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**Tasty rewards for ants: differences in elaiosome and seed
metabolite profiles are consistent across species and reflect
taxonomic relatedness**

RNDr. Thesis

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Annotation

The reward for ants in myrmecochory is a nutrient-rich appendage, the elaiosome. This study evaluates the metabolite composition of five groups (i.e. amino acids, fatty acids, organic acids, polyols, and sugars) in elaiosomes compared to the respective seeds. Two plant species from each of the four families (Amaryllidaceae, Boraginaceae, Papaveraceae, and Poaceae) were examined, with each species represented by three populations. The hierarchically designed data enabled the decomposition of variability in metabolite composition at three levels: family, species, and population. Another aspect studied was the consistency of elaiosome metabolite composition across unrelated species from monocotyledonous and dicotyledonous families with different elaiosome origins, which may reflect the convergent evolution of elaiosomes.

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České Budějovice, 30.6.2023

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Souhlasím s výše uvedeným prohlášením.

Za všechny spoluautory

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Prof. RNDr. Jan Lepš CSc.



Tasty rewards for ants: differences in elaiosome and seed metabolite profiles are consistent across species and reflect taxonomic relatedness

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Abstract

Diaspores of myrmecochorous plants consist of a seed (or fruit) and an attached appendage (elaiosome) which attracts ants. The elaiosome is a food resource for ants, whereas the seed is an energy source for subsequent germination and plant establishment. Although myrmecochory occurs in many phylogenetically unrelated lineages, multiple phylogenetic lineages display similar variation in elaiosome and seed metabolite composition due to convergent evolution. We focused on four families (Amaryllidaceae, Boraginaceae, Papaveraceae and Poaceae) each represented by two species from different genera. Diaspores of three populations per species were sampled and concentrations of 60 metabolites from five groups (amino acids, fatty acids, organic acids, polyols and sugars) were determined for both elaiosomes and seeds. Variability in metabolite composition was decomposed by hierarchical ANOVA and variation partitioning using redundancy analysis (reflecting both species nested within families, crossed with seed vs. elaiosome). Differences in the metabolite composition of elaiosomes and seeds were consistent across multiple phylogenetic origins (with more pronounced differences at the level of individual metabolites than at the level of metabolite groups) and supported the idea of convergent evolution under strong selection pressure. Elaiosomes contained higher amounts of easily digestible metabolites (especially amino acids) than seeds. Fatty acids were not more concentrated in elaiosomes, which contradicts the literal translation of “elaiosome” (= oil body). The differentiation of metabolite composition closely reflected taxonomic relatedness, particularly at the family level. Differences among populations within species were small, so the metabolite composition can thus be considered as a trait with relatively low intraspecific variability.

Keywords Myrmecochory · Convergent evolution · Variation partitioning · Ant–plant mutualism · Seed dispersal

Introduction

Myrmecochory is a type of plant dispersal conditioned by a plant–ant mutualism.

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Dispersal by ants helps myrmecochorous plants to reduce (1) density-dependent seed predation (Heithaus 1981), (2) competition among seedlings (Canner et al. 2012) and (3) parent–offspring competition (Giladi 2006). The ants are rewarded with a food source in the form of an elaiosome, a nutrient-rich appendage of the seed or fruit (Sernander 1906; Brew et al. 1989; Mark and Olesen 1996; Gorb and Gorb 2003). The seed or fruit together with the elaiosome, form the diaspore. For simplicity and to follow the terminology typically used in papers on myrmecochory, we will always use the term “seed” to refer to the diaspore but less the elaiosome (regardless of whether the elaiosome is appended to a seed or fruit). Elaiosomes can originate from seed, fruit or floral tissues (Ciccarelli et al. 2005; Mayer et al. 2005). Myrmecochorous plants have been identified in 77 plant families (with at least 101 independent evolutionary origins) and occur on all continents, except Antarctica (Lengyel et al.

2010). The attractiveness of diaspores to ants varies among plant species and is principally determined by elaiosome metabolite composition (Lanza et al. 1992; Reifenrath et al. 2012).

Brew et al. (1989) proposed three possible explanations for diaspore removal by mutualistic ants based on diaspore chemical composition: (1) elaiosomes are a nutrient source, a hypothesis that is supported by the preferential feeding of elaiosomes to larvae (Fischer et al. 2005) (2) elaiosomes are a source of essential chemical metabolites including fatty acids and sterols which cannot be synthesised by ants (Gammans et al. 2005) and (3) removal is triggered by the presence of chemical signals. The search for the main chemical diaspore-removal trigger for ants has been the topic of many studies—various free fatty acids or those bound in storage lipids have been among the most promising candidates, primarily oleic acid (Boulay et al. 2006; Lopez-Riquelme et al. 2006; Pfeiffer et al. 2010; Turner and Frederickson 2013). According to Fischer et al. (2008), oleic acid was the most abundant elaiosome fatty acid in the majority of their study species. Other lipids that have been shown to trigger the removal of diaspores by ants include linoleic acid (Diez et al. 2014), ricinoleic acids (Bresinsky 2014) triolein (Brew et al. 1989) and 1,2-diolein (Brew et al. 1989). Gammans and Bullock (2006) proposed that the diaspore-removal trigger may be 1,2-diolein, a diglyceride of oleic acid and major component of hemolymph (Hughes et al. 1994). Moreover, the overall fatty acid composition of elaiosomes is more similar to that of insect hemolymph than that of the respective seeds (Hughes et al. 1994).

To the best of our knowledge, only Fischer et al. (2008) have expanded on the previously studied metabolite spectrum by studying three groups of compounds in detail: amino acids, fatty acids and soluble carbohydrates (sugars and polyols), total proteins and starch.

The metabolites that are attractive for ants should be more concentrated in elaiosomes than in seeds, which should be dispersed unharmed to serve as an energy source for seedling growth. Due to the different functions of the two diaspore parts and convergent evolution of the elaiosome, we can expect differences in metabolite composition between elaiosomes and seeds to vary in a consistent manner. Elaiosomes should have higher concentrations of metabolites attractive for ants across multiple phylogenetic lineages. Previous studies have generally focussed on elaiosome chemical composition only (Lanza et al. 1992; Schmeer et al. 1996; Boulay et al. 2006; Peternelli et al. 2008; Boeiro et al. 2012), with relatively few (Hughes et al. 1994; Fischer et al. 2008; Chen et al. 2016) comparing metabolites in seeds and elaiosomes.

