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What shapes amino acid and sugar composition in Mediterranean floral nectars?

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We studied the amino acid (AA) composition of the floral nectars of 73 plant species occurring in a phryganic (East Mediterranean garrigue) community and investigated whether AA and sugar composition is shaped by evolutionary (plant phylogeny), ecological (flowering time as a direct effect of summer drought) and coevolutionary (pollinator partnership) constraints. Our study utilised an extensive plant–pollinator matrix compiled in the same area where the plants had been sampled.

Using HPLC we detected 22 AA compounds/groups of compounds, out of which 15 were commonly present in almost all nectars. Among all AAs, phenylalanine was the most abundant, especially in keystone ("cornucopian") plant species visited by many insect species, such as the majority of the Lamiaceae. Amino acid quantities were transformed into percentages (% of each AA over the total AA content of a flower). Sugar composition was similarly expressed as % of each of the three sugars (glucose, fructose, sucrose) over the total content of these sugars; a number of other sugars, occurring in only a few plant species and in very low quantities were disregarded. The number of insect species of a particular family or guild was taken as a measure of the attraction of a nectar compound for such a family (guild).

We found that taxonomical plant group had a weakly significant effect on nectar composition while neither life form nor flowering season had a discernable effect. Pollinators' preference had the most important effect, with phenylalanine being the most consequent discriminatory compound for the response of the nectar consumers in phrygana, predominantly for long tongued bees, especially for Megachilidae. Gamma-aminobutyric acid (GABA) had a similar, even stronger influence on bees (long tongued bees, Anthophoridae, Andrenidae) and flies (Syrphidae and other Diptera), whereas asparagine behaved as a general repellent together with tryptophane (rather as repellent). Considering total sugar and AA contents, as well as the volume of nectar, we found that total AA content was positively related to the number of species of long tongued bees and included families visiting the phryganic species; nectar volume was negatively related to flies (both hover flies and remaining Diptera), whereas total sugar content was not significant for any guild. We argue that due to the highly concentrated nectars in the dry Mediterranean communities that are characterised by outstanding melittophily, sugars play a less important role as phagostimulants compared to AAs in floral nectars. This is why phenylalanine, a phagostimulant tested earlier on honeybees, appears to be of high importance in phrygana, especially with long tongued bees and Megachilids as the main selective agents for phenylalanine-rich nectars. The role of GABA, a strongly NaCl-dependent AA, may be similar, probably because of the associated presence of NaCl.

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Functioning almost exclusively as floral reward to pollinators, nectar compostition is likely to be subject to selection pressures imposed by the nectar feeders. As a consequence, and due to "convergent coevolution", nectar characteristics tend to be similar for plants exhibiting the same pollination syndrome and different between closely related plants with different pollinators (Pyke and Waser 1981, Baker and Baker 1982). At the same time, nectar traits are subject to influence by phylogenetic affiliations and ecological factors imposed by the habitat (Corbet 1990, Petanidou and Smets 1996, Petanidou et al. 1999, 2000, Petanidou 2005).

Most of the nectar literature focuses on sugars, the major components of floral nectars. The amounts and sugar concentration of nectar have been related to pollinator type (Percival 1961, 1965, Baker and Baker 1975), especially considering the sucrose/hexose ratio (Wykes 1952, Percival 1961, Baker and Baker 1979, 1982, 1983, 1990, Southwick 1982, Stiles and Freeman 1993, Petanidou et al. 1996, 2000, Petanidou 2005). Amino acids (AAs) in nectars, and their evolutionary significance, have also received attention (Baker and Baker 1973a, 1973b, 1982, 1986, Baker et al. 1978, Gottsberger et al. 1984, 1989, 1990, Pais et al. 1986, Erhardt 1991, 1992, Petanidou et al. 1996, 2000). In a long series of studies based on the analysis of ca 1500 species, Baker and Baker (1973a, 1973b, 1982, 1986) proposed that the amount of AAs present in nectars are directly in connection with their pollination systems. These conclusions were disputed by Gottsberger et al. (1984, 1989), but the debate has never been concluded on the basis of hard evidence from a large scale study. Today the "consensus view" is that plants adapted to pollination by butterflies have high concentrations of AAs compared to plants that are bird or bee pollinated (Gardener and Gillman 2002).

Dafni and Kevan (1994) proposed that AAs in nectar may have a positive effect in attracting pollinators. When reviewing the recent literature on AA preference of flower-visiting insects, Gardener and Gillman (2002) also concluded that not only honeybees but also flies show a preferential response to AAs contained in nectar-mimicking solutions. The authors emphasize the contribution of AAs to the taste of nectar (rather than the nutritive value), an aspect that has been neglected by the nectar literature. This is related to their finding that the amounts of AAs in nectars are greatly variable in nature whereas their composition (i.e. % contribution of the particular AAs) is much less variable (Gardener and Gillman 2001). This implies that low AA quantities may act mainly as attractants or phagostimulants in the natural floral nectars found in nature. In this respect, the phagostimulatory effect of phenylalanine which was found to alter the response of honeybees among several single AAs tested, appears to be very significant (Inouye and Waller 1984).

In this study we investigate the factors shaping the AA and sugar (AA&S) composition in floral nectars. Because of the type and diversity of the factors explored, it was important that our approach should be carried out at a community level and encompass an entire plant–pollinator community. The study investigates the pollinator preference for nectar AA&S as recorded in nature at the community level and within the actual plant–pollinator (p-p) interaction network. The novelty of this study in comparison to earlier ones is that it explores:

1) AA&S nectar composition in a number of native plant species being under the same ecological constraints, and
2) the preference for AA&S nectar composition by bees and other insects visiting the plants at the community level, considering their taxonomy and guild membership on the basis of all plant–pollinator interactions recorded.

The study was conducted in a phrygana, a typical low shrub occupying the driest parts of the precipitation gradient in the East Mediterranean, comparable to the garrigue in the West Mediterranean. Its physiognomy is determined by seasonally dimorphic woody plants, such as *Thymus capitatus, Sarcopoterium spinosum,* and *Satureja thymbra,* but in terms of species diversity, annual plants are the predominant life form (Petanidou and Vokou 1993, Petanidou et al. 1995). Despite the first impression of precariousness and poverty, phrygana bears a rich flora, and many nesting possibilities for ground and twig nesting bees (Petanidou and Ellis 1993, Potts et al. 2004). This high diversity is maintained despite of strong environmental pressures, in the first place the prolonged summer drought of the Mediterranean climate, exacerbated by grazing and fires. In fact, the Mediterranean basin, and especially its low scrublands, has been considered as one of the world's centres of bee speciation (Michener 1979, 2000) supporting some of the most diverse plant–pollinator communities (Petanidou 1991, Petanidou and Ellis 1993, 1996, Petanidou et al. 1995, Petanidou and Potts 2006). The fact that background data is available on flowering and on p-p food web at the community level, together with the high insect (especially bee) diversity of phrygana, and the fact that ecological constraints play a significant role in shaping many pollination and nectar attributes in the community (Petanidou et al. 1995, 2000), make phrygana an excellent research habitat.

Considering that Mediterranean communities are strongly influenced by ecological limitations that may be more influential than phylogenetic ones (Petanidou et al. 1995), we ask whether AA&S composition is

influenced by phylogenetic or/and ecological constraints (life form, time of flowering) or/and whether it is a result of coevolution with floral nectar consumers. The study was carried out on the largest possible number of entomophilous plant species within the community studied. Yet, because of the consistency in their ecological origin (all were typical phryganic plant species, no cultivated or invading species) we assume that all species have undergone similar constraints, which allows us to draw conclusions concerning whether AA&S composition is shaped by phylogenetic, ecological or coevolutionary constraints.

