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Variation in nectar quality across 34 grassland plant species

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ABSTRACT

- Floral nectar is considered *the* most important floral reward for attracting pollinators. It contains large amounts of carbohydrates besides variable concentrations of amino acids and thus represents an important food source for many pollinators. Its nutrient content and composition can, however, strongly vary within and between plant species. The factors driving this variation in nectar quality are still largely unclear.
- We investigated factors underlying interspecific variation in macronutrient composition of floral nectar in 34 different grassland plant species. Specifically, we tested for correlations between the phylogenetic relatedness and morphology of plants and the carbohydrate (C) and total amino acid (AA) composition and C:AA ratios of nectar.
- We found that compositions of carbohydrates and (essential) amino acids as well as C:AA ratios in nectar varied significantly within and between plant species. They showed no clear phylogenetic signal. Moreover, variation in carbohydrate composition was related to family-specific structural characteristics and combinations of morphological traits. Plants with nectar-exposing flowers, bowl- or parabolic-shaped flowers, as often found in the Apiaceae and Asteraceae, had nectar with higher proportions of hexoses, indicating a selective pressure to decelerate evaporation by increasing nectar osmolality.
- Our study suggests that variation in nectar nutrient composition is, among others, affected by family-specific combinations of morphological traits. However, even within species, variation in nectar quality is high. As nectar quality can strongly affect visitation patterns of pollinators and thus pollination success, this intra- and interspecific variation requires more studies to fully elucidate the underlying causes and the consequences for pollinator behaviour.

INTRODUCTION

Many flowering plant species need flower-visiting insects for pollination. They often provide rewards (mostly nectar and occasionally pollen) to attract pollinators (Agthe 1951; Baker 1963; Kearns *et al.* 1998; Klein *et al.* 2007; Ollerton *et al.* 2011). Although most macronutrients, which are essential for organisms, are located in pollen (in particular protein and fat) (De Groot 1953; Roulston & Cane 2000; Keller *et al.* 2005; Weiner *et al.* 2010), nectar represents the main source of carbohydrates and additionally contains variable concentrations of amino acids (Heinrich 1981; Wcislo & Cane 1996; Carter *et al.* 2006; Venjakob *et al.* 2020). In fact, some pollinators, *e.g.* butterflies, even entirely depend on nectar as sole nutrient source (Erhardt & Rusterholz 1998) and rely on its amino acid content to compensate for nitrogen deprivation during larval development (Mevi-Schütz & Erhardt 2005). In other groups, *e.g.* parasitoid wasps, nectar can increase longevity, fecundity and mobility (Winkler *et al.* 2006). Nectar was therefore considered *the* most important floral reward for attracting pollinators (Simpson & Neff 1983; Somme *et al.* 2015; Parachnowitsch *et al.* 2019).

From the pollinators' perspective, nectar needs to meet nutritional requirements, outbalance foraging costs (Pyke *et al.* 1977; Waddington 1982) and provide sufficient energy for mobility and thermoregulation (herein McCallum *et al.* 2013; Hendriksma *et al.* 2014). From the plants' perspective, nectar needs to attract pollinators at minimum production costs (Pyke 1991; Nepi & Stpiczyńska 2008), deter non-mutualists, *e.g.* nectar robbers and micro-organisms, from stealing or degrading nectar (Adler 2000; González-Teuber & Heil 2009; Escalante-Pérez & Heil 2012) and ideally manipulate pollinator behaviour to their advantage (Pyke 2016). These different requirements render the nutritional composition of nectar (henceforth referred to as nectar quality) a multifunctional trait, which can be subject to conflicting interests (Parachnowitsch *et al.* 2019; van der Kooi *et al.* 2021). For example, different flower-visiting and pollinating species (henceforth all referred to as pollinators) prefer different sugar concentrations, *i.e.* bees prefer nectar with 50–60% sugar concentration and butterflies and birds prefer nectar with 35% sugar concentration, likely due to different ways of consumption (*i.e.* dipping *versus* suction) (Kim *et al.* 2011). Also, nectar sugar

concentration negatively correlates with yeast abundance (Herrera *et al.* 2009) which may in turn affect pollinator preferences (Vannette & Fukami 2016; Schaeffer *et al.* 2017). Consequently, nectar quality can directly and indirectly affect visitation patterns of pollinators.

Several biotic and abiotic factors were found to be related to variation in nectar quality, including age and damage to flowers (Gottsberger *et al.* 1990), soil conditions (Baude *et al.* 2011; Becklin *et al.* 2011), genetic relatedness (*i.e.* phylogeny) (Baker & Baker 1976; Nicolson & Van Wyk 1998; Perret *et al.* 2001), flower morphology (Gusman & Gottsberger 1996; Torres & Galetto 2002), microbial communities (Vannette *et al.* 2013), selection by specific pollinators (Petanidou *et al.* 2006; Willmer 2011; Chalcoff *et al.* 2017; Tiedge & Lohaus 2017; Silva *et al.* 2020) and various interactive effects [see Parachnowitsch *et al.* (2019) for a complete review of factors affecting nectar quality]. For example, phylogenetically related plant species, *i.e.* belonging to the same tribe or family, can show similar chemical characteristics of nectar, such as a clear dominance of hexose or sucrose (Percival, 1961; Bernardello *et al.* 1994; Wolff 2006) and similar amino acid profiles (Baker & Baker 1986). However, so far, only a few studies have investigated heritability and evidence for selection in nectar traits, such as sugar concentration and content (Parachnowitsch *et al.* 2019). Similarities in nectar quality may alternatively be due to similar morphological traits, *e.g.* shape of flower tubes, and thus similar requirements for protection against evaporation (Witt *et al.* 2013). For example, plant species with long tubular flowers typically contain sucrose-rich nectar (high sucrose proportion), whereas plant species with more open flowers typically contain hexose-rich nectar (high hexose proportion) to reduce evaporation *via* increased osmolality (Percival 1961; Bernardello 2007; Witt *et al.* 2013), because the loss of water in nectar leads to increased viscosity, which decelerates nectar uptake or even prevents pollinators from consumption (Köhler *et al.* 2010; Kim *et al.* 2011). In fact, several studies found a strong preference of long-tongued pollinators, such as butterflies, moths and long-tongued bees, for sucrose-rich nectar, and of short-tongued bees and flies for hexose-rich nectar (Baker & Baker 1983; Petanidou *et al.* 2006; González-Teuber & Heil 2009), indicating that floral morphology and nectar quality interact with pollinator preferences. Despite its importance as floral reward and source of nutrients for pollinators, the precise factors driving variation in nectar quality, such as the partial roles of phylogenetic relatedness, pollinator preferences and floral morphology, are still largely unclear (Parachnowitsch *et al.* 2019).

