Plant Ecol (2013) 214:799–809
DOI 10.1007/s11258-013-0204-z

Intrapopulation heterogeneity in floral nectar attributes and foraging insects of an ecotonal Mediterranean species

Daniele Nocentini · Ettore Pacini · Massimo Guarnieri · Diego Martelli · Massimo Nepi

Received: 14 January 2013 / Accepted: 17 April 2013 / Published online: 16 May 2013

Abstract A population of *Buglossoides purpurocaerulea* (L.) I.M. Johnst. (Boraginaceae–Lithospermeae) located in Lecceto (Siena Province, Tuscany, central Italy) has been studied to compare floral nectar attributes and forager species between sun-exposed and shaded plants. Flower anthesis and maturity of sexual organs were also investigated. Average flower anthesis lasted 3–4 days. Stigma receptivity and anther dehiscence occurred on the first day. Nectar production also began on the first day and maximum production occurred on second-third day. Significantly greater volumes and total sugars were recorded in individuals exposed to the sun. Nectar HPLC analysis showed a similar hexose-dominant sugar profile for all the individuals with percentages of sucrose, glucose and fructose around 5, 48 and 47 %, respectively. Protein amino acids represent the 90 % of the overall free amino acids profile. Significant differences between relative percentages of serine and proline were found between sun-exposed and shaded individuals. *Empis pennipes* and *Bombilyus major* were the most frequent insect visitors to shaded and sun-exposed individuals, respectively. The hexose dominance of the nectar of *B. purpurocaerulea*, an exception among the Mediterranean Lithospermeae, may be related to the habitat where this plant generally grows, i.e. the forest-edge, and to pollination mainly performed by dipterans.

Keywords *Buglossoides purpurocaerulea* · Boraginaceae · Pollination ecology · Nectar attributes · Foraging insects

D. Nocentini (✉) · E. Pacini · M. Nepi
Biodiversity and Conservation Network (BIOCONNET), Department of Life Sciences, University of Siena, Via P.A. Mattioli 4, 53100 Siena, Italy
e-mail: nocentini19@unisi.it

M. Guarnieri · D. Martelli
Department of Life Sciences, University of Siena, Via P.A. Mattioli 4, 53100 Siena, Italy

Introduction

Nectar is a common secretion of angiosperms (Bernardello 2007), produced by special organs called nectaries. It is a complex and dynamic fluid with important ecological functions, being involved in interactions with a wide range of animals (Pacini et al. 2003; Heil 2011; Nepi et al. 2011).

Nectar attributes, namely volume, concentration and composition, vary widely, qualitatively and quantitatively (Faegri and van der Pijl 1979; Cruden et al. 1983), during production, presentation and consumption depending on heterogeneous factors. Many environmental parameters, such as light intensity, temperature, relative humidity, water availability and CO_2 level can affect nectar properties (Beutler 1953; Huber 1956; Corbet 1990; Jakobsen and Kristjánsson 1994; Petanidou and Smets 1996; Pérez-Bañón 2000). Variations in microclimate between and

within individual plants may affect flower nectar production (Nicolson and Nepi 2005) as well as pollinator assemblages (Herrera 1995). The influence of these parameters is not equivalent in different species as morphological features of flowers can modulate their effect (Pacini and Nepi 2007). More than a single biotic factor may influence nectar characters. Some biotic factors can be intrinsic to plant itself: differences in flower age/gender may cause variations in nectar secretion rate (nectar volume) and sugar concentration between flowers of a given plant (Pacini and Nepi 2007). The dynamics of nectar production are related to pollinator requirements (Nicolson 2007a; Pacini and Nepi 2007) and certain sugar profiles are correlated with particular classes of pollinators (Baker and Baker 1982, 1983a; Nicolson and Thornburg 2007). Amino acid concentrations seem to be greater if nectar is the only or the predominant source of protein-producing substances as for example is the case for butterflies (Baker and Baker 1983b).

These biotic and abiotic parameters give rise to broad inter- and intra-species variability of nectar characteristics between flowers of the same plant, between plants of a population and between populations. Although there is wide variability, it is important for the plants to balance the nectar chemistry to attract generalist and/or specialist pollinators as both sugars and amino acids can influence the choice of insects. Sucrose and the monosaccharides fructose and glucose are the most powerful feeding stimulants for some herbivorous insects (Shoonhoven et al. 2006). Amino acids contribute to the taste of nectar stimulating specific labellar chemoreceptors on the insect (Gardener and Gillman 2002).