Our predictions are as follows: if AA&S composition is shaped by phylogenetic constraints, this must be reflected in differences of AA&S composition among high level plant taxonomical groups. If ecological constraints shape AA&S composition, this composition is expected to differ among life forms or/and to depend on flowering season. Finally, if AA&S composition is governed by coevolutionary constraints (i.e. pollinators), then plants will differ in AA&S composition according to the insect guilds and families they is pollinated by (Petanidou et al. 1995, Petanidou 2005).

In a separate study Petanidou (2005) explored the driving forces shaping the sugar composition alone within the floral nectars of the phryganic plants in the same community. She found that both ecological and evolutionary attributes are significant in shaping sugar composition in the nectars of the same phryganic plants. In particular she found that sugar composition in the nectars, measured as sucrose/hexose ratio, is associated with flowering season (ecological constraint), plant family membership (evolutionary constraints), as well as with particular pollinator guilds, with the bee family Megachilidae constituting a major selective factor for "high sucrose" nectars.

Material and methods

Study site and species

The study was conducted in a phryganic community within the nature reserve "I. and A. Diomedes Botanical Garden of Athens University". Detailed description of the site is available in earlier studies (Petanidou and Ellis 1993, 1996, Petanidou et al. 1995).

All major nectar producing species (73) occurring in the reserve were considered in the study, comprising ca 65% of the local nectariferous flora (Petanidou et al. 1995, Petanidou and Smets 1995, Table 1).

Nectar measurements and analyses

Nectar measurements and collection were carried out during 1992 and 1993 and, in very few cases, in 1994 during the flowering peak of each species (Petanidou et al. 1995, Petanidou and Smets 1995). Because AA quantity increases with senescence, we used flowers at their first day of anthesis (Petanidou et al. 1996).

All nectars analyzed in this study were collected at the same time and from the same plants used to measure nectar volume (Petanidou and Smets 1995) and to analyse for nectar sugars (Petanidou 2005). Laboratory AA chemical analyses were conducted on the same nectars used to carry out sugar analyses. This is why nectar volume and sugar data used in this study originate from the above two studies, respectively.

Flowers were selected at random and covered in bud stage with bridal veil in the eve of the collection day to prevent nectar removal and contamination by insects. Nectar was collected towards noon to early afternoon (11:00-14:00 h) except for the nocturnally flowering species *Capparis spinosa* sampled between 9:30 and 10:00 h. The nectar of each flower was picked up directly on a Whatman No. 1 small paper wick prepared in advance. During the collection, particular care was taken to avoid any contamination of the nectar by pollen or other structural plant tissue. After nectar collection the paper wicks were fixed on stainless steel pins that had been carefully cleaned with acetone, then placed on styrofoam blocks and left to air-dry. Finally they were stored at $\pm 5^{\circ}$C in air-tight containers over silica gel until analysis. Touching with the fingers or other possible contaminating sources was carefully avoided (Petanidou et al. 1996). Number of flowers analyzed per plant species varied between 1 and 51.

AA analysis of nectar samples was carried out by means of high pressure liquid chromatography (HPLC). Before analysis, nectar content of each wick was dissolved in 1 ml of distilled water in a microcentrifuge tube by intermittent vortexing at room temperature for at least one hour. Finally, the tubes were centrifuged to remove paper particles (Petanidou et al. 1996).

For chemical analysis, 100 µl of the diluted nectar were treated for 3 min with 50 µl of 0.02 (w/v) o-phthaldialdehyde and 0.02 (v/v) 2-mercaptoethanol in 0.1 M $NaHCO_3$, pH 9.5, immediately before injection. Twenty µl of the AA derivatives were then injected on a 200×3 mm ChromSpher C8 (Chrompack, Middelburg, the Netherlands) HPLC-column. Elution of the column occurred at a constant flow rate of 0.8 ml min^{-1} using a linear gradient from 20 to 100% (v/v) of solvent B over 15 min and continuing with solvent B for 10 min. Elution solvents were: A = methanol/H_2O/tetrahydrofuran (2/96/2) containing 50 mM Na_2HPO_4 and 50 mM sodium acetate (neutralized to pH 7.5 with acetic acid), and B = methanol/H_2O (65/35 v/v) (Petanidou et al. 1996). The derivatives were fluorometrically detected (1700 Fluorescence Monitor, Bio Rad, USA;

Table 1. The plant species of phrygana studied for their nectar amino acids and sugars using HPLC analysis.

Date of nectar collection	Plant species[1]	Abbreviation	Life form[2,3]	Nectar volume[3] µl flower^{-1}	Midpoint of flowering[3] calendar day
	Monocotyledons				
	Amaryllidaceae				
21.10.92	*Sternbergia lutea* Orph. ex Nym. subsp. *sicula* (Tin. ex Guss.) D.A.Webb	Sg	geo	1.33	297
	Iridaceae				
17.11.92	*Crocus cancellatus* Herb.	Ce	geo	0.19	298
25.2.94	*Romulea linaresii* Parl. subsp. *graeca* Bég.	Ro	geo	0.07	33
	Liliaceae				
30.4.92	*Allium subhirsutum* L.	Ab	geo	0.03	106
20.10.92	*Asparagus acutifolius* L.	Af	geo	0.02	268
6.4.92	*Asphodelus aestivus* Brot.	Am	geo	2.44	85
25.2.94	*Fritillaria graeca* Boiss. & Spruner subsp. *graeca*	Fg	geo	0.06	85
24.2.94	*Muscari commutatum* Guss.	Mu	geo	0.01	54
24.2.94	*Muscari neglectum* Guss. ex Ten.	Mn	geo	0.01	39
14.4.93	*Ornithogalum exscapum* Ten.; *O. graecum* C. Zahariadi	Oc	geo	0.05	70
21.10.92	*Scilla autumnalis* L.	Su	geo	0.01	290
6.9.92	*Urginea maritima* (L.) Baker	Um	geo	0.64	261
	Campanulids				
	Apiaceae				
16.7.92	*Eryngium campestre* L.	Ey	herb	0.004	193
7.4.92	*Scandix australis* L. subsp. *australis*	Sc	ther	0.03	73
4.6.92	*Thapsia garganica* L.	Tg	herb	0.02	136
9.4.92	*Tordylium apulum* L.	Ta	ther	0.01	105
	Asteraceae				
13.4.93	*Calendula arvensis* L.	Ca	ther	0.01	80
14.6.93	*Centaurea orphanidea* Heldr. & Sart. ex Boiss. subsp. *orphanidea*	Co	ther	0.01	165
25.4.92	*Centaurea raphanina* Sibth. & Sm. subsp. *mixta* (DC.) Runemark	Cr	herb	0.21	117
3.5.92	*Chrysanthemum coronarium* L.	Cc	ther	0.01	128
13.7.92	*Echinops microcephalus* Sibth. & Sm.	Ec	herb	0.13	190
9.8.92	*Echinops sphaerocephalus* L. subsp. *albidus* (Boiss. & Spruner) Kozuharov	Es	herb	0.16	220
2.6.92	*Helichrysum stoechas* DC. subsp. *barrelieri* (Ten.) Nyman	Hs	woody	0.001	136
26.4.92	*Hypochaeris achyrophorus* L.	Ha	ther	0.01	114
4.6.92	*Pallenis spinosa* (L.) Cass.	Ps	ther	0.001	144
1.5.92.	*Phagnalon graecum* Boiss. & Heldr.	Pg	woody	0.01	125
28.4.92	*Reichardia picroides* (L.) Roth	Rp	herb	0.03	107
1.5.92	*Tragopogon porrifolius* L. subsp. *porrifolius*	Tp	ther	0.01	110
	Campanulaceae				
27.4.92	*Campanula drabifolia* Sibth. & Sm. subsp. *drabifolia*	Cf	ther	0.03	118
	Capparidaceae				
13.6.94	*Capparis spinosa* L. var. *inermis* Turra	Cs	woody	42.05	188
	Dipsacaceae				
5.6.92	*Pterocephalus papposus* (L.) Coult.	Pp	ther	0.03	123
12.5.93	*Scabiosa atropurpurea* L.	Sa	ther	0.01	125
27.4.92	*Tremastelma palaestinum* (L.) Janch.	Tm	ther	0.05	116
	Lamiids				
	Boraginaceae				
7.4.92	*Alkanna tinctoria* (L.) Tausch	At	herb	0.34	95
13.4.93	*Anchusa variegata* (L.) Lehm.	Av	ther	0.45	62
3.6.92	*Echium creticum* L.	Ea	herb	2.89	178
12.7.92	*Heliotropium europaeum* L.	He	ther	0.05	276
13.7.92	*Heliotropium hirsutissimum* Grauer	Hh	ther	0.06	277
	Convolvulaceae				
13.7.92	*Convolvulus arvensis* L.	Cv	herb	0.05	170
8.7.92	*Convolvulus cantabrica* L.	Cn	herb	0.06	169
	Ericaceae				
1.11.93	*Erica verticillata* Forssk.	En	woody	0.003	363
	Globulariaceae				
11.4.93	*Globularia alypum* L.	Ga	woody	0.01	77
	Lamiaceae				
11.6.93	*Ballota acetabulosa* (L.) Benth.	Ba	herb	0.14	160
26.2.94	*Lamium amplexicaule* L. subsp. *amplexicaule*	La	ther	0.20	74
15.5.93	*Phlomis fruticosa* L.	Pf	woody	2.52	108
28.4.92	*Prasium majus* L.	Pm	woody	7.48	114
14.4.93	*Salvia triloba* L.f.	St	woody	7.74	94