To contribute to a better understanding of the parameters which affect variation in nectar quality, we analysed potential correlations between the phylogenetic relatedness and morphology of plants and the amino acid and carbohydrate composition of their nectar for 34 grassland species. We hypothesized that: (i) plant species generally differed both qualitatively and quantitatively in their amino acid and carbohydrate composition, but that (ii) related plant species (*i.e.* species within the same families) would show a more similar nectar composition (with regard to concentrations and proportions of sugars and amino acids as well as their ratios) than plant species from different families due to phylogenetic and/or morphological constraints.

MATERIAL AND METHODS

Experimental field site

The study was conducted within the framework of the Jena Experiment, which is a grassland biodiversity experiment in Thuringia, Germany (50°55′ N, 11°35′ E; 130 m a.s.l.) established in 2002, comprising 60 native plant species of the plant association Arrhenatherion, with 16 grass species and 44 flowering herb species (Roscher *et al.* 2004). The study field is situated near the river Saale and encompasses 82 plots containing different plant species mixtures, forming a plant species richness gradient. We collected nectar of 34 flowering plant species from 22 of those plots, preferentially from species' monocultures, but also from plots with higher levels of plant species richness, when monocultures comprised insufficient numbers of flowering individuals. We selected all flowering plant species for which we could access a minimum of 1 µl, *i.e.* 34 out of 41 species. For more details on the design of the Jena Experiment, see Roscher *et al.* (2004).

Nectar sampling

In 2011, we sampled nectar of a total of 34 flowering plant species on sunny to cloudy days. Flowers were bagged in cotton gauze 1 day before nectar sampling (mesh size 0.80–1.00 mm) to exclude pollinators (Klein *et al.* 2003) and ensure that nectar was not depleted by insects prior to sampling. Accumulated nectar was collected in the morning using a microcapillary pipette (1 µl holding volume) equipped with a pipetting aid (for more details see Corbet 2003; Venjakob *et al.* 2020). We aimed to collect a minimum of 1 µl nectar per sample. When 1 µl could not be obtained from one flower, additional flowers were sampled. We collected samples from at least seven plant individuals per species. Exceptions with less samples for carbohydrate analysis were *Geranium pratense* L. (6 samples), *Trifolium fragiferum* L. (6), and *Trifolium pratense* L. (3). Six plant species (*Anthriscus sylvestris* Hoffm., *Campanula patula* L., *Glechoma hederacea* L., *Primula veris* L., *Ranunculus repens* L. and *Veronica chamaedrys* L.) were re-sampled in 2012 because nectar was too dilute due to heavy rain during the flowering period or because of insufficient amounts of nectar. Microcapillaries for nectar sampling were stored in Eppendorf tubes (see Venjakob *et al.* 2020) and kept at −20 °C until analyses.

Nectar preparation

Prior to analysis, microcapillaries (32 mm) with nectar were rinsed with 100 µl 99.8% ethanol and centrifuged for 5 min to remove nectar from capillaries into 2-ml Eppendorf tubes (see Venjakob *et al.* 2020). Eppendorf tubes with nectar were kept in a DURAN® desiccator to evaporate ethanol, and nectar was subsequently re-dissolved in 50 µl ultra-pure water and centrifuged for 3 min (Venjakob *et al.* 2020). Finally, 48 µl of the supernatant were transferred into 1-ml glass vials equipped with 250 µl pulled-point glass inserts and stored at −20 °C (for details see Venjakob *et al.* 2020).

Amino acid and carbohydrate analysis

Analyses of amino acids and carbohydrates were performed with high performance liquid chromatography (HPLC: Agilent

Technologies 1260 Series; Agilent, Böblingen, Germany). The LC was equipped with an Agilent 1260 Infinity Quaternary Pump (G1311C, Agilent), an Agilent 1260 Infinity Standard Autosampler (G1329B) and an Agilent 1260 Infinity Thermostatted Column Compartment (G1316A). Oven temperature was 40 °C for amino acids and 30 °C for carbohydrates.

Of each nectar extract, 8 µl (amino acids) and 30 µl (carbohydrates) were injected into the system. Prior to analysis, amino acids were derivatized using either orthophthalaldehyde (OPA; Agilent, for primary amino acids) or 9-fluorenylmethyl chloroformate (FMOC; Agilent, for secondary amino acids, *e.g.* proline). Separation of amino acids was achieved on an Extend-C18 column (Zorbax: 3.0 × 150 mm, 3.5 µm; Agilent) preceded by an Extend-C18 guard column (Zorbax: 2.1 × 12.5 mm, 5 µm; Agilent). We used a solvent gradient to separate amino acids with a buffer (1 l ultra-pure water, 10 mM Na_2HPO_4, 10 mM $Na_2B_4O_7$, 0.5 mM NaN_3, pH 8.2) as polar and acetonitrile–methanol–water (45%, 45%, 10% [vol/vol], all CHROMASOLV®; Sigma-Aldrich, Taufkirchen, Germany) as non-polar phase. A Diode Array Detector (DAD; Agilent 1260 Infinity System, G4212B) was used for detection. Each run started with a 2–98% non-polar to polar phase. The ratio was then gradually changed to 57% to 43% over 13 min and finally to 100% non-polar phase, which was kept for about 2 min. Solvent flow rate was constant at 0.75 ml min^{-1}.