Here, we study floral nectar attributes and forager guild in the ecotonal species *Buglossoides purpurocaerulea* (L.) I.M. Johnst. (Boraginaceae, Lithospermeae). This Mediterranean species is a perennial herb widely distributed in southern Europe and Italy. It is found predominantly in thermophilous deciduous forests, scrub and oak coppices and it is considered ecotonal, i.e. it grows in the transition area between the woods and adjacent open fields. This particular habitat allows examination of differences in nectar characters and pollinator assembly between individuals subjected to different microclimates. A second reason for choosing this species is due to preliminary analysis of its floral nectar sugars that revealed a hexose dominance, which is an exception among the insect-pollinated Lithospermeae (Nocentini et al. 2012; Dupont et al. 2004). Individuals of the studied population are distributed in an ecotone along a gradient of sun exposure: from the shade of the forest to open sun irradiance outside the forest. Thus the aim of this study is to test the differences between sun-exposed and shaded plants in: (1) flower phenology, (2) nectar production dynamics and composition (3) visitor guilds.

Materials and methods

Population sites

Data were collected in May to June 2011 from a wild population of *B. purpurocaerulea* in Lecceto (Siena Province, Tuscany, central Italy; 43°18′ 27″ N, 11°16′ 10″ E, 329 m a.s.l.). This population is located at the verge of a forest and we examined shaded individuals (hereafter SIs) growing just on the edge of the wood and rarely exposed to sun as well as sun-exposed individuals (hereafter EIs) growing in full sun. Maximum sunlight intensity in a series of ten contemporaneous measurements at the two sites, recorded with a LI-191SA line quantum sensor (LI-COR, inc. Lincoln, NE, USA), was 315.3 in SIs and 1660.7 μmol s^{-1} m^{-2} in EIs.

Flower phenology and maturation of sexual organs during anthesis

Flower phenology was investigated by monitoring ten inflorescences in both sites. The mean period of anthesis (in days) from opening of the bud to senescence and shedding of the corolla was determined. Stigma receptivity and dehiscence of anthers were determined for at least five flowers in each of the following stages: early (day 1 of anthesis), mature (day 2 of anthesis) and senescent (day 3–4 of anthesis). Gynoecium receptivity was monitored by a colorimetric test responsive to peroxidases on the stigma surface (Dafni and Motte-Maues 1998). Anther maturity was evaluated on the basis of dehiscence and the presence of pollen.

Nectar production

The presence of nectar was verified in ten flower buds in both sites. Nectar production was studied by

enclosing flowers with tulle nets (mesh 1.0 mm Ø) to prevent access by foragers. Based on the previous study of anthesis, three sets of buds were selected for nectar collection in the day 1–3 flower stages. Totals of 44 and 39 flowers were sampled for SIs and EIs, respectively. Nectar measurements were performed on each flower only once at 18h00 in order to allow the daily volume of nectar to accumulate. Nectar presence was also verified in a few flowers that persisted on the fourth day.

Nectar was withdrawn using 5.0 µl graduated glass microcapillary tubes (accuracy ± 0.5 mm; Blaubrand, intraMark) and its volume was calculated from the length of the nectar column.

Total sugar (µg) per flower was determined by the product of volume (µl) times total sugar concentration (sucrose + glucose + fructose; µg μl^{-1}) according to HPLC determinations (see below).

Determination of nectar sugars and amino acids

Thirty-two nectar samples (16 for each site) were stored at −20 °C in vials containing 1.5 ml 70 % ethanol to prevent growth of microorganisms (Herrera et al. 2008, 2009) until analysis by high performance liquid chromatography (HPLC). Prior to analysis the samples were thawed, air-dried in a Speedvac centrifuge (Jouan RC 1010) to eliminate the alcohol, and then diluted with distilled water (1:25).

Sugar analysis was performed by isocratic HPLC with a Waters Sugar-Pak I ion-exchange column (6.5 × 300 mm) at 85.0 °C and a Waters 2410 refractive index detector. Water (MilliQ, pH 7.0) was used as mobile phase at a flow rate of 0.6 ml min^{-1}; 20 µl of sample and standard solutions of sucrose, glucose and fructose were also injected.