The origin of the elaiosome is usually the same within a genus or family (Mayer and Svoma 1998; Bresinsky 1963), but it differs among taxonomic groups (i.e., it is

phylogenetically conservative, as is the chemical composition). Still, previous studies were focused on a limited number of populations of one species (Hughes et al. 1994; Boulay et al. 2006; Peternelli et al. 2008; Boeiro et al. 2012). Very few studies compared elaiosomes and seeds across multiple species (Hughes et al. 1994) and families (Fischer et al. 2008). Following Fischer et al. (2008), we broadened the usual focus from fatty acids to a wider metabolite spectrum. We included additional metabolite groups which are involved in central metabolism and are thus potentially important for ants, specifically amino acids, organic acids, sugars and related polyols. Moreover, we also focused on the metabolite variability in elaiosomes and seeds across various levels of taxonomic relatedness. This, until now, had not been studied, likely due to the requirement of balanced sampling at various taxonomic levels. To do this, we selected four families in the Czech flora for metabolite analysis, each represented by two myrmecochorous genera with well-developed elaiosomes. Each genus in our selection was represented in our study by one species, thus in the analyses and proceeding text we call this taxonomic level “species” (so that the variation explained by this level corresponds to the difference between two non-congeneric species). We aimed to answer the following questions:

1. What are the compositional differences between elaiosomes and seeds in a wide spectrum of metabolites?
2. Do differences in metabolite composition in elaiosomes and seeds reflect taxonomic relatedness (i.e., populations within species, species within families and families themselves)?
3. Are these differences consistent across unrelated species from monocotyledonous and dicotyledonous families with different elaiosome origins, thus supporting the hypothesis of convergent evolution?

Materials and methods

Selected species, diaspore collection and storage

The selected metabolite groups (amino acids, fatty acids, organic acids, polyols and sugars) were analysed in eight myrmecochorous species from the flora of the Czech Republic. Pairs of plant species with comparable diaspore weight were selected from different genera within four families (the delineation of families according to APG (Stevens 2016): Boraginaceae and Papaveraceae from dicotyledons (dicots), and Amaryllidaceae and Poaceae from monocotyledons (monocots). The target families and species also differed in terms of the tissue from which their elaiosomes originated (fruit, seed or floral tissues): Boraginaceae *Pulmonaria officinalis* and *Symphytum officinale* (fruit origin—pericarp base), Papaveraceae *Corydalis cava* and *Chelidonium majus*

(seed origin—raphe), Amaryllidaceae *Galanthus nivalis* and *Leucojum vernum* (seed origin—chalaza) and Poaceae *Danthonia decumbens* and *Melica nutans*. Both species have two elaiosomes originating from fruit, i.e., the part of the lemma which becomes fleshy, in addition, *Melica nutans* has one extra elaiosome of floral origin—arising from a sterile fleshy spikelet (called bud in the following text).

Diaspores of selected myrmecochorous plants were collected in 2013 and 2014. Diaspores from several plant individuals were used for one sample, thus representing the whole population. The number of analysed elaiosomes in one sample was species-specific due to different elaiosome sizes (minimal weight needed for chemical analyses was 5 mg of dry mass). For example, in the case of Amaryllidaceae the sample contained less than ten elaiosomes, in contrast, the *Chelidonium majus* sample contained more than one hundred elaiosomes. Three local populations of each species were sampled, with each population being at least 1 km from each other (Supplementary material Online Resource 1). The diaspores were stored in a freezer (−35 °C) to preserve their chemical composition. Storage lasted the whole period between sampling and metabolite analysis with the exception of the time spent separating the two diaspore parts (using a binocular microscope and a scalpel). Each diaspore was divided into two parts for the analysis—elaiosome (E) and seed (S) (Supplementary material Online Resource 2). The only exception was the presence of two different types of elaiosomes on the caryopsis of *Melica nutans*. These diaspores were thus divided into three samples—S, E (two lemma-originated elaiosomes), and B (a bud-originated elaiosome). Metabolite composition of both elaiosome types was analysed, but to obtain comparable samples within the family Poaceae, only data from the lemma-originated elaiosome were used in most statistical analyses.

Metabolite analysis

Each elaiosome and seed was lyophilized and afterwards divided into three parts for triplicate analysis with a minimal weight of 5 mg and 10 mg for elaiosomes and seeds, respectively. A method described by Fischer et al. (2008) was adapted for metabolite extraction. Each sample was homogenized in an Eppendorf tube with 1 ml of cold hexane and a steel ball in a Qiagen Tissue Lyser II, (5 min, 40 oscillations s^{−1}). After centrifugation (4 °C, 10 min, 10,000 rpm), the extraction was repeated and the supernatant was collected and stored at −20 °C for fatty acid analysis.

The residual pellet was dried in vacuo and extracted 5 times with 200 µl ethanol–water (70:30). The upper organic layer was collected, centrifuged (4 °C, 10 min, 10,000 rpm), divided into 100 µl aliquots and used separately for amino and organic acid analysis and determination of sugars and

polyols. Each aliquot was dried by evaporation and stored at −20 °C prior to further analysis.

Fatty acids were measured by gas chromatography with flame ionization detection (GC/FID) as described earlier by Zahradníčková et al. (2014) after their release from the extracted glycerides by transesterification yielding fatty acid methyl esters (FAMES). Briefly, a 100 µl aliquot of the hexane extract was mixed with 10 µl of tetracosane (IS, 10 nmol) in 50 µl hexane. 2 M sodium methoxide in methanol (100 µl) was added and the mixture was shaken intensively for 10 s and then left to stand, with occasional mixing for 15 min. Then, hexane (100 µl) and 1 M HCl (250 µl) were added and stirred to create an acidic pH. The upper hexane phase was withdrawn and after another 200 µl hexane addition, mixing and upper phase transfer, both hexane extracts were combined, dried by a nitrogen stream, diluted with an isooctane volume thus adjusting the concentration to 1 ml per mg of each sample. Finally, 1 µl of the extract was injected into a GC/FID system (Shimadzu 17A, Prague, Czech Republic).