Carbohydrates were eluted on a NH2 column (Zorbax: 4.6 × 250 mm, 5 µm; Agilent) preceded by a NH2 guard column (Zorbax: 4.6 × 12.5 mm, 5 µm; Agilent) under isocratic conditions. We used an elution buffer containing 78% [vol/vol] acetonitrile and 22% [vol/vol] ultra-pure water at a constant flow rate of 1.5 ml min^{-1}. Detection was achieved with a Refractive Index Detector (RID; Agilent 1260 Infinity, G1362 A).

After every five samples, we ran an external standard containing 17 amino acids (Amino Acid Standard solution; Sigma-Aldrich) or three carbohydrates (sucrose, fructose and glucose, HPLC grade; Sigma-Aldrich) in four different concentrations. Using the Agilent ChemStation software for LC 3D systems (Agilent), amino acids and carbohydrates were identified and quantified by matching retention times and areas of calibrated standard compounds with retention times and areas of compounds found in nectar samples. Note that we cannot measure glutamine, arginine and tryptophan with our method due to either their destruction during acid hydrolysis (tryptophan) or a lack of standards (asparagine, glutamine).

Statistical analysis

We investigated whether nectar from the 34 plant species and the four most abundant plant families differed in the composition of carbohydrates and/or amino acids using permutation tests (PerMANOVA, using 10,000 permutations), which were based on Bray-Curtis distances between substances (R package: vegan; Adonis command). Separate permutation tests were performed for concentrations (in mg ml^{-1}) and proportions of all carbohydrates and amino acids. We obtained proportions of individual compounds by dividing the concentration of each individual carbohydrate/amino acid by the total concentration of all carbohydrates/amino acids analysed.

We tested for plant species-specific differences in total carbohydrate and amino acid concentrations (*i.e.* the sum of all individual compounds), the total concentration and proportion of all essential amino acids and the total concentrations of all non-essential amino acids, as well as the ratio of all carbohydrates to all amino acids. Due to the nested plot design from which the samples were taken, we always tested first whether sample plot influenced the explained variance by composing both a generalized linear model (GLM) and generalized linear mixed effect models (GLMMs), with plant species entered as fixed factor and the plot from which the sample was taken as random factor. Due to the lack of a phylogenetic signal for all nutrient groups (see Table 1) we did not use GLMMs corrected for phylogenetic relatedness. Models were compared using the Akaike information criterion (AIC) and likelihood ratio tests (R package: lme4; anova command; following Zuur *et al.* 2009). We always provide results for the GLM if it did not have a significantly higher AIC value than the GLMM. For tests on plant species-specific differences, GLMs were always better than GLMMs, which renders the application of permutation tests for plant species-specific differences in compound compositions valid despite the nested plot design.

All analyses were repeated for plant family-specific differences between Asteraceae, Apiaceae, Fabaceae and Lamiaceae, as well as for different morphological flower traits. We confined family analyses to these four plant families, because they were the only families with sufficient numbers of plant species sampled per family ($N \geq 3$). Information on floral traits composed for the same plots was obtained from Fornoff *et al.* (2017) and included flower symmetry (binomial: actinomorphic or bilateral), nectar access (binomial: open or hidden), inflorescence area (mm^2) and flower height (mm). All morphological traits were obtained as mean per plant species. We therefore also calculated mean per plant species for amino acid and carbohydrate concentrations and proportions when analysing the effect of family and traits on nectar chemistry and for the equivalent permutation tests. We again used generalized linear (mixed effect) models (GL(M)Ms) and compared GLMs with GLMMs, with plot included as random factor. GLMMs often had significantly higher AIC values than GLMs, indicating that a high proportion of the observed variance was explained by plot identity. We additionally extracted marginal R^2 values for the different models to compare the variance explained by morphological traits and by plant family.

Following Junker *et al.* (2017) and Ruedenauer *et al.* (2019), we tested for a phylogenetic signal in the total, essential and non-essential content of amino acids, as well as the content of sugars and the carbohydrate (C) to total, essential and non-essential amino acid (AA) ratios (*i.e.* C:AA ratios) using

Table 1. Results of Blomberg's *K* tests for a phylogenetic signal within total/essential/non-essential amino acid and carbohydrates content, as well as the carbohydrate to amino acid ratios of 34 plant species.

nutrient	*K*	*P*
Total amino acids	0.101	0.325
Essential amino acids	0.094	0.476
Non-essential amino acids	0.132	0.177
Carbohydrates	0.112	0.331
Carbohydrate to amino acid ratio	0.304	0.107
Carbohydrate to essential amino acid ratio	0.306	0.097
Carbohydrate to non-essential amino acids ratio	0.160	0.265

Blomberg's *K*. The underlying phylogenetic tree was based on the phylogeny of Zanne *et al.* (2014).

Response variables were always tested for normality and homogeneity of variances using graphical tools, as suggested by Zuur *et al.* (2009) and either log- or square root- (concentrations, ratios) or arcsine square root (proportions)-transformed when these requirements were not met. Due to multiple use of the same dataset, we only considered $P < 0.01$ as being significant. We finally used the mean carbohydrate and amino acid proportions and concentrations of each plant species to visually assess similarities within plant families using cluster dendrograms (R package vegan), also based on Bray-Curtis distances between substances. All analyses were performed in R version 3.1.3 (R Development Core Team 2015).