Table 1 (*continued*)

Date of nectar collection	Plant species[1]	Abbreviation	Life form[2,3]	Nectar volume[3] µl flower^{-1}	Midpoint of flowering[3] calendar day
9.4.92	*Salvia verbenaca* L.	Sb	ther	0.33	85
16.5.93	*Satureja thymbra L.*	Sj	woody	0.05	137
2.6.92	*Stachys cretica* L. subsp. *cretica*	Sy	herb	0.59	137
5.6.92	*Teucrium chamaedrys* L.	Td	woody	0.50	135
4.6.92	*Teucrium polium* L. subsp. *capitatum* (L.) Arcang.	Te	woody	0.06	156
8.7.92	*Thymus capitatus* (L.) Hoffmanns. & Link	Tc	woody	0.10	171
	Malvids				
	Brassicaceae				
19.4.93	*Eruca vesicaria* Cav. subsp. *sativa* (Mill.) Thell.	Ev	ther	0.13	113
2.5.92	*Sisymbrium orientale* L.	So	ther	0.01	119
	Cistaceae				
30.4.92	*Cistus parviflorus* Lam.	Cp	woody	0.05	125
2.5.92	*Cistus salvifolius* L.	Ci	woody	0.02	104
	Malvaceae				
5.6.92	*Alcea pallida* (Willd.) Waldst. & Kit.	Ap	herb	2.54	160
	Resedaceae				
27.4.92	*Reseda alba* L.	Re	herb	0.10	104
	Rutaceae				
5.6.92	*Ruta graveolens* L.	Rg	herb	0.32	145
	Thymelaeaceae				
27.2.93	*Thymelaea hirsuta* (L.) Endl.	Th	woody	0.000	25
	Lower dicotyledons				
	Caryophylaceae				
25.4.92	*Petrorhagia velutina* (Guss.) P.W.Ball & Heywood	Pv	ther	0.04	106
9.4.92	*Silene colorata* Poir.	Si	ther	0.06	95
	Ranunculaceae				
10.7.92	*Delphinium peregrinum* L.	Dp	ther	0.52	185
5.6.92	*Nigella arvensis* L.	Ng	ther	0.30	169
13.4.92	*Ranunculus sprunerianus* Boiss.	Ra	geo	0.08	98
	Fabids				
	Cucurbitaceae				
15.7.92	*Ecballium elaterium* (L.) A. Rich.	Ee	herb	0.03	214
	Euphorbiaceae				
11.4.93	*Euphorbia acanthothamnos* Heldr. & Sart. ex Boiss.	Eu	woody	0.25	79
	Fabaceae				
16.5.93	*Anthyllis hermanniae* L.	Ah	woody	0.01	132
8.4.92	*Astragalus monspessulanus* L.	Ao	herb	0.28	102
17.4.93	*Hymenocarpos circinnatus* (L.) Savi	Hc	ther	0.01	96
29.4.92	*Psoralea bituminosa* L.	Pso	herb	0.24	135
8.4.92	*Trifolium stellatum* L.	Ts	ther	0.04	98

[1] Nomenclature used is according to "The International Plant Names Index" (2004)
[2] Life forms are: geophytes (geo), therophytes or annuals (ther), herbaceous perennials (herb), and woody perennials (woody)
[3] from Petanidou et al. (1995) and Petanidou and Smets (1995)

excitation at 350 nm, emission at 440 nm), and quantified by automatic integration after calibration of the system with known AA quantities. Although proline is known to be important to pollinators, it could not be identified by this method; the same held for cysteine (same as in Petanidou et al. 1996, 2000). Because the pairs glutamine and histidine, glycine and threonine, as well as alanine and tyrosine, could not be well separated in many plant species, these AAs will be referred to as AA pairs in this paper.

Plant flowering time, life form and phylogeny

In order to investigate the importance of flowering time during the year in shaping nectar AA&S composition, we determined the midpoint of flowering time of the plant species studied. Species having their midpoint in calendar days 61-151 were assigned as spring-, those between 152-240 as summer-, between 241-320 as autumn- and between 321-60 as winter-flowering (Petanidou et al. 1995). The life form types are described in Petanidou et al. (1995).

Conceivably, the AA&S values might be determined by the phylogeny of the plants, rather than by ecological constraints. To test this we grouped the plant species into a limited number of supra-ordinal categories, as defined by A.P.G. (1998) and Soltis et al. (2005). These groups, in "rising" taxonomical order, are: monocots, lower dicots, fabids, malvids, lamiids and campanulids.

Flower visitors and plants used in the analyses

In order to detect whether pollinator species had a differential response to the AA&S composition of flower nectars, we used the community data matrix from Petanidou (1991) that was also used as a basis in similar studies (Petanidou and Ellis 1996, Petanidou 2005, Petanidou and Potts 2006). The data were collected from March 1983 to May 1987 from all entomophilous plant species of the same community where the nectars were collected. As "pollinators", all flower visitors were considered if visiting the flowers repeatedly, irrespectively of their "quality" (pollinator effectiveness), as normally done in such community studies (Waser and Ollerton 2006). The matrix contains the plant–pollinator interactions for 70 out of the 73 plant species analyzed here (no data for the species *Echinops sphaerocephalus* subsp. *albidus*, *Teucrium chamaedrys* and *Romulea linaresii*, which were excluded from the plant–pollinator interaction analyses). In addition, another three plant species had to be excluded from all the data analyses because either their nectar value was taken from the chemical analysis of one flower only (*Fritillaria graeca*), or because there was a high risk of nectar contamination (*Thymelaea hirsuta* and *Crocus cancellatus*). Therefore, our analysis is based on 67 plant species visited by 565 insect species, resulting in 1865 plant–pollinator interactions which were basically considered in the calculations.