For saccharide and polyol analysis, the second aliquot obtained by the ethanolic sample extraction (described above) was mixed with 120 µl of methanolic xylitol solution (internal standard = IS, 10 nmol), and the mixture was dried by evaporation using a nitrogen stream. Oximation and silylation of the residue and the subsequent GC-FID were accomplished as described earlier (Košťál et al. 2011a).

The third aliquot of the ethanolic extract was used in the analysis of amino acids and organic acids by an ethyl chloroformate derivatization method with subsequent GC–MS analysis (Košťál et al. 2011b).

Statistical analysis

For all analyses, concentrations of metabolites were expressed in µg per 1 mg of elaiosome or seed dry mass (i.e., µg mg^{−1}). Furthermore, as concentrations varied by orders of magnitude, they were log transformed using the formula $Y' = \log(Y+1)$ due to the presence of zero values in the data. The log transformation converts multiplicativity on the original concentration scale to additivity on the log scale (approximately, due to the addition of one). As the metabolite analysis of some polyols and sugars in one *Danthonia decumbens* elaiosome sample failed, the missing values were replaced by average of the other two populations to keep a balanced design needed for the multivariate analyses.

The metabolite composition was characterized at two resolution levels: first, as total content of all measured metabolites within each group (called as metabolite group sums in the following, i.e., amino acids, organic acids, fatty acids, sugars and polyols) and, second, considering the individual metabolites. The total content within each metabolite group was analysed by separate univariate analysis (i.e., by nested

design mixed effect ANOVA; type III—orthogonal sum of squares decomposition; Statistica programme StatSoft) for each metabolite group and for all groups together, in one multivariate analysis. All multivariate analyses were done using the framework of constrained ordinations (Šmilauer and Lepš 2014) using the programme Canoco 5 (ter Braak and Šmilauer 2012). The individual metabolites (in total 60 metabolites) were analysed using the same multivariate analyses both separately for metabolites in each of the five major metabolite groups, and also all the individual metabolites together. In all analyses (both univariate and multivariate), we aimed to decompose the variability into the differences between the elaiosome and seed and the variability explained by the taxonomic relatedness of species (and the interactions of these two components).

The decomposition of the variability in concentration sums of each individual target metabolite group was done by a nested design mixed effect ANOVA simultaneously for all hierarchical levels, i.e., *family*, *species(family)*, *part*, and *species(family) × part* interaction. The brackets signify nesting, so that *species(family)* indicates species nested in *family*, since the variability explained by *species* is related to the variability of species within families, i.e., the difference of the two confamilial species. The factor *species* (and also its interaction with *part*) was considered as a factor with a random effect. The *species(family) × part* interaction reflects the non-additivity of the main effects of factors *species* and *part*—in other words, low variability explained by this interaction means that the differences between elaiosome and seed are consistent across various species. The residual variability corresponds to variability among populations of the same species. The variability explained was characterized by mean square (MS), because the total amount of explained variability, characterized by sum of squares (SS), is dependent on degrees of freedom (DF) of the corresponding explanatory variables.

The multivariate analyses (redundancy analysis—RDA, which can be understood as a multivariate extension of general linear models) were used to analyse the sums of metabolite groups, individual metabolites within groups, or all of the individual metabolites together. In each case, the concentrations of individual metabolites were centred and standardized (resulting in a variable with zero mean and variance of one). The significance was determined by a Monte Carlo permutation test (9999 permutations). To identify the main differences between diaspore parts for both concentration sums and individual metabolites, we applied a partial RDA with a population factor as the categorical covariate. This category was also used as a block to define permutations. Because a single multivariate analysis does not enable simultaneous variability decomposition, we used a series of analyses with various combinations of explanatory variables and covariables to decompose the variability (Šmilauer

and Lepš 2014). As in the previously mentioned univariate analyses, explained variability was expressed by MS (similar to the method of variation partitioning implemented in the Canoco 5 package, ter Braak and Šmilauer (2012). MS was obtained by dividing the explained variability (trace, corresponds to SS in univariate analyses) by respective DF. Hierarchical decomposition of variability for individual metabolites was examined separately for elaiosomes and seeds (unlike in previous univariate analysis) at two levels (species and family). At the species level, partial RDA was used with family as a categorical covariate and model-based permutations, i.e., in this case freely exchangeable, were performed. The family level analysis was performed by RDA with a hierarchical permutation design. The variability among individual populations was calculated by subtracting the variability explained by the two previous levels from one (i.e., from the total variability). The combinations of explanatory variables and covariables to estimate individual components of variability are shown in (Online Resource 3); note that variability among populations equals to the residual variability (i.e., variability not explained by any explanatory variables).

To visualize the differences in diaspore parts among individual species (and also among families), we used RDA with the *species × part* interaction as the only explanatory variable. The interactions, as implemented in Canoco5 multivariate analyses, also include the corresponding main effects. In our case, the interaction is a categorical variable, where individual categories are all possible combinations of species and part (each category is then shown by its centroid in the ordination diagram). Because the distances in the ordination diagram correspond to compositional dissimilarity, we can compare not only the differences between diaspore parts with differences between species, but also infer the (in)consistence of the seed–elaiosome differences: if the differences across species are consistent, the elaiosomes in the ordination diagram will be shifted from the seeds in the same direction in all species. In this analysis, the balanced design was not strictly necessary, and we thus used both elaiosome types of *Melica nutans*.

Results

Differences in chemical composition of elaiosomes and seeds

In total, 60 metabolites were quantified in diaspores of the target myrmecochorous species (26 amino acids, 13 fatty acids, 6 organic acids, 9 polyols and 6 sugars—Online Resource 4). We found significant differences between elaiosomes and seeds in terms of metabolite composition characterized by the five metabolite group sums using RDA