RESULTS

Differences between plant species

The 34 plant species sampled showed species-specific compositions for both carbohydrates (PerMANOVA: concentrations: $R^2 = 0.69$, $P < 0.001$; proportions: $R^2 = 0.75$, $P < 0.001$) and amino acids (concentrations: $R^2 = 0.65$, $P < 0.001$; proportions: $R^2 = 0.82$, $P < 0.001$) with significant differences between species in the concentrations and proportions of all individual carbohydrates and amino acids (GLMs: *P*-values always ≤0.001; Tables S1, S2, Fig. 1). Intraspecific variation was also pronounced, particularly for amino acids (see standard variations in Table S1). Interestingly, related species (*i.e.* species within the same family) could be either chemically similar (*e.g.* Apiaceae species with regard to amino acid compositions, except *Pimpinella major*) or chemically distinct (*e.g.* Lamiaceae species with regard to amino acid compositions) or both (Asteraceae species with regard to amino acid compositions) as assessed by dendrograms and chemical distances between species (see Table S2a–d, Fig. S1). Also, some plant species (*e.g. Crepis biennis* or *Ajuga reptans* with regard to amino acid compositions, *Ranunculus acris* and *Sanguisorba officinalis* with regard to carbohydrate compositions) showed very distinct nutritional profiles which strongly deviated from most other plant species (Table S2a–d, Fig. S1). None of the nutrient contents, proportions and ratios showed a phylogenetic signal (Table 1).

Nectar of the 34 plant species also differed in the concentrations of the sum of all amino acids (GLM: $F = 18.93$, $P < 0.001$), all essential amino acids (GLM: $F = 17.15$, $P < 0.001$) and all non-essential amino acids (GLM: $F = 25.69$, $P < 0.001$), as well as in the proportion of all essential amino acids (GLM: $F = 35.44$, $P < 0.001$; Table S1, Fig. 1). *Crepis biennis* L., *Tragopogon pratensis* L., *Leontodon hispidus* L. (all Asteraceae) and *Sanguisorba officinalis* L. (Rosaceae) had nectar with the highest overall amino acid concentrations (all mean values >20 mg ml^{-1}; Table S1, Fig. 1). Nectar of these plant species also contained most essential amino acids, while highest concentrations of all non-essential amino acids were found in *Tr. pratensis* (Asteraceae), *T. campestre* Schreb., *T. hybridum* L., *T. pratense* L. (all Fabaceae) and *P. veris* (Primulaceae) (all mean values >10 mg ml^{-1}; Table S1, Fig. 1). Essential amino acids made up the highest proportion of all amino acids in *C. biennis* (Asteraceae), *Ajuga reptans* L, and *Prunella vulgaris* L. (both Lamiaceae) (all mean values ≥80%; Table S1, Fig. 1), while they were generally low in Fabaceae (*T. campestre*, *T. hybridum*, *T. pratense*, *T. repens* and *Vicia cracca*) and in *P. veris* (all mean values <40%; Table S1, Fig. 1).

The total carbohydrate amount in nectar also differed between plant species ($F = 18.95$, $P < 0.001$), with highest overall carbohydrate concentrations in *T. campestre*, *Lotus corniculatus* L. (both Fabaceae), *A. reptans* (Lamiaceae) and *Tr. pratensis* (Asteraceae) (all mean values >100 mg ml^{-1}; Table S1, Fig. 1). Overall carbohydrate concentrations were low in *Daucus carota* L. and *Pimpinella major* (L.) Huds. (both Apiaceae) (all mean values <10 mg ml^{-1}; Table S1, Fig. 1). Individual amino acids and carbohydrates followed similar trends (Table S1, Fig. 1).

The C:AA ratios in nectar of the 34 plant species were generally carbohydrate-biased, but ranged from equal mean ratios of C:AA = 1:1 [for *A. sylvestris* (Apiaceae) and *L. hispidus* (Asteraceae)] to mean ratios largely dominated by carbohydrates, C:AA = >20:1 [for *Geranium pratense* L. (Geraniaceae), *A. reptans* and *P. vulgaris*] and differed between plant species (GLM: $F = 19.17$, $P < 0.001$; Fig. S1, Fig. 2). The same was true for the ratio of all carbohydrates to all essential amino acids (henceforth referred to as C:EAA) (GLM: $F = 17.83$, $P < 0.001$).

Differences between plant families and related to morphological traits

Asteraceae, Apiaceae, Fabaceae and Lamiaceae differed in the composition of all amino acids when concentrations were considered (PerMANOVA: $P^2 = 0.27$, $P = 0.007$; Fig. S2a), and marginally when proportions were considered ($R^2 = 0.25$, $P = 0.02$; Fig. S2b). Essential amino acids also tended to show family-specific profiles (concentrations: $R^2 = 0.27$, $P = 0.01$; Fig. S2c; proportions: $R^2 = 0.27$, $P = 0.02$; Fig. S2d). However, visual inspection of chemical similarities showed that separation by family was not strict and mostly driven by differences between Fabaceae and Asteraceae (Fig. S2). Moreover, some species of different families (*e.g. Centaurea* and *Anthriscus*) were more similar to each other than to other species within their respective plant families (Fig. S2, Table S2a–d). The proportion of all essential amino acids (proportion: GLMM: $F = 4.090$, $P = 0.003$; Fig. 3c) also differed between the four plant families, whereas the overall amino acid concentrations did not (GLMM: $F = 1.425$, $P = 0.241$; Fig. 3a). Nectar of Asteraceae and Lamiaceae generally contained the highest concentrations and proportions of essential amino acids (Fig. 3b, c). Concentrations and proportions of 11 out of 17 single amino acids (*e.g.* glutamic acid, histidine, arginine. . .) also differed or tended to differ between the four plant families (Table S3).

When examining the effect of morphological traits of flowers, we found the concentration and proportion of lysine differed between symmetric and non-symmetric flowers (concentration: GLMM: $F = 11.329$, $P = 0.007$; proportion: $F = 38.488$, $P < 0.001$). The proportions of three other amino acids also tended to differ between the two flower types, while the remaining species were not affected by any floral traits (Table S3).