For each plant species, we counted the number of species visiting them, grouped into guilds and into major bee families. We recognized the following guilds: Coleoptera, Hemiptera, Lepidoptera. Syrphidae, other Diptera, Apidae, long tongued (solitary) bees (viz. Anthophoridae and Megachilidae), short tongued bees (viz. Andrenidae, Halictidae and Colletidae; the single observation of a melittid species was disregarded), and wasps (i.e. other Hymenoptera). The numbers so obtained (referred to below as "visitors per guild" or "visitors per family") were taken as indicative of the importance of a plant for a guild or family.

Data analysis

The AA values from all laboratory analyses were calculated as amounts of AA per flower (= AA content). This was the average of several separate runs (mainly 3-11; Table 2), each one on the basis of one flower where nectar secretion was sufficient (e.g. in Lamiaceae). In cases where nectar secretion per flower was too low, due to the little nectar secreted by a flower (Petanidou and Smets 1995), the nectar samples were pooled for HPLC analysis (Table 2). Information about chemical and data analysis for sugars is given in Petanidou (2005).

In all statistical analyses we used the % composition of a particular AA (i.e. proportion of a single AA over the total AA content in the nectar of a plant species; in pmoles) instead of using absolute AA concentrations. Similarly, values of glucose, fructose and sucrose (in nmoles) were transformed into percentages over the total sugar content. The use of proportions reduces problems of concentration changes due to evaporation and inter-flower variability of the total AA content, therefore it may account for more reliable results both in chemical and biological aspect (Lanza et al. 1995, Gardener and Gillman 2001).

In order to investigate the effects of ecological or phylogenetic factors on nectar composition, avoiding at the same time any possible autocorrelations within our data set, we used a multifactorial approach (multivariate ANOVA, stepwise multiple regressions, PCA). In the analyses we considered volume, all AAs, and only the three major sugars (viz. sucrose, glucose, fructose, i.e. discarding minor sugars found in traces) of the nectars.

For statistical analysis, all nectar values (viz. AA and sugar percentages, nectar volumes) and numbers of visitors were effectively normalized by transforming them to their ln values ($x' = \ln(x+1)$). Statistical analyses were done using the Data Desk package (Velleman 2005).

Whenever used in the text, values are followed by their SE. SE values are omitted in Table 2 in order to avoid confusion (SE would give the true flower-to-flower variability only in cases where nectar was analysed per flower, as it was in the case of the family Lamiaceae; Petanidou et al. 2000).

Results

In Table 1 we have listed all of the 73 plant species studied in the community. Twenty-two AA compounds or groups of AA compounds were found in their floral nectars (Table 2). Out of these, 19 were identifiable on the basis of the AA references used in the HPLC analysis. The remaining three non-identified AAs are summed up and listed under "unknown" in Table 2.

Phenylalanine was the most abundant AA in the nectars (mean value 0.72 ± 0.233 nmoles flower^{-1}, $n = 70$, Table 2). It also had the the highest proportion in the nectars over all plant species studied (% of phenylalanine: 17.1 ± 2.65, $n = 70$). Within the whole community, 21 plant species were phenylalanine-rich (having nectars with $>30\%$ phenylalanine), among them all the Lamiaceae (47.2% in average, including *Lamium amplexicaule* having a very low value). Among the most prominent phenylalanine-rich species were *Stachys cretica* (80%), *Phlomis fruticosa* (55%), *Satureja thymbra* (48%), *Urginea maritima* (73%), *Asphodelus aestivus* (46%) and *Thapsia garganica* (46%), all keystone species within

Table 2. Amino acid and sugar (only the main three sugars) composition of the floral nectars of phryganic plants after HPLC analyses.

Plant species[1]	N (N′)[2]	Amino acids																				Sugars[6]			
		Total AAs[3]	Asp[4]	Glu	Asn	Ser	His+ Gln[5]	Gly+ Thr[5]	Arg	Tyr+ Ala[5]	Gaba	Trp	Met	Val	Phe	Ile	Leu	Orn	Lys	H-Ser	Unknown	β-Ala	Total sugars[3]	Glucose	Fructose	Sucrose
		pmoles/flower																				nmoles/flower			
Ab	10 (3)	869	49	50	14	67	191	80	16	121	0	63	9	66	30	3	22	47	4	0	39	0	300	122	125	53
Af	55 (6)	583	16	21	54	91	108	54	5	38	3	16	3	25	17	7	9	3	12	0	16	84	222	93	127	1
Ah	11 (4)	240	7	4	3	19	15	40	3	15	0	11	0	94	0	4	25	0	0	0	0	0	42	0	0	41
Am*	3 (3)	8880	90	50	62	600	534	472	82	293	1291	50	25	142	4097	77	90	61	69	0	795	0	21326	7845	8035	5447
Ao	13 (6)	1621	29	19	263	128	23	119	15	26	94	13	28	74	6	14	19	26	24	150	552	0	1188	265	25	898
Ap*	3 (3)	5758	280	154	574	195	445	473	237	1610	0	75	0	287	9	17	50	140	82	0	1129	0	22637	10456	11351	831
At	6 (4)	456	18	7	11	32	7	53	5	173	36	9	0	52	39	0	12	0	0	0	0	0	1136	421	148	567
Av	13 (5)	2030	54	75	1285	47	76	101	76	75	14	84	0	26	40	6	13	22	9	0	27	0	731	216	130	385
Ba*	10 (10)	2620	70	30	0	370	160	430	0	250	0	0	30	100	920	20	50	140	50	0	0	0	338	47	12	279
Ca	12 (3)	1336	16	14	33	56	316	69	20	46	46	46	0	151	262	57	51	32	109	0	10	0	106	50	52	3
Cc	24 (5)	436	20	9	4	24	17	43	18	131	0	12	0	7	56	3	7	29	14	0	43	0	111	58	50	4
Ce	13 (3)	18033	597	442	707	1610	2866	7000	138	2429	0	167	170	737	232	242	230	249	55	0	162	0	537	219	210	109
Cf	6 (3)	1271	25	24	13	51	23	85	26	148	0	109	0	0	534	15	31	62	53	0	72	0	1195	308	358	529
Ci	10 (3)	9663	1518	781	285	630	1569	539	578	1007	212	410	24	437	324	276	264	475	237	0	96	0	3648	1442	1627	579
Cn	10 (4)	1080	23	26	38	41	57	66	17	76	35	38	13	27	546	8	20	0	0	0	50	0	639	293	336	10
Co	13 (4)	2673	26	7	92	102	89	1739	11	61	0	33	0	222	16	17	0	260	0	0	0	0	130	12	8	110
Cp	10 (3)	8172	1041	867	147	1025	537	725	232	1138	9	689	20	313	387	145	272	272	311	0	42	0	3389	763	806	1821
Cr	11 (3)	1323	36	14	39	214	114	343	12	170	0	41	4	51	26	12	20	136	62	0	31	0	568	61	15	491
Cs*	5 (5)	24529	9852	281	2094	889	3793	1441	138	1107	697	0	1519	804	348	273	568	369	282	0	18	56	70313	27972	27243	15099
Cv	6 (3)	1832	21	34	124	75	54	183	14	117	29	31	62	761	0	25	56	98	23	0	126	0	898	325	369	204
Dp	10 (3)	1717	49	29	22	147	435	145	25	91	97	53	0	39	430	8	15	104	26	0	0	0	887	13	76	798
Ea*	5 (5)	5094	49	37	3	108	47	225	4	630	70	42	0	0	3458	38	65	196	124	0	0	0	4493	652	157	3684
Ec	8 (5)	642	19	16	3	41	82	85	42	87	44	17	3	57	0	6	7	4	4	0	125	0	1654	443	1201	10
Ee	14 (3)	1375	25	60	287	113	54	182	62	230	41	74	0	25	116	17	11	46	11	0	21	0	163	47	47	69
En	6 (5)	334	14	7	23	26	34	55	33	19	4	11	4	72	10	6	8	2	9	0	0	0	9	5	5	0
Es*	4 (4)	1430	40	26	109	60	186	79	59	148	44	96	0	49	443	12	8	57	12	0	0	0	755	285	281	190
Eu	14 (2)	294	5	6	11	28	23	41	4	13	15	13	21	36	45	3	5	16	2	0	6	0	216	84	111	21
Ev	5 (3)	715	41	47	58	57	142	81	40	54	10	24	0	42	57	11	14	0	38	0	0	0	956	469	483	4
Ey	12 (1)	273	6	2	7	15	15	35	9	57	0	12	0	56	1	2	9	16	8	0	23	0	267	113	142	12
Fg*	1	7346	129	219	520	528	1260	683	816	330	954	13	0	683	119	57	217	485	200	0	132	0	305	18	175	112
Ga	6 (1)	355	6	2	6	26	14	67	4	19	3	0	25	118	14	3	12	15	14	0	5	0	103	51	35	17
Ha	51 (3)	762	38	18	11	55	70	72	19	207	88	27	0	43	35	5	16	12	10	0	35	0	218	104	111	4
Hc	13 (2)	1317	46	25	157	100	54	128	86	76	2	27	0	212	220	51	34	63	34	0	0	0	100	30	34	35
He	8 (3)	867	45	82	47	66	77	72	104	97	47	32	7	45	30	19	28	45	13	0	11	0	286	134	137	15
Hh	8 (3)	1946	75	149	62	121	112	137	572	272	12	46	17	71	72	29	92	0	105	0	0	0	487	146	153	188
Hs	40 (4)	170	2	2	5	8	9	0	0	12	8	6	4	31	0	1	1	80	0	0	0	0	102	31	38	33
La*	4 (4)	3690	200	50	30	1000	180	890	20	290	0	0	20	60	70	70	190	200	330	0	0	90	202	68	14	120
Mn*	11 (11)	4472	184	160	893	278	375	280	130	220	217	35	455	203	150	75	123	381	165	0	149	0	252	116	134	2
Mu*	11 (11)	3462	115	93	636	143	95	167	71	122	123	26	292	155	90	194	72	789	246	0	32	0	121	68	51	2
Ng*	3 (3)	576	25	15	9	30	34	39	4	26	0	8	0	49	299	6	15	0	17	0	0	0	317	3	27	287
Oc*	3 (3)	1514	82	39	31	234	181	284	71	59	65	23	6	165	37	34	47	105	36	0	14	0	392	262	123	8
Pf*	10 (10)	5770	140	80	60	270	510	230	30	890	0	0	40	150	3150	50	60	70	40	0	0	0	4084	267	1258	2559
Pg	12 (1)	594	6	3	10	17	20	32	41	19	29	3	0	103	222	5	8	38	26	0	11	0	124	58	63	3
Pm*	9 (9)	2020	70	30	10	140	60	210	20	180	220	0	60	80	750	20	50	40	80	0	0	0	4900	980	267	3653
Pp	14 (2)	307	7	7	17	25	54	45	4	35	12	17	5	40	5	4	10	2	1	0	16	0	434	151	155	128
Ps	18 (2)	138	2	1	2	7	6	17	2	13	3	6	5	18	14	0	1	35	6	0	0	0	7	4	2	1

