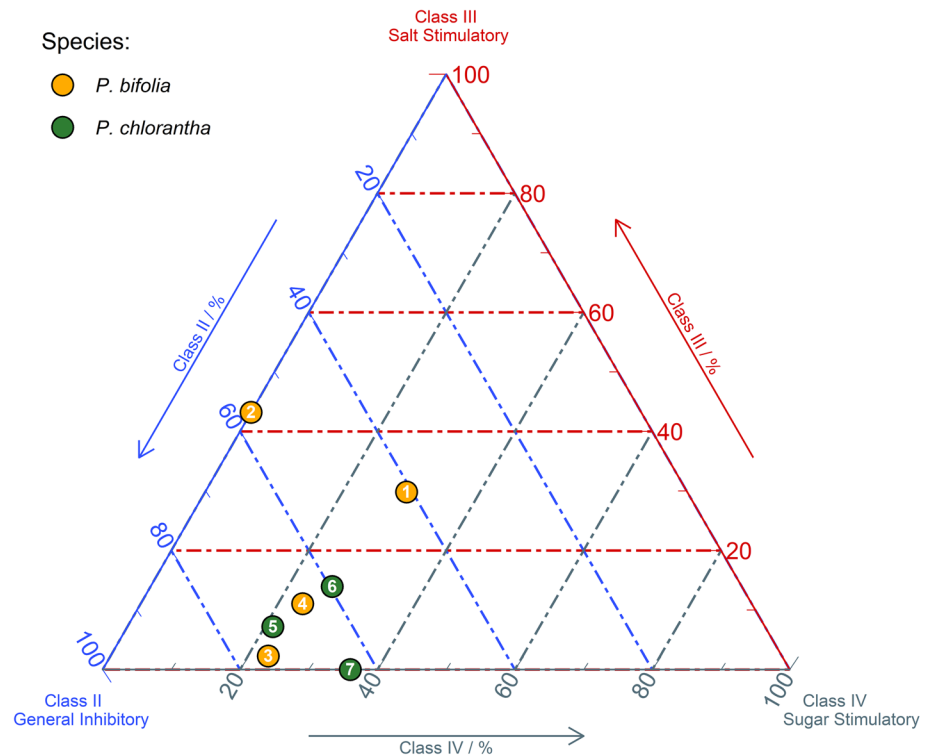


Fig. 3 Amino acid taste profiles for *Platanthera* populations: 1—POG, 2—BC, 3—SMOL, 4—POB1, 5—BON, 6—BF, 7—LIN. Classes represent the effect of AAs on insect chemoreceptors. Possible simulation of insect chemoreceptors by AAs in nectars indicates classes: II (Asp, Glu, His, Arg, Lys), III (Hyp, Pro) and IV (Val, Met, Trp, Phe, Ile, Leu). The first class of AAs (Asn, Gln, Ala, Cys, Gly, Ser, Thr, Tyr) has no effect on the chemoreceptors of fly (data not shown)



explained (as in other studies) by the longer visitation of inflorescences with more reward, i.e. higher nectar column in flowers (Hodges 1995; Maad and Reinhammar 2004). Simultaneously, such pollinator behaviour may have negative consequences such as increased autogamous pollination that causes decreased progeny fitness (de Jong et al. 1993; Jersáková and Johnson 2006). The relationship between nectar column length and the level of fruiting also suggests that in the studied populations, in which pollination is dominated by moths with relatively short tongues, there is a required higher level of nectar in spurs. Among the two main pollinator groups of *P. bifolia* and *P. chlorantha*, Sphingidae and Noctuidae, representatives of the second group usually possess shorter tongues. Most noctuids from the temperate zone have 10–20-mm-long tongues; sphingids' tongues may even exceed 40 mm (Boberg et al. 2014). On the other hand, large variations of the nectar column within populations provide the possibility that raising nectar by pollinators has a wider range of proboscis length than noctuids alone. The role of other factors that influence pollinator activity cannot be excluded. Flower morphology plays an important role in attracting pollinators and reproductive success because phenotypic selection is dependent on the mutual match between pollinator and flower traits (Moré et al. 2012). In long-spurred plants, such as *Platanthera*s, one of the most important flower characters that influence pollinator activity and RS is spur length. Despite the fact that we did not measure this trait, its importance was documented for many plants, including *Platanthera* species (Maad 2000; Boberg and Ågren 2009; Nilsson 1988; Alexandersson and Johnson 2002; Little et al. 2005; Moré et al. 2012; Boberg et al. 2014). Moreover, the size of the spur is often linked to nectar production. It should also be noted that characteristics of individuals, such as their high and inflorescence size, as well as their spatial distribution, can influence pollinator activity and behaviour, which often translate to the level of RS (Maad 2000; Vallius and Salonen 2006; Duffy and Stout 2008).