However, variation in nectar amino acid concentrations and proportions was always better explained by plant family than by flower morphology ($0.1 < R^2 \leq 0.92$ for 32 out of 36 models on the effect of plant family on variation in amino acid concentrations and proportions; $0.1 < R^2 \leq 0.13$ for 3 out of 144

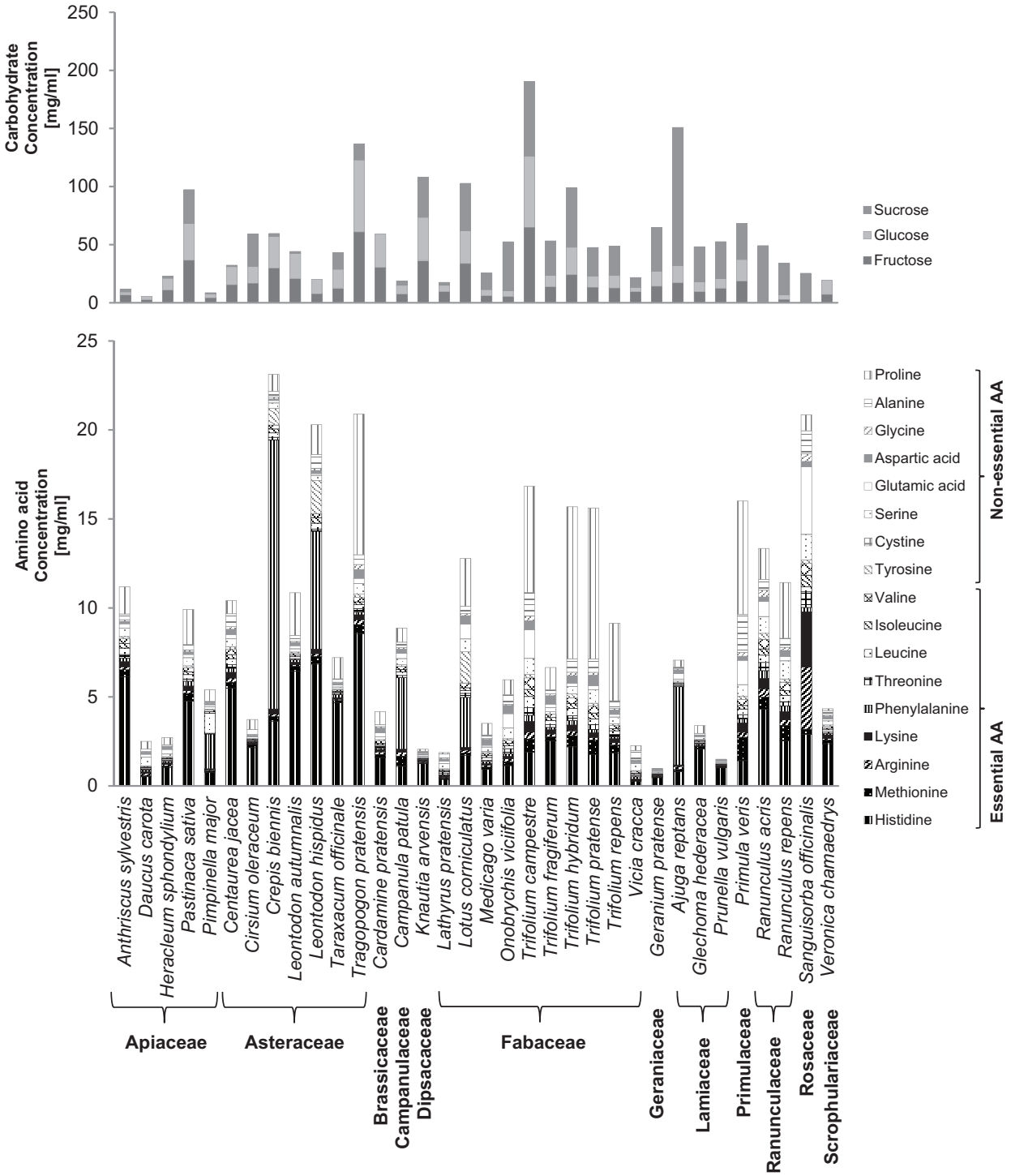

Fig. 1. Nectar carbohydrate and amino acid concentrations in mg ml^{-1} of 34 plant species, grouped by family in alphabetical order. Grey amino acids are non-essential amino acids, while black amino acids are amino acids considered essential for honeybees (following De Groot 1953).

models on the effect of morphology on variation in amino acid concentrations and proportions; Table S3).

The four plant families also had specific nectar carbohydrate profiles with regard to compound concentrations (PerMANOVA: $R^2 = 0.32$, $P = 0.003$; Fig. 4) and particularly proportions ($R^2 = 0.61$, $P < 0.001$; Fig. 5). Sucrose proportions were generally higher in Lamiaceae and Fabaceae and lower in Asteraceae and Apiaceae (GLMM: $F = 7.580$, $P < 0.001$; Fig. 5a). This pattern was reversed for glucose (GLMM: $F = 5.805$, $P < 0.001$; Fig. 5b) and fructose (GLMM: $F = 3.813$, $P = 0.004$; Fig. 5c). Single carbohydrate concentrations did not significantly differ between plant families (Fig. 4a–c), and neither did

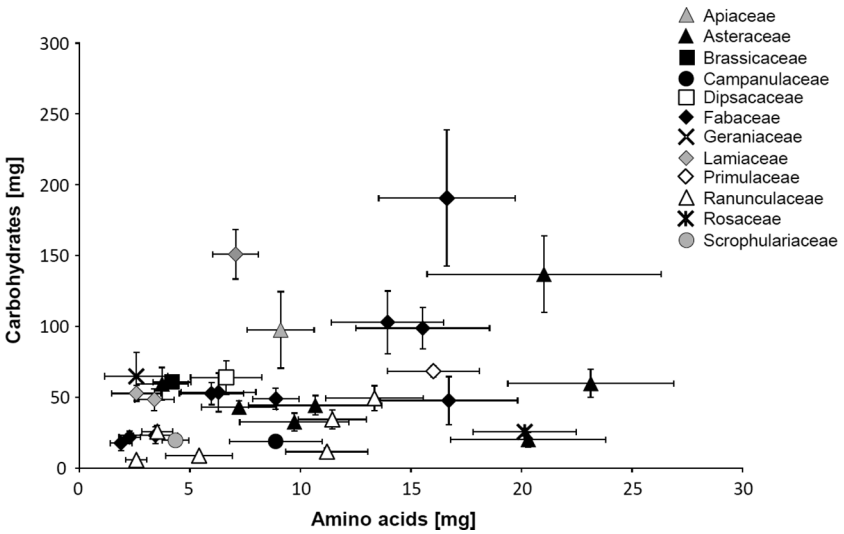

Fig. 2. Mean amounts [± SE] of total carbohydrates and amino acids in nectar of 34 plant species. Each symbol represents one plant species, with plant species having the same symbol belonging to the same plant family.

the total carbohydrate concentration (GLMM: $F = 0.585$, $P = 0.820$; Fig. 4d).