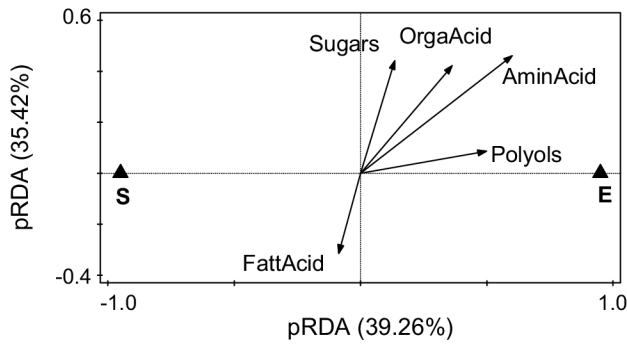


Fig. 1 Results of partial RDA of the five target metabolite group sums with part as the explanatory variable and population identity as the covariate. The effect of the explanatory variable, i.e., the difference between seeds (S) and elaiosomes (E) in the concentration of five groups of metabolites (AminAcid=amino acids, FattAcid=fatty acids, OrgaAcid=organic acids, Polyols and Sugars) is highly significant ($F=15.5$; $p=0.0004$). Numbers in axes labels are percentages of partial variability explained by individual axes

analysis (Fig. 1, Online Resource 5). While amino acids, organic acids and polyols were highly positively correlated and significantly more concentrated in elaiosomes (significance of each group determined by separate ANOVA, Table 1), sugars and fatty acids were not significantly different between elaiosomes and seeds, and were negatively correlated with each other ($r = -0.415$).

The greatest difference between diaspore parts was detected in the amino acids, which were on average 8.9 times more concentrated in elaiosomes than in seeds of the respective species, (the only exception was *Galanthus nivalis*). Similarly, significantly higher concentrations of organic acids (exceptions are *Galanthus nivalis* and *Danthonia decumbens*) and polyols (exception *Chelidonium majus*) were found in elaiosomes. Fatty acids in seeds exhibited the highest total concentration within our metabolite groups across all species (Fig. 2). The fatty acids were the only metabolite group with (nonsignificantly) higher average concentrations in seeds (apart from *Leucojum vernum* and *Danthonia decumbens*), whereas in elaiosomes they were as concentrated as sugars with by far the highest amount found in the *Galanthus nivalis* elaiosome (Fig. 3).

The sums of metabolite groups were significantly different between diaspore parts in only three out of five groups (amino acids, organic acids and polyols). However, when taking the individual metabolite composition into account, RDA revealed significant differences in all five groups (Online Resource 6) and accordingly, if all the individual metabolites were analysed together, all individual amino acids and organic acids were more concentrated in elaiosomes (corresponding well to the fact that their sums were highly different between elaiosomes and seeds). Despite having no significant difference between

Table 1 Results of nested ANOVAs of the concentrations of the five target groups of metabolites (NS— $p > 0.05$, * $0.01 < p < 0.05$, ** $0.001 < p < 0.01$, *** $p < 0.001$)

Effect	DF	Error DF	Amino acids			Fatty acids			Organic acids			Polyols			Sugars			p					
			SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F						
Family	Fixed	3	4	1.40	0.47	0.65	NS	9.05	3.02	7.54	*	1.25	0.42	1.26	NS	0.36	0.12	0.41	NS	6.93	2.31	24.70	**
Part	Fixed	1	7	4.57	4.57	12.20	*	0.01	0.01	0.03	NS	0.96	0.96	6.69	**	0.97	0.97	20.18	**	0.19	0.19	1.31	NS
Species(family)	Random	4	7	2.86	0.71	1.91	NS	1.60	0.40	1.60	NS	1.32	0.33	2.30	NS	1.16	0.29	6.01	*	0.37	0.09	0.64	NS
Species(family) × part	Random	7	32	2.62	0.37	9.92	***	1.75	0.25	0.94	NS	1.00	0.14	1.68	NS	0.34	0.05	1.44	NS	1.03	0.15	2.50	*
Residual			32	1.21	0.04		8.48	0.27		2.73	0.09		1.07	0.03		1.88	0.06						

The significant terms are shown in bold
Residual comprises combined variability among populations and population × part interaction

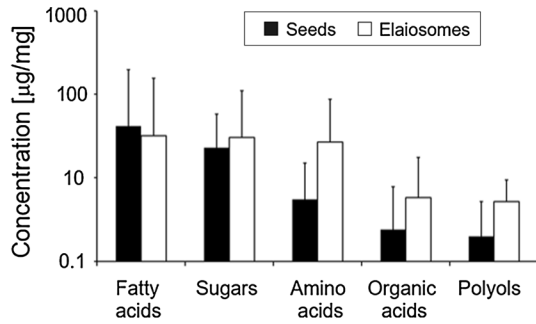


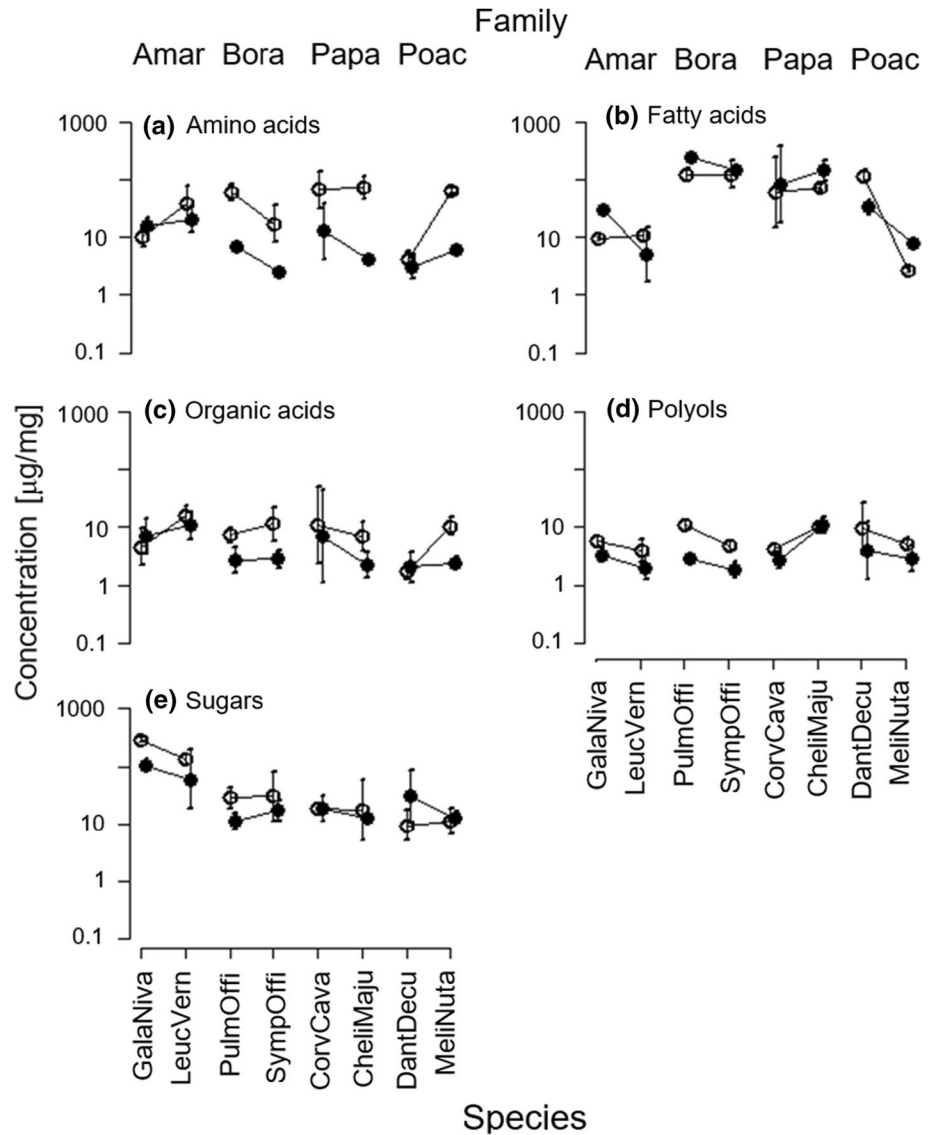
Fig. 2 Mean concentration sums of the five metabolite groups across all species based on dry mass [µg/mg]. Note log scale of vertical axis. The error bars represent variability among species, calculated as standard deviation based on log-transformed concentrations