The presented results confirm the reports of some authors that sucking feeders, including butterflies, prefer less concentrated nectar (with sugar concentrations of 20–25%) than bees or birds (Kim et al. 2011; Tiedge and Lohaus 2017). Low concentrations of sugars in nectar for hawk moth-pollinated flowers (19%) have also been reported (Pyke and Waser 1981; Baker and Baker 1982; Heyneman 1983). On the other hand, Willmer (2011) reported that the optimal feeding rates for Lepidoptera are at higher sugar concentration (35–45%) and Josens and Farina (2001) found that hawk moths achieve peak intake at 34% sucrose. In both *Platanthera* species, sugars constituted around 13% of the nectar in five of the seven populations. In one *P. chlorantha* population, the sugar content exceeded 20% and in one *P. bifolia* population, the sugar content equalled only 9%. These values

are within the range recorded by Stpiczyńska and Pielecki (2002) for the *P. chlorantha* (27.5% during the first days of secretion and 5.6% during the resorption stage). The percentage of sugars in *P. chlorantha* nectar during maximum secretion (19.37%), which was measured by Stpiczyńska and Pielecki (2002), is almost identical to our measurements (20.68%) in the BF population, which exists in the same type of community (broadleaved forest, *Tilio–Carpinetum*) as *P. chlorantha* plants used in Stpiczyńska's and Pielecki's experiments. In all populations of both *Platanthera* species, nectar was dominated by hexoses, which contrasts with the prevalent opinion that sucrose dominates in nectar (Petanidou 2005; Nepi et al. 2010; Antoń et al. 2017). This hypothesis was previously put forth in studies on orchids (Gottsberger et al. 1984; Pais et al. 1986; Stpiczyńska and Pielecki 2002; Gijbels et al. 2014). Even in Danish *P. bifolia* and *P. chlorantha* populations, Lindqvist et al. (2018), who was testing a novel technique, found that the nectar was dominated by sucrose. Stpiczyńska and Pielecki (2002) also found domination of sucrose in *P. chlorantha* nectar, while fructose and glucose occurred in a 1:1 ratio. Moreover, these authors noted that relations between particular sugars were the same in different phases of flower's lifespan. Our results also contrast with the statements of many authors that suggest high sucrose content in nectar is associated with a specialized pollination mode and that long-tubed flowers are adapted to the preference of long-tongued pollinators and use sucrose-dominated nectar (Baker and Baker 1990; Torres and Galetto 2002; Nicolson and Thornburg 2007; Johnson and Nicolson 2008; Witt et al. 2013; Tiedge and Lohaus 2017). Moreover, Baker and Baker (1983) observed that moths belong to a group of pollinators that prefer nectar that is rich in sucrose, and with sucrose:hexoses ratios that are > 0.5. Also, Johnson and Nicolson (2008) point out that there is a clear distinction between nectar sucrose content of specialized (40–60% of total sugar) and generalized (0–5%) bird-pollinated species. In our studies, sucrose:hexoses ratios were < 0.5 in all populations. We did not find selection on the total sugar content at the population level, which may indicate that carbon content in soils (where both species exist) is enough to produce the required nectar components. It was documented that nutrient availability in soil influences plant metabolism and nectar production (Gardener and Gillman 2001). Some authors found that nectar sugar concentration increases with increasing soil organic matter (Baude et al. 2011; Gijbels et al. 2014). Among three sugars detected, only an increase in fructose content in populations increased both MRS and FRS ($r = 0.82$ and $r = 0.79$, respectively). Fructose may be preferred by pollinators due to its easier absorption and/or lower viscosity, thus allowing better flux (Heyneman 1983). Preferring lower sugar concentration by at least some *Platanthera* species pollinators reveals that some of them possess the ability to dilute

more concentrated nectar through a salivating technique, e.g. *Autographa gamma* from the Noctuidae (Wei et al. 1998).

Despite the fact that many data document the importance of AAs in nectar for pollinators attraction and their behaviour on the grounds of plant fitness (Baker and Baker 1986; Gijbels et al. 2014), some studies do not support that there is selection on the AAs content of nectar (Gijbels et al. 2015a, b). All correlations, which were noted between AA characteristics and the level of reproductive success, were negative and concerned almost exclusively MRS parameters. Increases in total AA content caused decreased levels of pollinaria removal in populations of *P. bifolia* and *P. chloantha*, which suggests that moths pollinating both *Platanthera* species prefer nectar with lower AA concentrations in contrast to some Lepidoptera preferring nectars with higher AA concentrations. It is well known that some AAs influence nectar taste, thus attracting or discouraging visitors (Gijbels et al. 2015a, b). Our results suggest that pollinators of *Platanthera* species are also sensitive to nectar taste. An interesting finding is that AAs from taste classes I and IV have a negative influence on MRS. Negative selection on Ile content and increases of this AA in populations caused decreases in both MRS and FRS. Insects can avoid or reject nectar with an overabundance of Ile because it can change the nectar taste to one that is not preferred by them. An excess in Ile may inhibit activity of other AAs that are important for insects' metabolism (Gardener and Gillman 2002).