Table 2 (*continued*)

Plant species[1]	N (N′)[2]	Amino acids																				Sugars[6]			
		Total AAs[3]	Asp[4]	Glu	Asn	Ser	His+ Gln[5]	Gly+ Thr[5]	Arg	Tyr+ Ala[5]	Gaba	Trp	Met	Val	Phe	Ile	Leu	Orn	Lys	H-Ser	Unknown	β-Ala	Total sugars[3]	Glucose	Fructose	Sucrose
		pmoles/flower																				nmoles/flower			
Pso	15 (3)	480	26	13	34	47	17	68	5	69	25	13	7	37	22	5	13	34	10	0	36	0	707	40	49	618
Pv	8 (2)	1296	53	36	79	60	94	90	131	124	0	33	0	52	392	19	19	26	18	0	69	0	815	376	435	4
Ra	5 (3)	1489	24	45	78	100	275	103	62	113	108	125	0	25	82	11	18	104	79	0	139	0	794	76	75	643
Re	5 (3)	3024	53	51	21	243	326	270	221	287	0	54	0	65	1167	23	56	113	50	0	23	0	1283	457	436	390
Rg	8 (3)	2318	19	25	34	81	71	203	171	812	0	18	0	178	19	4	37	124	9	0	515	0	3418	1146	2047	225
Ro	4 (4)	2599	80	152	38	243	365	263	111	285	0	21	187	118	90	24	198	344	0	0	80	0	377	155	180	41
Rp	20 (3)	433	25	27	3	44	62	50	16	70	0	22	0	61	3	6	15	3	4	0	21	0	202	78	78	46
Sa	8 (3)	567	20	9	7	38	76	60	4	46	26	16	0	143	18	10	4	39	45	0	9	0	361	155	157	48
Sb*	7 (7)	3200	0	0	0	50	40	120	10	40	60	0	20	60	2670	20	20	30	60	0	0	0	752	244	109	399
Sc	9 (4)	324	12	4	23	37	8	63	8	43	0	7	0	53	10	4	13	15	5	0	17	0	321	148	121	52
Sg	8 (3)	6874	127	94	1715	462	1375	473	79	744	0	76	7	224	202	83	116	502	419	0	175	0	922	313	319	290
Si*	4 (4)	395	4	3	0	16	5	42	13	139	0	6	0	36	2	9	5	55	31	0	29	0	178	91	85	3
Sj*	6 (6)	4280	90	30	20	360	120	370	30	690	0	0	20	110	2060	20	60	180	120	0	0	0	391	62	97	232
So	5 (3)	1866	54	130	56	136	183	222	57	238	328	55	44	91	34	17	21	41	25	0	134	0	603	283	315	5
St*	5 (5)	4900	170	100	100	360	330	390	330	310	40	0	140	350	1830	180	80	80	90	0	20	0	6614	974	1406	4233
Su	8 (3)	633	15	18	114	65	44	39	25	74	10	124	0	19	17	10	25	0	34	0	0	0	165	83	82	0
Sy*	10 (10)	17240	150	60	130	340	230	380	400	640	0	0	590	0	13780	70	70	140	220	0	40	0	2048	155	384	1509
Ta	15 (4)	166	7	3	0	17	8	32	5	21	3	7	0	45	7	1	10	0	0	0	0	0	125	50	58	17
Tc*	8 (8)	2570	60	10	0	260	60	230	10	210	0	30	70	140	770	20	30	200	460	0	10	0	348	51	176	121
Td*	3 (3)	9310	560	130	20	110	80	130	0	1270	0	0	80	40	6660	50	60	40	40	0	30	10	3262	559	692	2011
Te*	10 (10)	2650	350	30	20	150	100	160	10	390	40	30	20	90	1030	10	40	70	80	0	30	0	1241	116	350	775
Tg	5 (3)	869	13	17	5	50	32	66	7	82	24	26	0	51	400	6	30	0	60	0	0	0	1223	513	690	20
Th	10 (2)	346	10	9	9	49	18	76	11	32	23	35	0	41	2	3	7	2	19	0	0	0	7	2	2	4
Tm	24 (3)	216	9	8	11	19	13	26	3	38	0	16	0	29	4	2	4	13	11	0	10	0	382	147	166	69
Tp	14 (3)	536	23	13	18	60	63	81	13	77	10	19	0	74	5	6	17	30	14	0	15	0	393	168	185	40
Ts*	3 (3)	364	17	14	53	40	19	53	7	50	0	6	0	50	5	4	7	18	17	0	6	0	246	79	89	78
Um*	4 (4)	2749	18	20	27	68	180	148	13	85	61	29	0	58	1994	33	16	0	0	0	0	0	2416	1376	1041	0
Mean valuel plant		*2908*	*236*	*71*	*157*	*184*	*265*	*309*	*78*	*277*	*73*	*45*	*56*	*126*	*699*	*36*	*54*	*102*	*67*	*2*	*71*	*3*	*2478*	*869*	*906*	*703*