elaiosome and seed total fatty acids and sugars, their individual metabolite compositions differed, with some of them more concentrated in elaiosomes, and some in seeds. This was particularly pronounced in sugars, where saccharose prevailed in seeds, whereas the other sugars were more abundant in elaiosomes. In general, the comparison showed a trend towards the accumulation of more easily digestible compounds in the elaiosome, particularly amino acids and to a lesser extent also fatty acids with shorter chains (Online Resource 6).

Influence of taxonomic relatedness

We found a pronounced effect of taxonomic relatedness (i.e., family and species within family) on metabolite composition. There was a typical descending pattern (with few exceptions) in the amount of explained variability from the

Fig. 3 Differences in the average content of the target metabolite groups within elaiosomes (open circles) and seeds (filled circles) at the species and family level. The error bars represent 95% confidence intervals. Family and species abbreviations are: Amar = Amar-yliaceae, Bora = Boragi-naceae, Papa = Papaveraceae, Poac = Poaceae; GalaNiva = *Gal-anthus nivalis*, LeucVern = *Leu-cojum vernum*, PulmO-ffi = *Pulmonaria officinalis*, SympOffi = *Symphytum officinale*, CoryCava = *Corydalis cava*, CheliMaju = *Chelidonium majus*, DantDecu = *Danthonia decumbens*, MeliNut = *Melica nutans*



family level followed by species and populations explaining the lowest portion of variability (Fig. 4a, Online Resource 7). In all metabolite groups the population level accounted for the smallest amount of variability (but still the variability is not negligible, Online Resource 8). Furthermore, species (within family) explained more variability than family in amino acids in elaiosomes and roughly the same amount in

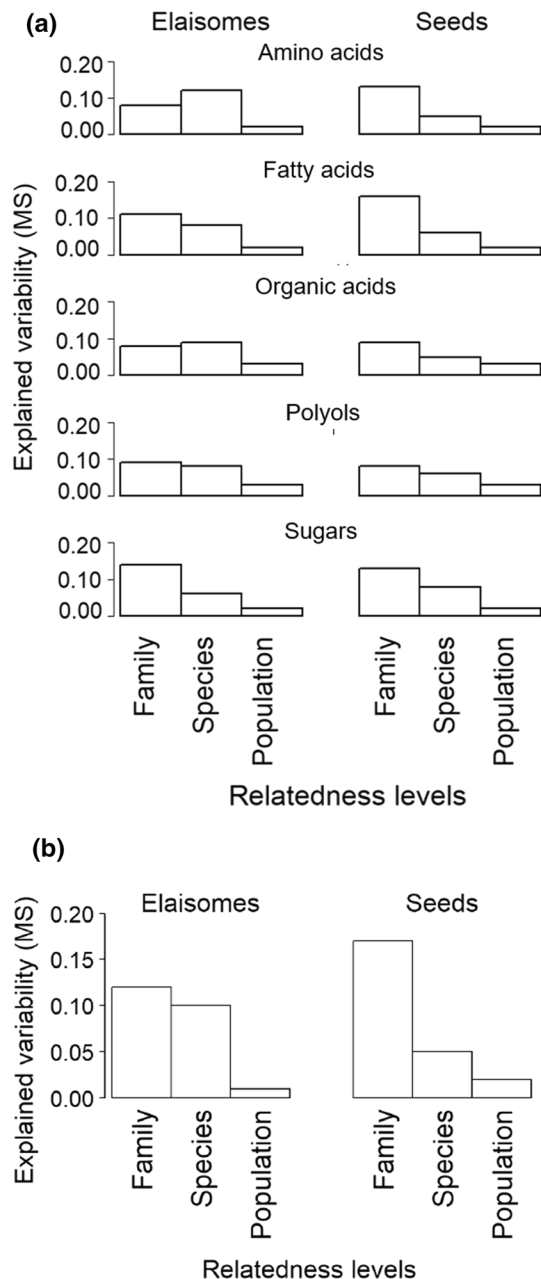


Fig. 4 Variability explained by individual taxonomic levels (separately for elaiosomes and seeds): **a** individual metabolites of five groups, **b** sums of metabolite groups. The explained variability is characterized by MS. The variability explained by the levels family and species is obtained from separate RDA analyses and MS for population level is derived from the residual variability

organic acids and polyols in elaiosomes. Despite the strong explanatory power of the family level, families significantly differed in total sugar and fatty acid content only (ANOVA $F_{3,4} = 24.70$; $p = 0.005$ and $F_{3,4} = 7.54$, $p = 0.04$, respectively; Table 1; Fig. 3), whereas the species level differed significantly in all metabolite groups (with the only exception of organic acids in seeds). The non-significant results for the family were quite likely the result of low test power determined by the corresponding denominator degrees of freedom in the F test and the use of species as a random factor. Considering species as a random effect indicates that the studied species represent a random selection of species from an indefinitely large number of species in a given family. When the species was treated as a fixed factor, differences between families were tested against the variability among populations, and were always highly significant (Online Resource 9). Considering *species* as a fixed factor would suggest that the objects of our interest were just the two selected species from each family only or reflect the fact that the number of native myrmecochorous species in some families is rather low. In the Amaryllidaceae, the two investigated species are the only native myrmecochorous species. For the sums of the metabolite groups, family explained slightly more variability than the species level in seeds, whereas in elaiosomes species explained considerably less than family (Fig. 4b).