Some non-proteogenic AAs, such as Sar, Cit, Orn, Hyp, AABA, BABA, and GABA, have been found in the nectar of *P. bifolia* and/or *P. chloantha* (Table 5). Although the role of non-proteogenic AAs is still unclear in nectar, they have different functions in plants that include antiherbivory, antimicrobial and allelochemical activities, protection against stress, signalling, nitrogen storage, and they are used as toxins against invertebrates and vertebrates (Bell 2003; Vranová et al. 2010; Nepi et al. 2012). They also play an important role in modulating insect behaviour. For example, GABA and β -Ala influence insect nervous system and muscle activity (Nepi 2014; Felicioli et al. 2018). In the nectar of some plants (e.g. *Nicotiana glauca* and *N. glauca*), GABA dominates, constituting even 39% of the total AAs (Kaczorowski et al. 2005). Taking into account the quantity of particular AAs in *Platanthera* nectar, Sar has the greatest importance, reaching (on average) > 20% in both species and even exceeding one-third of the total AAs in two populations (one of each species).

The next three AAs that exceeded 10% of the total AAs content are Pro, Ser and Glu and the three AAs that constitute ca. 7–8% are Asp, His and Ala. Many of these AAs function as nutritional sources. For instance, Ala and Ser influence insects' growth (Gijbels et al. 2014) and Glu,

Ser and Asp influence pollinators' behaviour. Additionally, Gln is useful for energetically expensive flight (Gardener and Gillman 2002; Gijbels et al. 2015b). One of the most common AAs in plant nectar, including the species studied here, is Pro (on average Pro accounts for 10% of all AAs in both species). Pro is the dominant AA in some species (Carter et al. 2006; Nepi et al. 2012), but in *Gynadenia conopsea*, an orchid that is pollinated by diurnal and nocturnal Lepidoptera, Pro has only a small contribution (Gijbels et al. 2014) and is absent in *Oenothera* species (Antoń et al. 2017). The accumulation of Pro is commonly interpreted as a plant's answer to stress factors, such as drought or frost (Carter et al. 2006). Pro plays an important role in nectar taste because it can trigger the normal insects' salt-receptor neurons, which can initiate feeding (Heil 2011; Willmer 2011; Nocentini et al. 2013). Moreover, Pro may be an important energy source due to its utilization over glucose and its fast production of ATP (Carter et al. 2006; Gijbels et al. 2014; Nepi 2014). Pro may be especially important in the context of low sugar concentrations in nectar from the two *Platanthera* species. Taking into account the positive influences of Pro, two questions, which are in contradiction with the importance of Pro for pollinators' attraction, arise: why is this AA absent in the two populations studied by us? And, why was the lowest fruiting noted in the *P. bifolia* BC population, which had the most abundant Pro in its nectar (21.6%)?

This study documents for the first time the sugar and AA composition in the nectar of natural populations of two sister species, *P. bifolia* and *P. chloantha*, which are the most common representatives of this genus in Europe in the context of reproductive success. Although the main sugar and AA components of *P. bifolia* and *P. chloantha* nectar are similar, detailed comparisons reveal some differences. The total sugar content in nectar was higher in *P. chloantha*. Moreover, although hexoses dominated in the nectar from both species, the nectar from *P. chloantha* contained more glucose and fructose than *P. bifolia*. The sucrose content was over two times higher in *P. bifolia*. Nectar of these two species also differed according to their sucrose:hexoses ratio. The ratio in *P. bifolia* is over threefold higher than *P. chloantha*. The number of AAs detected and their total content was approximately at the same level in both species. Similarly, no species-specific AAs were reported. On the other hand, the ratio of AA sum to sugar sum was higher in *P. bifolia*. Additionally, the presence of AAs from taste class III was found to be three times higher in *P. bifolia* than in *P. chloantha*, while the percentage of AAs from taste class IV was higher in *P. chloantha*. To verify if the above-mentioned differences are truly species specific, or, if these results are simply due to the particular populations studied, further studies are needed. These studies should include more populations

from other habitats and regions. Similarly, to assess the needs and preferences of *P. chlorantha* and *P. bifolia* pollinators to nectar offered by these orchids, additional studies are also necessary and should include nectar analysis at the level of individuals.

Author contribution statement EB conceived the study and wrote the manuscript. AB performed statistical analysis and the nectar analysis. EB and AB read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that the research was conducted in the absence of any commercial relationships, and there was no potential conflict of interest.

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