[1] Except few cases of relatively high nectar yielding plants (indicated with *), in ca 2/3 of the plant species several flowers were pooled for analysis
[2] The amounts (pmoles flower^{-1}) given in the table are means over N' runs (in parentheses), calculated on the basis of the mean values per run. N is the total number of flowers analyzed
[3] Total value is the sum of all particular AAs (pmoles flower^{-1}) or sugars (nmole flower^{-1}) within a plant species. Mean value/plant is the average of all values (AAs, sugars; particular or total) of all plant species within the community
[4] The order of AA presentation follows the compound appearance in the chromatograms. Abbreviations are: Ala-alanine, Arg-arginine, Asn-asparagine, Asp-aspartic acid, β-Ala-β-alanine, Gaba-γ-aminobutyric acid, Gln-glutamine, Glu-glutamic acid, Gly-glycine, His-histidine, H-ser-homoserine, Ile-isoleucine, Leu-leucine, Lys-lysine, Met-methionine, Orn-ornithine, Phe-phenylalanine, Ser-serine, Trp-tryptophane, Tyr-tyrosine, Val-valine. Plant abbreviations are as in Table 1
[5] Because in most chromatograms they were inseparable, the groups: His+Gln and "unknown" were calculated as His equivalent; Gly+Thr as Thr equivalent; Tyr+Al as Tyr equivalent; and β-Ala as Ala equivalent. Under "unknown" the total amount of three different unknown compounds are grouped
[6] Data from Petanidou (2005)

the p-p food web of the community (Petanidou 1991, Petanidou and Ellis 1996). Each of the remaining particular AAs contributed with less than 20% to the total AA composition in all the plant families studied.

Phylogenetic and ecological constraints

Using Wilk's lambda test criterion in a multivariate ANOVA, in which the AA&S percentages in the nectar of each plant species were taken as dependent variables, and plant taxonomic group, life form and season as factors (fixed, discrete), we found that only plant taxonomic group has a significant, although quite weak, effect, whereas life form and season no discernable influence (Table 3). Scheffe's post hoc tests revealed that weak difference were discernable in asparagine between lamiids and fabids and glutamic acid between malvids and campanulids. Remarkably, phenylalanine, the most abundant AA, and all the "three abundant" sugars did not vary notably among any of the categories.

Pollinators' preferences

Figure 1 and 2 give the results of PCAs for flower-visiting guilds and bee families respectively. Note that because of the generally high phenylalanine percentages most of the other AAs are pushed to one side of the plots. The majority of the bee families and some guilds, most clearly the long tongued bees, and among these the Megachilidae and Anthophoridae, are centred in the phenylalanine-rich part of the plot. Gamma-aminobutyric acid (GABA) seems to have a similar, however a weaker and lees focused effect spread among a wide range of insect guilds. In general it is surprising that the behaviour of the pollinator guilds is so uniform, with wasps, short tongued bees, other Diptera and Coleoptera falling in one overlapping cluster. It is also striking that the long tongued bees, the most specialised pollinators of all, stand the most isolated in their preference for a an extremely high-phenylalanine nectar in which sucrose is the dominant sugar and nectar volume has some effect. Other families and guilds, most clearly the Syrphidae, prefer a "broth" high in phenylalanine, but also containing many other AAs, like tryptophane, arginine, glutamic acid and histidine-glutamine in hexose-rich nectars. AAs that seem to be avoided by some insect groups are asparagine (by all guilds and bee families) and glycine-threonine, H-serine, serine, β-alanine, valine, leucine (by most bee families).

To analyse this in somewhat more detail we performed stepwise multiple regressions of the number of visitors per family and guild upon the nectar values (i.e. AA&S percentages and nectar volume; Table 4). From the table, like from the PCAs, it is apparent that asparagine and H-serine have a generally negative partial coefficient, while few others (methionine, valine, leucine) have negative effects on individual insect groups. Tryptophane has both a negative (viz. for long tongued bees and Anthophoridae), and a positive effect (viz. for Coleoptera and Syrphidae). Phenylalanine and GABA were the only AAs having a generally positive effect on insect groups, especially for long tongued bees with partial effect on Megachilidae (phenylalanine) and Anthophoridae (GABA).

As the PCA plots show, phenylalanine is loosely associated with sucrose-rich nectars, while most AAs of the "broth" predominantly occur in low volume, hexose-rich nectars. Voluminous nectars apparently are favoured by Megachilidae, while most other guilds and families seem to prefer low-volume, i.e. concentrated, nectars. We also calculated multiple regressions of the number of visitors per guild and family upon the three main parameters of nectar: volume, total AA content and total sugar content (Table 5). The table shows that among all insect guilds only long tongued bees, and in particular Anthophoridae, showed a significant dependence upon the total AA content of nectars. Total sugar had no effect at all, whereas nectar volume had only negative impacts, especially on flies and wasps. Lepidoptera were not significantly associated with any of the three parameters.

Discussion

Phenylalanine: an important amino acid in the phryganic nectars

Although in low concentration in floral nectars, particularly when compared to sugars, AAs are commonly present in the floral nectars of phrygana, where they were found to be represented by 22 AAs or AA groups (Petanidou et al. 1996, Petanidou 2005). Among them phenylalanine dominated in many respects. The dominance was particularly obvious within most members of the Lamiaceae family, where it contributed by over 50% to the total AA amount. Such extreme quantities of phenylalanine were also found in the nectars of *Satureja thymbra* and *Salvia fruticosa* in Israel (phenylalanine accounting for 71% and 52% of the total AAs, respectively). However, phenylalanine content in the nectars of another two species of the same study, viz. *Thymus capitatus* and *Rosmarinus officinalis*, was at community average levels (Dafni et al. 1988).

Moreover phenylalanine, together with GABA, was the only AA in the phryganic nectars that is clearly attractive to certain insect guilds (i.e. long tongued bees; Fig. 1) and families (i.e. Megachilidae, Anthophoridae; Fig. 2). Other families and guilds, most clearly the Syrphidae, prefer a hexose-rich "broth" which is high in phenylalanine and rich in other AAs, in particularly

Table 3. Results of a MANOVA with nectar AA and sugar percentages, as well as volume as dependent variables and plant taxonomic group, life form and flowering season as independent variables, using Wilk's lambda test criterion. All data were transformed ($x' = \ln(x+1)$). In post hoc results the groups with the highest values are given first.