Consistency of elaiosome–seed trends across species

Differences between diaspore parts in terms of the sums of the five metabolite groups were consistent across species and are shown in the ordination diagram (Fig. 5). With the exception of *Danthonia decumbens*, there was a negative shift on the first ordination axis from the seed to elaiosome, corresponding to an increase of amino acids, organic acids, and sugars in elaiosomes. A similar pattern was also revealed for individual metabolite composition (Fig. 6a, b). Nevertheless, when each of the metabolite group sums was analysed separately by ANOVA, the significant species \times part interaction revealed that the differences in log-transformed concentrations (i.e., in concentration ratios) between elaiosomes and seeds for amino acids and fatty acids varied across species (Table 1; Fig. 3). The two elaiosome types of *Melica nutans* differed in the content of our target metabolite groups, with the exception of sugars, which were comparable in all three parts of the diaspore (Figs. 5 and 6, Online Resource 5). Amino acids were approximately three times more concentrated in the bud (which was not included in the main variability decomposition analysis) than in seed, whereas they were 13.8 times higher in the lemma. Polyol and organic acid concentrations were lower in the

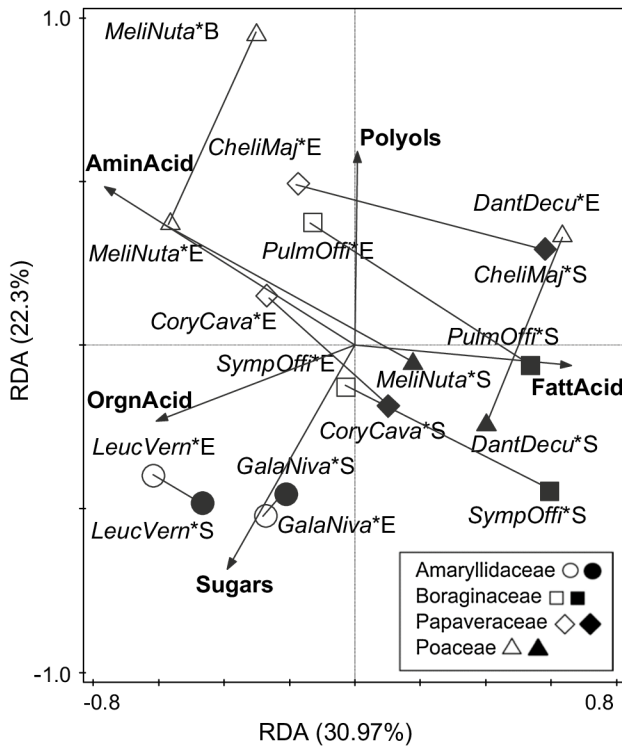


Fig. 5 RDA of the five target metabolite groups sums (arrows) with the *species* × *part* interaction as the explanatory variable. The effect of interaction is highly significant ($F=8.4$; $p=0.0001$). Note that the effect of interaction also includes the main effects (i.e., *species* and *part*). Numbers in axes labels are percentages of total variability explained by individual axes. Lines connect elaiosomes and seeds of one species (the bud elaiosome [B] is taken as separate category). Numbers in brackets are percentages of variability explained by individual axes; the shapes of the centroids signify the species membership in a family, Amaryllidaceae (circle), Poaceae (triangle), Papaveraceae (diamond), and Boraginaceae (square); filled ones are for seeds and open for elaiosomes. Species abbreviations are GalaNiva=*Galanthus nivalis*, LeucVern=*Leucojum vernum*, PulmOffi=*Pulmonaria officinalis*, SympOffi=*Symphytum officinale*, CoryCava=*Corydalis cava*, CheliMaju=*Chelidonium majus*, DantDecu=*Danthonia decumbens*, MeliNut=*Melica nutans*

bud-originated elaiosome and were similar to those of the seed. Both elaiosome types together contained approximately half of the amount of fatty acids found in seeds. This resulted in Poaceae having the greatest among species differences within the same family, whereas Amaryllidaceae species differed the least. Elaiosomes were, for several species, more similar to each other across different families than to seeds of the respective species. For instance, the elaiosome of *Melica nutans* from Poaceae had a rather similar metabolite composition to that of *Leucojum vernum* from the Amaryllidaceae family, and that of *Corydalis cava* to that of *Chelidonium majus*—both from the Papaveraceae family.