The concentrations of fructose (GLMM: $F = 4.347$, $P = 0.045$) and glucose (GLMM: $F = 4.537$, $P = 0.041$), as well as the total carbohydrate concentration (GLMM: $F = 4.629$, $P = 0.039$), also tended to differ between plants with open nectar access and plants with restricted nectar access. Proportions of glucose (GLMM: $F = 4.325$, $P = 0.046$) and sucrose (GLMM: $F = 4.276$, $P = 0.047$) also tended to increase with inflorescence area.

As found for amino acids, plant family explained most of the variance in nectar carbohydrate concentrations and proportions ($0.1 < R^2 \leq 0.67$ for all models on the effect of plant family on variation in carbohydrate concentrations and proportions; Table S4). Some variation was also explained by nectar access, *i.e.* variation in fructose ($R^2 = 0.12$), glucose ($R^2 = 0.13$) and total carbohydrate ($R^2 = 0.12$) concentrations, and by inflorescence area, *i.e.* variation in glucose ($R^2 = 0.12$) and sucrose ($R^2 = 0.12$) proportions (Table S4).

Interestingly, all plant species within a specific family, *i.e.* Asteraceae, Apiaceae, Fabaceae and Lamiaceae, showed similar C:AA ratios (Fig. 2), although total amounts of carbohydrates and amino acids per ml nectar differed between species (Table S1). Thus, C:AA ratios were family-specific, with significant differences between families (GLMM: $F = 9.16$, $P < 0.001$; Fig. 2). These differences could be attributed mainly to the Lamiaceae, whose nectar contained two to four times more carbohydrates (mean C:AA = 19:1) than nectar of the other plant families (Asteraceae 6:1, Apiaceae 5:1, Fabaceae 8:1; Fig. 2).

DISCUSSION

As expected, nectar of the 34 studied plant species and four most abundant plant families of a mesotrophic grassland community differed in carbohydrate and amino acid composition. However, despite significant differences between families, nectar composition was not significantly influenced by phylogenetic relatedness, indicating that additional factors affected variation in floral nectar quality.

Interestingly, when comparing the explanatory power of floral morphology and plant family, family continuously explained a larger proportion of the variation in nectar chemistry, *e.g.* up to 92% for the concentration of lysine, than the investigated morphological parameters. Differences between Apiaceae, Asteraceae, Fabaceae and Lamiaceae (all very attractive for pollinators: Ebeling *et al.* 2008; Venjakob *et al.* 2016) were particularly pronounced for carbohydrates, with higher hexose concentrations and proportions in Apiaceae (open flowers) and Asteraceae, and higher sucrose concentrations and proportions in Fabaceae and Lamiaceae (closed/tubular flowers). This finding agrees with previous studies indicating that nectar carbohydrate composition largely correlates with floral morphology (Percival 1961; Bernardello 2007; Witt *et al.* 2013) and thus likely with constraints imposed through osmolality. However, Asteraceae also have tubular flowers (ray or disc flowers) composed in radially symmetrical flower heads (Jäger *et al.* 2013), often formed as bowl- or even parabola-shaped flower heads (Kevan 1989). They nevertheless had mostly hexose-rich nectar, except for three species, which had relatively similar proportions of sucrose, fructose and glucose (Fig. 1). The hexose-rich nectar in most Asteraceae may be explained by the stronger tendency of the flowers to heat up as a consequence of the bowl- or parabola-shaped flower heads (Percival 1961). Different combinations of family-specific morphological aspects of flowers determining overall inflorescence shape and structure therefore need to be taken into account when addressing variations in nectar chemistry. The importance of such family-specific structural characteristics and combinations of morphological traits may also explain why 'nectar access' (a parameter based on the morphology of individual flowers in our study) had overall less explanatory power than 'plant family', and why we found strong family-specific differences in nectar chemistry but no in the phylogenetic signal. In fact, the two differently related plant families, Fabaceae and Lamiaceae, showed similarities in nectar carbohydrate composition, likely due to structural similarities of the flowers. The two more closely related plant families, Asteraceae and Apiaceae, also showed similarities in nectar carbohydrate composition, despite different flower structures. Moreover, within plant families, the nectar composition of individual species still varied independent of their relatedness.