	Lambda	P	F	df, error df	Dependent (nectar) and independent variable groups responsible for the difference[1]	Pairs of differring independent variable groups (Scheffe's post hoc results)
Constant	0.004	0.000	374	24, 32		
Plant taxonomy group	0.034	0.042	1.34	120, 162	Asn-fabids ($P < 0.0176$) Glu-malvids ($P = 0.0013$)	fabids-lamiids ($P < 0.0206$) malvids-campanulids ($P < 0.0358$)
Life form	0.152	0.227	1.18	72, 96		
Season	0.125	0.086	1.35	72, 96		

[1] We give only those pairs that finally give significant Scheffé's post hoc results

tryptophane. Interestingly, short tongued bees lie within these guilds, being comparable in behaviour with wasps rather than longed tongued bees (cf. Table 4). Apidae differentiate from the other long tongued bees, i.e. Megachilidae and Anthophoridae, probably because they visit systematically and almost indiscriminately the majority of the available flora within the community (Petanidou 1991).

Whereas we have no reference from other studies on the effect of GABA and asparagine, the above results are in agreement with previous studies showing that phenylalanine is an AA of particular importance in the context of pollination ecology. Although highly variable in floral nectars, phenylalanine is one of the most common AAs in floral honeys (Bose and Battaglini 1978), one of the ten essential AAs for honeybees (Groot 1953, Chapman 1983, Dafni and Kevan 1994) and a precursor of the specific aroma component phenyl-ethanol (Thawley 1969). Most important, phenylalanine has been found to be the only AA with a strong phagostimulatory effect on honeybees at the highest concentrations tested (Inouye and Waller 1984). The latter finding is very relevant to our results, implying that bees may detect and respond to differences in concentrations of some AAs in solution (Inouye and Waller 1984), as do hummingbirds (Hainsworth and Wolf 1976).

In contrast with the phrygana, phenylalanine had a significantly lower concentration (M-W U test, $P < 0.0001$) in 30 British plant species analysed by Gardener and Gillman (2001). We attribute the dominance of phenylalanine in the Mediterranean to the high number of bees, especially longed tongued ones, and we argue that in the Mediterranean such bees might have acted as crucial selective factors for phenylalanine-rich nectars (Michener 1979, 2000, O'Toole and Raw 1991, Petanidou and Ellis 1993, 1996, Dafni and O'Toole 1994,

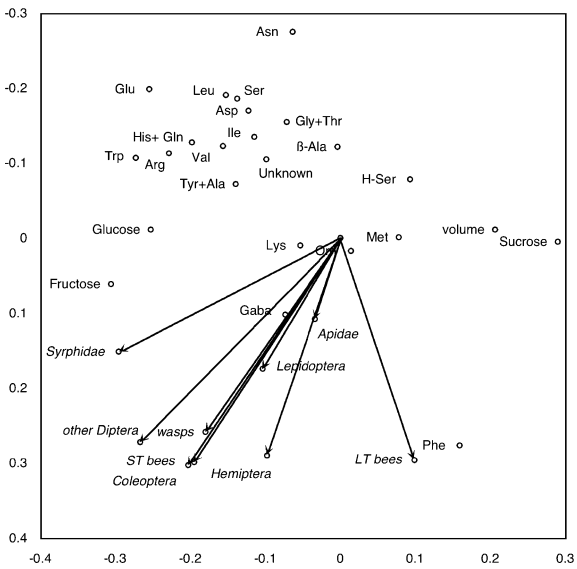

Fig. 1. Principal components analysis (PCA) run on the basis of the nectar attributes of the 67 plant species (AAs and sugar percentages; nectar volume) and all insect species per guild visiting each plant species. First and second component axes account for 15.4% and 13.2% of the total variance. Origin is at (0,0).

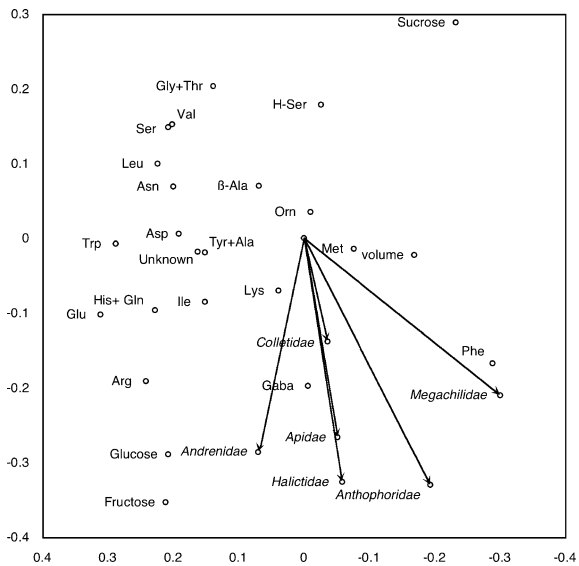

Fig. 2. Principal components analysis (PCA) run on the basis of the nectar attributes of the 67 plant species (AAs and sugar percentages; nectar volume) and all visiting bee species shown per family. First and second component axes account for 16.5% and 10.2% of the total variance. Origin is at (0,0).

Table 4. Relationship of different flower visiting insect groups (guilds and bee families) to different nectar parameters of the floral nectars of the phryganic plants (AAs, major sugars, nectar volume) visited by each group. We give multiple regression results (adjusted overall R^2 and partial coefficients) of flower visitors (numbers of visitor species of a certain insect group per plant species) over the proportions of different AAs (% of each AA over the total amount of AAs) and the proportions of different sugars (% over the total amount of sugars) contained in the nectars of the same plant species. The analysis was made on 67 plant species. All data were transformed ($x' = \ln(x+1)$). We give only significant values: *: $P < 0.05$, **: $0.05 < P < 0.01$, ***: $0.01 < P < 0.001$.

Nectar traits	Coleoptera		Hemiptera		Long-tongued bees (excl. Apidae)		Other Diptera		Short-tongued bees		Syrphidae		wasps		Andrenidae		Anthophoridae		Colletidae		Megachilidae	
	Overall coefficients																					
	$R^2 = 34.9$, 63 df		$R^2 = 4.4$, 65 df		$R^2 = 38.1$, 61 df		$R^2 = 41.2$, 62 df		$R^2 = 11.4$, 64 df		$R^2 = 32.6$, 61 df		$R^2 = 33.6$, 63 df		$R^2 = 8.8$, 64 df		$R^2 = 33.4$, 62 df		$R^2 = 8.8$, 65 df		$R^2 = 32.3$, 63 df	
	Partial coefficients																					
	b	P	b	P	b	P	b	P	b	P	b	P	b	P	b	P	b	P	b	P	b	P
Volume							−0.484	***					−0.282	*								
Glucose													−0.490	**								
Fructose	0.391	**					0.421	***	0.310	*	0.443	***	0.613	***								
Sucrose					0.168	*																
Asn	−0.516	***	−0.124	*			−0.342	**	−0.286	*			−0.330	**					−0.158	**	−0.271	*
Gaba					0.371	**	0.302	**			0.269	*			0.251	*	0.331	**				
Trp	0.363	*			−0.468	**					0.320	*					−0.558	***				
Met															−0.270	*						
Val																	−0.289	*				
Phe					0.189	*															0.183	*
Leu																					−0.551	*
H-Ser					−0.962	**											−0.786						

Table 5. Relationship of pollinator guilds and families to different nectar parameters of the floral nectars of the phryganic plants visited by each pollinator group. We give multiple regression results (adjusted overal R^2 and partial coefficients) of flower visitors (numbers of visitor species of a certain insect guild visiting a plant species) over the total nectar volume, total AA content and total sugar content (glucose + fructose + sucrose) contained in the nectars of the plant species visited. All data were transformed ($x' = \ln(x+1)$). No significance was found for: total sugars and the insect guilds and families missing from the table. *: $P < 0.05$, **: $0.05 < P < 0.01$, ***: $0.01 < P < 0.001$.