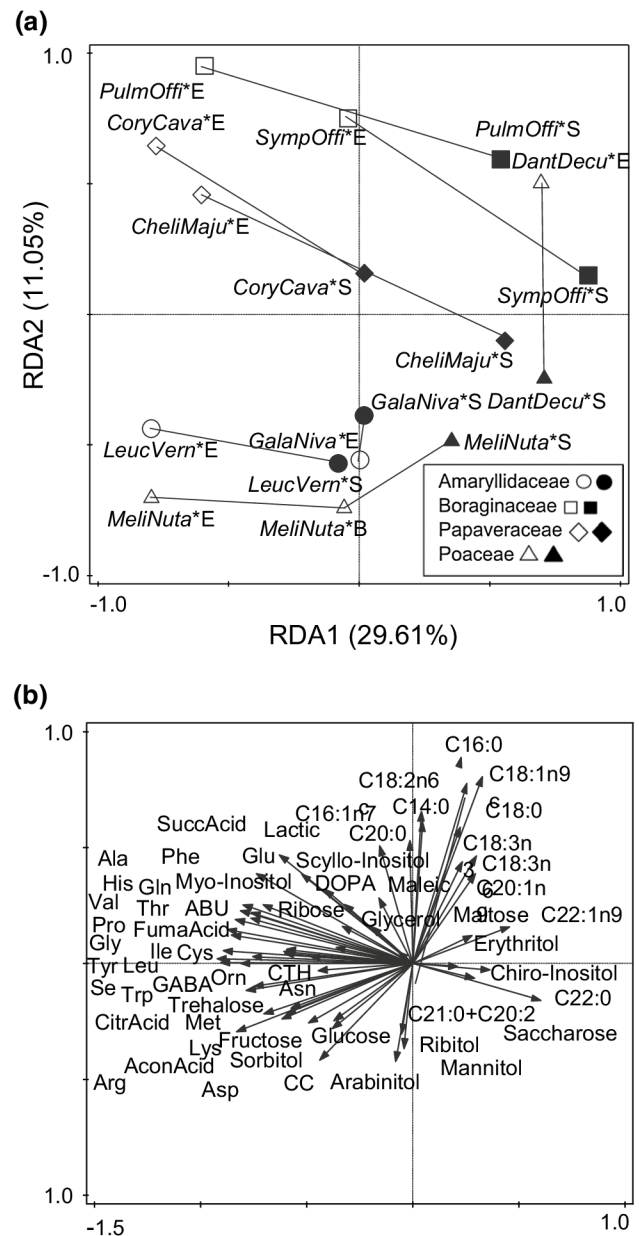


Fig. 6 RDA of individual metabolites with the *species* × *part* interaction as explanatory variable (a) lines connect elaiosomes and seeds of one species (the bud elaiosome [B] is taken as separate category) (b) corresponding ordination with individual metabolites (arrows). The effect of the interaction is highly significant ($F=6.3$; $p=0.0001$). Note that the effect of the interaction also includes the main effects (i.e., *species* and *part*). Numbers in axes labels are percentages of partial variability explained by individual axes; the shapes of the centroids signify the species membership in a family, filled ones are for seeds and open for elaiosomes

Discussion

The total concentrations of three out of our five target metabolite groups were significantly higher in elaiosomes than in seeds (amino acids, organic acids and polyols). Moreover, all

five groups (including also fatty acids and sugars) differed significantly between elaiosomes and seeds in terms of the composition of individual metabolites. This suggests that not only the total amount, but also the identity of metabolites is important for ants. These trends were consistent across species indicating the adaptive value of metabolite composition for ant dispersal. The accumulation of more easily digestible metabolites (e.g., fatty acids with shorter chains) in elaiosomes produces the tasty reward for ants. The metabolite composition closely reflected taxonomical relatedness, with particularly large differences between families. Small differences among populations indicated relative stability in metabolite composition within species.

Differences in chemical composition of elaiosomes and seeds

There was a clear trend across all studied species that the total concentration of amino acids, organic acids, and polyols was higher in elaiosomes than in seeds. Fischer et al. (2008) reported the same trends for amino acids, whereas the other two groups have not been studied separately. Fischer et al. (2008) examined polyols grouped together with sugars within a soluble carbohydrate group, and reported no significant difference between elaiosome and seed in the concentration of these metabolites. Similar to Fischer et al. (2008), we did not find any consistent differences in total concentrations of sugars and fatty acids.

The total content of amino acids was on average 8.9 times higher in elaiosomes than in seeds, representing the most pronounced difference among all the metabolite groups. This trend was consistent for all individual amino acids. Similarly, Fischer et al. (2008) and Reifenrath et al. (2012) found higher amino acid content in elaiosomes (7.5 and 3.6 times, respectively).

Fatty acids are frequently considered as ant attractants or a constituent thereof (Lopez-Riquelme et al. 2006; Boulay et al. 2006; Pfeiffer et al. 2010; Reifenrath et al. 2012). However, in our study, their content was slightly, but not significantly, higher in seeds than in elaiosomes. The exception to this was *Leucojum vernum* and one population of *Danthonia decumbens*. Significantly higher fatty acid content in the elaiosome than the seed has been reported in only two other species. Fischer et al. (2008) found significantly higher (two times) lipid content in elaiosomes in only one out of 15 species (*Knautia arvensis*), and Chen et al. (2016) in *Stemona tuberosa*. In some cases, the fatty acid content can even drop under 5% of elaiosome dry mass (Fischer et al. 2008). In our study, fatty acid content was lowest in one *Leucojum vernum* population (2.5%); however, the mean across all species was 16% of dry mass. Therefore, neither the above-mentioned studies, nor ours, support the meaning of the word elaiosome, which literally translates as “oil body”.

The low concentrations of fatty acids in elaiosomes cannot be explained by incomplete diaspore maturity, as suggested by Ciccarelli et al. (2005), because we collected only mature diaspores directly from plants. On the other hand, we measured fatty acids in the nonpolar lipid fraction only (similarly as Fischer et al. 2008). While they represent the most abundant group of storage lipids occurring in elaiosomes (Paternelli et al. 2008), other forms such as triglycerides and diglycerides (monoglycerides less frequently) occur (Boulay et al. 2006; Paternelli et al. 2008; Chen et al. 2016). However, these fatty acids typically constitute only a minor component of elaiosome fatty acids and are thus unlikely to reverse the trend. While lipid content has been shown to be important for diaspore removal (e.g., Diez et al. 2014; Bresinsky 2014), not one single compound has been consistently identified. For example, Boulay et al. (2006) showed that ants prefer diaspores with relatively high concentrations of oleic acid but low concentrations of palmitic and linoleic acids. In our study, linoleic acid was the most abundant fatty acid in elaiosomes, followed by palmitic acid and oleic acid. However, Fischer et al. (2008) found that linoleic acid and oleic acid were dominant in elaiosomes and seeds, respectively. Seeds in our study contained mainly arachidic acid, in accordance with the findings of Hughes et al. (1994). Therefore, which fatty acids (if any) might act as attractants for ants remains unclear.

A detailed analysis of elaiosome polyols is presented here for the first time. Previously, only Fischer et al. (2008) gave mention to two polyols, which were included in a group of soluble carbohydrates, together with sugars. However, we found that these two groups follow different trends. Higher sums of polyols across target species were associated with elaiosomes (all species except *Chelidonium majus*), whereas sugars were roughly equally represented in both diaspore parts. Similar to Fischer et al. (2008), we found higher myoinositol content in elaiosomes than in seeds. Nevertheless, Fischer et al. (2008) found a higher glycerol concentration in seeds, whereas we noted a clear association of glycerol with elaiosomes. We hypothesize that the higher levels of polyols in elaiosomes could serve as an anti-freezing agent (many myrmecochorous plants have an early phenology, with night frost quite likely to occur during seed ripening). Furthermore, polyols could also serve as a protectant against dehydration, thus keeping the elaiosome longer fresh and attractive for ants.