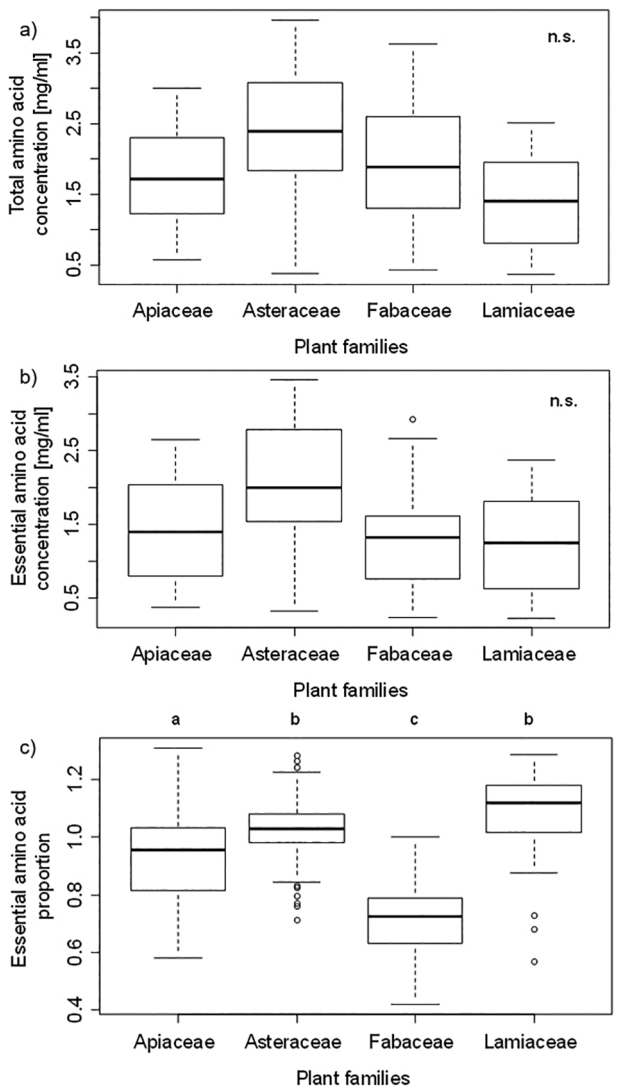

Fig. 3. Differences in (a) total concentrations (mg ml^{-1}) of amino acids, (b) concentrations (mg ml^{-1}) of all essential amino acids (both datasets were log-transformed) and (c) proportions of all essential amino acids (data were (arcsin(sqrt(x))-transformed)).

Alternatively or additionally, the observed differences in nectar chemistry may be related to different pollination syndromes. In fact, morphological and/or functional traits of specific pollinators can reliably predict the spectrum of flowering plants visited and thus specific links in pollination networks (Rosas-Guerrero *et al.* 2014). Besides preferences for specific flower colours or shapes, pollinators can also show preferences for specific nectar profiles. For example, many flower visitors prefer sugar solutions with amino acids (*i.e.* nectar) over pure sugar solutions, *e.g.* butterflies (Alm *et al.* 1990; Mevi-Schütz & Erhardt 2005; Beck 2007), flies (Shiraishi & Kuwabara 1970; Potter & Bertin 1988), honeybees (Inouye & Waller 1984; Alm *et al.* 1990; Carter *et al.* 2006; Bertazzini *et al.* 2010) and solitary bees (Petanidou *et al.* 2006). Honeybees further prefer sugar solutions with essential amino acids over sugar solutions with non-essential amino acids (Hendriksma *et al.* 2014). Moreover, essential amino acids significantly support their feeding gland and flight muscle development (Hendriksma *et al.* 2019). Increasing concentrations and proportions of (essential) amino acids or of amino acids known to be perceived by pollinators (Ruedenauer *et al.* 2020) may consequently be a useful tool to increase attractiveness to potential pollinators and/or distract them from collecting pollen. In fact, Asteraceae plants in our study tended to have the highest concentrations and proportions of all (essential) amino acids and of the essential amino acid histidine, which may (among others) explain why Asteraceae were frequently visited by many different pollinators and other flower-visiting insects (Table S5; also Ebeling *et al.* 2008; Venjakob *et al.* 2016). However, some amino acids can also deter insects (Bell *et al.* 1996; Toshima & Tanimura 2012), as can several plant secondary metabolites (Stevenson *et al.* 2017). Moreover, nectar often contains additional substances in relatively low amounts, such as minerals or fatty acids (Nicolson & Thornburg 2007), which may also affect nectar preferences (Parachnowitsch *et al.* 2019). Notably, nectar of the Lamiaceae species investigated in our study did not show as high concentrations of the essential amino acid phenylalanine as Lamiaceae nectar of plants from the Mediterranean phryganic community (Petanidou *et al.* 2006), suggesting that different selection pressures may act on the nectar amino acid composition of related plant species growing in different habitats.

Besides individual compounds and compound groups (*e.g.* carbohydrates, C, or amino acids, AA), ratios (e.g. C:AA) between different compound groups were recently found to correlate with pollen foraging preferences, *e.g.* in bumblebees (Vaudo *et al.* 2016). Ratios have, to our knowledge, hitherto not been related to phylogeny or other floral traits. We found C:AA and C:EAA ratios in general to be carbohydrate-biased and variable, but also to show some plant species and family specificity. The carbohydrate-biased ratios meet the nutritional needs of most flower visitors, *e.g.* adult honeybee and bumblebee workers, which typically prioritize carbohydrate over (essential) amino acid intake, even over-consuming amino acids to obtain sufficient carbohydrates, and perform generally better on carbohydrate-rich diets (Paoli *et al.* 2014; Stabler *et al.* 2015; Austin & Gilbert 2021). However, (in particular female) butterflies prefer nectars rich in amino acids, and thus likely having lower C:AA ratios, because amino acids increase their fecundity (Mevi-Schütz & Erhardt 2005). Nutritional requirements of different pollinator groups (*e.g.* butterflies *versus* bees) might thus exert different selection pressures on the nectar chemistry of insect-pollinated plants (Jervis & Boggs 2005), and might affect C:AA ratios (*e.g.* towards amino acids/proteins in flowering plant species pollinated by butterflies). In our dataset, C:AA ratios were lowest in Apiaceae and Fabaceae and high in Lamiaceae, which might explain (among others) why Lamiaceae (with their proportionally lower amino acid content) were most frequently visited by bees, while Apiaceae and Fabaceae (with their proportionally higher amino acid content) attracted all sorts of flower visitors (including butterflies; see Tables S3, S4).