Nectar traits	LT bees		Anthophoridae		Megachilidae		Apidae		Other Diptera		Syrphidae		Wasps	
	$R^2 = 13.4$, 65 df		$R^2 = 21.6$, 65 df		$R^2 = 4.3$, 65 df		$R^2 = 8.2$, 65 df		$R^2 = 12.0$, 65 df		$R^2 = 6.8$, 65 df		$R^2 = 4.3$, 65 df	
	b	P	b	P	b	P	b	P	b	P	b	P	b	P
Volume									−0.522	**	−0.393	*	−0.330	0.052
Total AAs	0.326	**	0.368	***	0.174	0.052	0.084	*						
Total sugars														

Petanidou and Potts 2006). Similarly, the dominance of phenylalanine in the Lamiaceae may be explained by the highly melittophilous character of this family, hence selected by the high number of bees visiting it (Petanidou and Vokou 1993). Such coevolution between long tongued bees and phenylalanine-rich nectars was less prominent in *Thymus capitatus* and the cultivated *Rosmarinus officinalis* studied in Israel by Dafni et al. (1988). Interestingly, *T. capitatus* had a low phenylalanine score also within the Lamiaceae of the phrygana studied (second lowest, second only to *Lamium amplexicaule*; Table 2). This is not surprising, because thyme flowers in a competition-free period (summer) in both communities, in which it functions as a "pollinator sink" visited by a large array of insects (viz. 123 species in the study phrygana, consisting of bees (24%), wasps (18%), flies (20%) and other insect groups; Petanidou 2004, Petanidou and Potts 2006). With a very extended flowering period in wintertime and visited mainly by honeybees (T. Petanidou, pers. obs.) *R. officinalis* seems also to face no competition for pollination – a major hint for coevolution at the community level. The case of *L. amplexicaule* might be partly explained by the cleistogamic character of its flowers (Lord 1982, Petanidou 1991).

On basis of the above we argue that phenylalanine constitutes a very important compound of the phryganic nectars and pollinator diet. Its importance appears to be highest for bees, especially the long tongued ones, and among them Megachilidae. What is the specific role phenylalanine plays in the floral nectars of phrygana? One possibility is that, by being one of the essential AAs for bees (Groot 1953), phenylalanine constitutes an AA source for bees, in addition to the pollen collected by the females. However, because no other essential AA in the floral nectars of phrygana has a comparable effect on bees as phenylalanine (GABA is a non-essential AA), we believe that a better explanation is offered by the strong phagostimulatory effect of phenylalanine that was found or discussed in earlier studies (Inouye and Waller 1984, Gardener and Gillman 2002). By acting as a phagostimulant phenylalanine constitutes a major coevolutionary mechanism in shaping the nectars within the Mediterranean area, with long tongued bees, and especially Megachilids acting as the main selective agents. In this respect, the evident significance of GABA, a neurotransmitter that is "absolutely dependent on sodium and chloride" (Keynan and Kanner 1988, Wolfersberger 2000), in the nectars of phrygana seems to be highly relevant. On the basis of the above, we argue that it may not be GABA alone, as evidenced by Inouye and Waller (1984), but its combination with NaCl – or even NaCl only that constitutes an important nectar trait that bees and other anthophilous insects are allured by. The combined phagostimulatory effect of GABA and NaCl, associated

with flowers yielding relatively low nectar (Fig. 1, 2) is certainly an interesting issue that merits special attention in future studies.

What does shape floral nectar composition?

We have evidence to support the hypothesis that nectar composition (AA&S + nectar volume) in phrygana appears to have been weakly shaped by phylogenetic relationships, not by ecological constraints. However, the most decisive players in shaping chemical composition in the floral nectars of these habitats appear to have been the flower visiting insects, primarily the long tongued bees and especially Megachilids in the supreme role of selective agents. This emphasises the importance of coevolution in the process of evolution at the community level in the Mediterranean area, which appears more overwhelming than the overriding influence of Mediterranean climate. In fact, climate has been found to be extremely important in shaping many other pollination and nectar characteristics detected independently (e.g. flowering, nectary size, nectar secretion; Petanidou et al. 1995, 2000).

Recent findings on the biology of other insects uphold the conclusion that coevolution between flower visiting insects and AA&S composition of nectar is widespread, although this concerns mainly sugars, rather than AAs (reviewed by Petanidou 2005). As to AAs, and according to the "consensus view", such a coevolution should especially apply to pollinators that have no alternative nitrogen resources, such as butterflies and moths (Baker and Baker 1986, Gardener and Gillman 2002). Indeed, experimental work on butterflies has shown that *Pieris rapae* females prefer nectar containing AAs over sugar-only nectars (Alm et al. 1990), *Pieris brassicae* females select for nectar rich in sucrose and AAs (Romeis and Wackers 2000), while females of the species *Inachis io*, *Pieris napi* and *Araschnia levana* detect and prefer nectar with high AA content, especially when they have been deprived of AAs during their larval stage (Erhardt and Rusterholz 1998, Mevi-Schutz and Erhardt 2003, 2004). The concluding view that floral nectar AAs favour butterfly fecundity and fitness (Mevi-Schutz and Erhardt 2005) has been criticized repeatedly by other authors suggesting that most of the egg AAs needed by female butterflies originate from nectar sugars, whereas essential AAs entirely from the larval diet (O'Brien et al. 2002, Jervis and Boggs 2005). Evidently our results do not support the above "consensus view", as no significant relation of Lepidoptera with any of the nectar variables tested was revealed (Fig. 1, Table 4, 5). An explanation can be that as a pollinator guild, Lepidoptera are relatively unimportant in the Mediterranean, therefore non influential in shaping nectar traits, at least much less important than bees (Herrera 1987).

A major result of this study is also that solitary bees, especially long tongued bees, respond positively to the total AAs of nectar in phrygana (Table 5). As far as we know, since the historical paper by Baker and Baker (1986), solitary bees alone have never been considered as selective agents for high AA content of nectar. According to Gardener and Gillman (2002) honeybees and flies show a preferential response to AAs contained in nectar-mimicking solutions, whereas nectar-feeding ants also show some preference for AAs (Bluthgen and Fiedler 2004). Tropical bees are exempted from this rule. For instance, the tropical bee *Trigona hockingsi* shows no preference for AAs in artificial nectars (Gardener et al. 2003). Similarly, AA solutions did not affect attractiveness to tropical bees of the subfamilies Euglossini, Meliponini, and Centridini as much as sugar solutions (Roubik et al. 1995). These results can be explained by the differential nectar choice of tropical bees in general, as tropical systems are dominated by species secreting relatively dilute nectar, therefore sugars may play a greater role in nectar choice rather than AAs (Cruden et al. 1983, Petanidou and Smets 1995). Yet, by having high quantities of nectar at their disposal, by the end of the day tropical bees get the AA quantity required to cover their needs.

Finally, why AAs appear more important than sugars in shaping the p-p interaction web within a community characterised by an outstanding melittophily? As inferred by Gardener and Gillman (2002) sugars "by far dominate the taste of nectars" because they represent the most abundant compound therein. But by being so dominant, sugars are expected not to constitute the main discriminatory compound for the response of the nectar consumers in phrygana, which is the case in other habitats (Gardener and Gillman 2002). This can be explained as in dry and hot Mediterranean habitats nectars have very high sugar concentrations (Petanidou and Smets 1995), therefore with a sweet taste that is generally too "strong". In such habitats, dominated by thick nectars, it is probable that AAs constitute the discriminant taste and take over as major taste stimulators and contributors to the overall nectar palatability. In this respect, the high contribution of phenylalanine, a well known phagostimulant (Inouye and Waller 1984) and the significant presence of GABA, a NaCl-dependent AA (Wolfersberger 2000), in the nectars of phrygana, appear to be highly meaningful.

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