Slightly (but nonsignificantly) higher total sugar content was found in elaiosomes compared with seeds across all target species, in line with Fischer et al. (2008) and Chen et al. (2016). The elaiosomes of Amaryllidaceae species *Galanthus nivalis* and *Leucojum vernum* are well-known for high sugar content. Sugars comprised above 10% of Amaryllidaceae elaiosome dry mass according to Fischer et al. 2008, while here they accounted for 23%. According

to Bresinsky (2014), the sugar content of the species *Galanthus nivalis* is sufficiently high to be recognizable by human taste. In fact, *Galanthus nivalis* had the highest elaiosome total sugar content recorded in this study. Bresinsky (2014) noted *Chelidonium majus* as one of the few examples of a species with a sugarless elaiosome. This is however in conflict with our study and also with Fischer et al. (2008). The descending order of detected sugar abundance was as follows: fructose, glucose, saccharose, maltose, trehalose, and ribose. Saccharose (a disaccharide) was the only sugar more abundant in seeds (suggesting that its primary role is energy storage in seeds), in agreement with Fischer et al. (2008). Bresinsky (2014) suggested that total hexose concentration (represented in our work by fructose, glucose and saccharose, three of the most abundant sugars in elaiosomes) may play an important role in myrmecochory.

Influence of taxonomic relatedness

Metabolite composition closely reflected taxonomic relatedness, with the largest proportion of variability explained by family, indicating that on the family level, it is highly phylogenetically conservative. The variability among populations of the same species was rather low. To reliably decompose the variability, we need a balanced design. In previous studies, families were generally represented by only one, or at most, three target species but not equally distributed across families (Fischer et al. 2008), which makes comparisons difficult.

The Amaryllidaceae were the most homogeneous family with regards to metabolite content (both in terms of the similarity between diaspore parts and among the species). This may be explained by both diaspore parts originating from the same tissue (seed tissue), but also by their close taxonomic relatedness (only *Galanthus* and *Leucojum* belong to the same tribe). On the other hand, the group which varied most was the other monocot family, Poaceae, which is the only one in which elaiosomes of the two species originated from different tissues. It suggests that the metabolite composition of elaiosomes is determined not only by taxonomical similarities, but also by the morphological origin of elaiosomes.

The variability among populations (i.e., the residual variability), was lowest in all five metabolite groups. In addition to differences between populations (Boulay et al. 2006, for fatty acid), differences among individuals within populations are known to occur (Boieiro et al. 2012, for fatty acids). Nevertheless, our data suggest that the intraspecific differences are rather small in comparison with interspecific variability, particularly in elaiosomes. It is possible that under favourable conditions plants invest more in seeds. This will always benefit germination success; however, investing more into elaiosomes will likely not bring any substantial additional benefits.

Consistency of elaiosome–seed trends across species

Corresponding to the different purposes of elaiosomes and seeds, ant attractant (Sernander 1906) and germination energy source, respectively, we found that differences in metabolite composition between these two diaspore parts were consistent across various species. These elaiosome and seed differences were present across the various tissue types (i.e., seed, fruit and floral tissue) from which elaiosomes originate (Mayer et al. 2005) and occurred across families despite multiple independent evolutionary origins. Moreover, differences between elaiosome and seed were often (particularly for amino acids and organic acids) more pronounced than the effect of taxonomic levels. In general, the metabolite composition of elaiosomes in unrelated species seems to be more similar to each other than to the composition of seeds of the same species, with the exception of two monocots, *Danthonia decumbens* and *Galanthus nivalis*. Thus, our results based on five metabolite groups, and also the findings of other studies examining lipid fatty acid content (Fischer et al. 2008; Boieiro et al. 2012) suggest convergent evolution of the elaiosome, which provides a selective advantage to myrmecochorous plants. This is in line with the findings of Dunn et al. (2007) demonstrating that the origins of myrmecochory are closely associated with an increase in the relative abundance of ants in the fossil record. As noted by Lengyel et al. (2010), the elaiosome might have originated as an adaptation for diaspore dispersal either due to strong selection pressure or because it is “cheap” to produce. Higher amino acid content in the elaiosome than in the seed was the most pronounced and widespread pattern we observed. This supports the idea of strong selection pressure, as amino acids are rather costly for plants.

The *species* \times *part* interaction was not significant in the majority of metabolite groups, indicating consistent concentration differences between elaiosomes and seeds. Amino acids were the only exception to this pattern. However, despite its statistical significance, the interaction MS was more than ten times smaller than the MS for the part level, showing that the differences in the ratio are relatively small.

Not very surprisingly, the composition and concentration of individual metabolites differed between the examined plant species. Therefore, the metabolome of elaiosomes and seeds is species-specific and thus the selection of species may have partially affected the results (e.g., relatively high total sugars in our study might partially reflect the high proportion of Amaryllidaceae in our species set). Nevertheless, the consistent differences between elaiosomes and seeds in the composition of metabolites across various distantly related families underpin the robustness of our results. The relatively small differences between populations of the same species indicate that metabolite composition can be

considered a trait for which intraspecific compared to interspecific variation is small and thus for ecological functioning (i.e., for triggering the ant removal behaviour), species identity is crucial (Lepš et al. 2011).

The consistency of the differences between elaiosomes and seeds indicates their adaptive significance. We can expect that the metabolites most important for ants are those with the highest ratio of elaiosome to seed concentrations. This indicates that amino acids (and perhaps also polyols and organic acids), and not storage lipids, are the key metabolites for attracting and rewarding of ants.

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Author contribution statement MK and JL designed the experiment, MK collected the plant diaspores and prepared separated samples of elaiosomes and seeds, MM and HZ conducted the chemical analyses, MK and JL carried out the statistical analyses. MK wrote the first draft of the manuscript with JL, and MM, HZ and PŠ contributed the chemical aspects. All the authors revised the text and agreed upon the final version.

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