However, studies investigating the importance of various plant traits in predicting niche partitioning in temperate interaction networks between plants and insect flower visitors found floral phenology, morphology (*e.g.* tube length), scent and colour, but not nectar chemistry, to most strongly determine choices of insect visitors (Junker *et al.* 2013; Rafferty & Ives

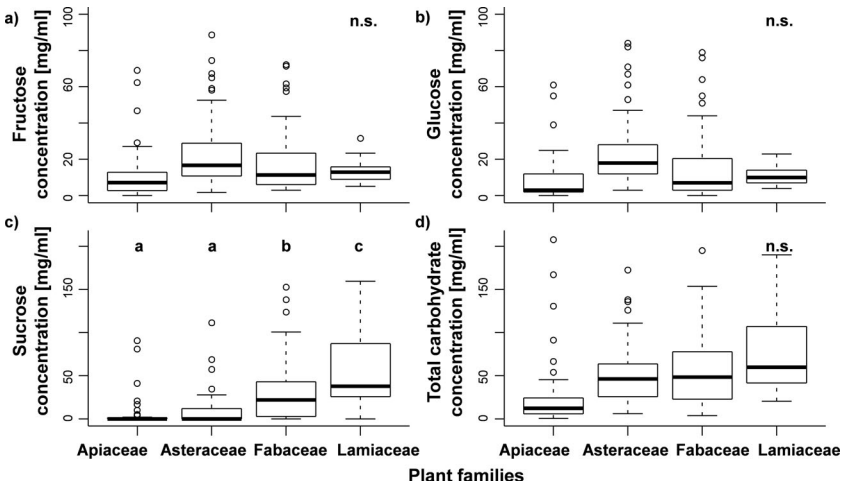

Fig. 4. Concentrations of fructose (a), glucose (b), sucrose (c) and all three (total carbohydrates) (d) (in mg ml^{-1}) in nectar of the most abundant plant families, Apiaceae, Asteraceae, Fabaceae and Lamiaceae. Different lowercase letters within each diagram indicate significant differences between the plant families, n.s. indicates no significance.

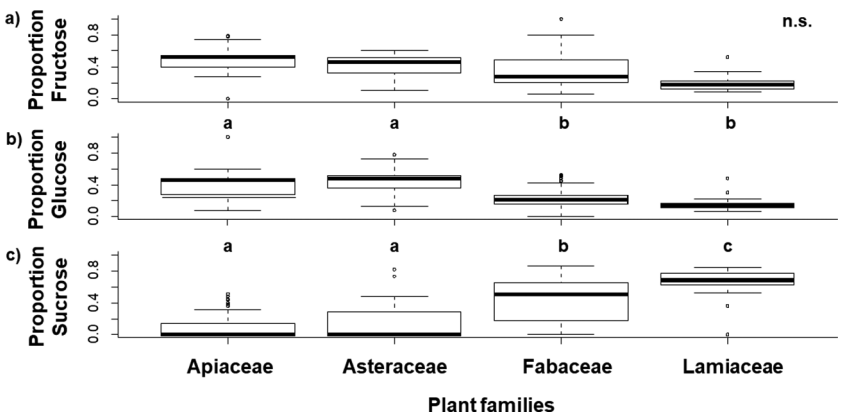

Fig. 5. Proportions of fructose (a), glucose (b) and sucrose (c) in nectar of the most abundant plant families, Apiaceae, Asteraceae, Fabaceae and Lamiaceae. Different lowercase letters within each diagram indicate significant differences between the plant families.

2013; Kantsa *et al.* 2018). Nevertheless, interestingly, different floral traits can interact. For example, bee-pollinated flowers often have a blue or yellow colour and UV marks (Wilson *et al.* 2004), known to be correlated with a comparatively high sugar content (Chittka & Menzel 1992) and to match the visual capacities of bees (Chittka & Menzel 1992). In fact, local variation in nectar sugar content might even drive selection for innate colour preferences (Raine & Chittka 2007). In turn, nectar volume in the bumblebee-pollinated *Aconitum gymnandrum* was found to be under strong selection pressure by pollinators (Zhao *et al.* 2016). These studies indicate that the plant–pollinator mutualism can affect nectar chemistry, but that directional outcomes are variable and likely depend on specific interactions. Our results suggest that these additionally depend on plant family-specific morphological constraints. Finally, an increasing number of studies highlight the role of specific microbiota in nectar (Fridman *et al.* 2012) and even of the foraging behaviour of pollinators themselves (Bogo *et al.* 2021) in affecting nectar chemistry, providing additional factors that may determine variation in floral nectar chemistry.

More thorough investigations of nutrient amounts and ratios found in floral resources, *i.e.* pollen and nectar, of plant species differing in phylogenetic relatedness, morphological characters, pollinator dependency and reward strategy, as well as their effect on pollinator preferences, should enable us to better disentangle the contributions of these various factors impacting floral resource chemistry. This knowledge is essential for better understanding the mechanisms underlying plant–pollinator interactions (Kantsa *et al.* 2018; van der Kooi *et al.* 2021).

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CONFLICTS OF INTEREST/COMPETING INTERESTS

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

CV, AMK and SDL designed the experiment. CV collected data; SDL and FAR performed statistical analyses. CV developed the first draft of the manuscript, and all authors contributed substantially to revisions.

AVAILABILITY OF DATA AND MATERIAL

All original data is submitted with this manuscript and will be deposited at the Jena Experiment upon publication.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Ratio of total amino acids (AA) in mg to total sugar in mg.

Fig. S2. Cluster dendrograms of the four most abundant plant families (Apiaceae, Asteraceae, Fabaceae and Lamiaceae).

Table S1. Nectar concentration and proportion of chemical components, such as amino acids and carbohydrates, measured *via* HPLC.

Table S2. Chemical distance matrix between 34 plant species based on Bray-Curtis distances between either: the (a,c) proportions (%) or (b,d) concentrations (conc) of amino acids and carbohydrates.

Table S3. Statistical results of generalized linear (mixed) models (GL(M)Ms) testing for effects of morphological factors (flower symmetry, nectar access, inflorescence area, flower height) and family for different single and total amino acid concentrations and proportions.

Table S4. Statistical results of generalized linear (mixed) models (GL(M)Ms) testing for effects of morphological factors (flower symmetry, nectar access, inflorescence area, flower height) and family for different single and total carbohydrate concentrations and proportions.

Table S5. Plant species studied (Roscher *et al.* 2004) for their nectar amino acid and carbohydrate composition (plant species in italic and families in bold letters) with flower visitors recorded at different plots of the Jena Experiment in 2011 (for further details see Venjakob *et al.* 2016).

Table S6. Morphological traits of flowers and accessibility of pollen and/or nectar.

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