Amino acid analysis was performed by gradient HPLC with an ion-exchange Novapak C18 (4.6 × 150 mm) column at 37.0 °C and a Waters 470 scanning fluorescence detector (excitation at 295 nm, detection at 350 nm). A solvent composed of TEA-phosphate buffer (pH 5.0) mixed with a 6:4 acetonitrile–water solution was used as mobile phase at a flow rate of 1.0 ml min^{-1}. According to the AccQtag protocol (Waters Corp.), the selected volume of each reconstituted sample was amino acid derivatized (Cohen and Micheaud 1993) with AQC fluorescent reagent and 0.02 M borate buffer (pH 8.6). Besides all the protein amino acids, standards of β-alanine, citrulline, L-homoserine, α-aminobutyric acid (AABA), γ-aminobutyric acid (GABA), hydroxyproline, ornithine and taurine were also used. Only 16 samples out of the 32 used for sugar determination were large enough for amino acid analysis. An equal number of samples per stage have been considered in the two sites: two samples from early flowers, three samples from mature flowers and three samples from senescent flowers (a total of eight samples per site). Thus, specific amino acid profiles for the different flower stages were not obtained, but rather, an overall estimation for the two sites. The data were presented grouping the protein amino acids in the four classes described by Gardener and Gillman (2002) and Nicolson and Thornburg (2007) based on the effect of the single amino acids on the labellar chemoreceptors of flies, although we are aware that the sensitivity is not the same for all insects. A fifth class called 'Other amino acids' were used for non-protein amino acids.

Flower visitors

On three consecutive days with suitable weather conditions (sunny with little wind), patches of plants were monitored by two operators simultaneously in order to identify insect visitors (four sessions at 9h00, 12h00, 15h00 and 18h00 each day for a total of 12 h of observation). Insects were classified as occasional or main visitors. Specimens of main visitors were captured for identification on the basis of morphological characters, as indicated by Prŷs-Jones and Corbet (2011), Intoppa et al. (1997), Pagliano (1994) and Tolman and Lewington (2008). Voucher specimens were deposited at the Department of Life Sciences, University of Siena. Successive field observations were performed to characterize the foraging behaviour of the main visitors during 5 days (four sessions at 9h00, 12h00, 15h00 and 18h00 each day for a total of 20 h of observation).

Statistical analysis

Normality of distribution of nectar parameters (volumes, total sugar concentrations, total sugar per flower) and HPLC parameters (sucrose, glucose, fructose concentrations and percentages, percentages of amino acid classes) was assessed by the *W* test of Shapiro–Wilk. As volume was not normally distributed, it was

analysed by the Mann–Whitney *U* test and Kruskal–Wallis ANOVA by ranks to evaluate the significance of differences in two or more sets of data respectively. In the latter case, differences between individual pairs of data were assessed by Dunn's test. All the other parameters were normally distributed and were analysed by the *t* test and one way ANOVA followed by Tukey's test for post hoc comparison of means to assess the significance of differences in two or more sets of data, respectively. Statistical tests were performed using the program Statistica (version 7.1) with α error set at 0.05. Data are reported as mean $\pm$ SD.

Results

Flower morphology and phenology

Hermaphrodite flowers of *B. purpurocaerulea* developed in an acropetal manner into scorpioid and terminal cymes. The mean period of flower anthesis was 3–4 days. Buds were generally pinkish purple changing colour as the flower matured. Flowers on day 1 had a fully open purplish blue corolla tube, the anthers began to dehisce and the stigma became receptive. On day 2, the flower was fully mature: the sexual organs were functional, the stigma receptive, the pollen presented on the anthers and corolla colour faded to pale blue. The mature corolla tube was funnel or crater shaped showing pentamerous actinomorphic symmetry and each flaring lobe had a plica at the base that contacted each other by the basal part of the rib reducing the diameter of the funnel opening. At the end of day 3, the anthers were generally devoid of pollen and the brown colour of the stigma indicated decreasing receptivity. In most flowers of SIs group, the corolla began to wither and was shed at the end of the day 3, few persisted on day 4. In EIs group, many flowers persisted until the end of day 4.

Nectar production

Buds about to open were devoid of nectar in both sites. Flowers in SIs showed variations in volume with flower age ($H_{2.44} = 19.53$; $p = 0.000$ according to Kruskal–Wallis ANOVA). Volume increased on day 1 reaching a maximum on day 2, then decreased in senescent flowers (day 3; Fig. 1a). Significant differences were recorded between nectar volumes in

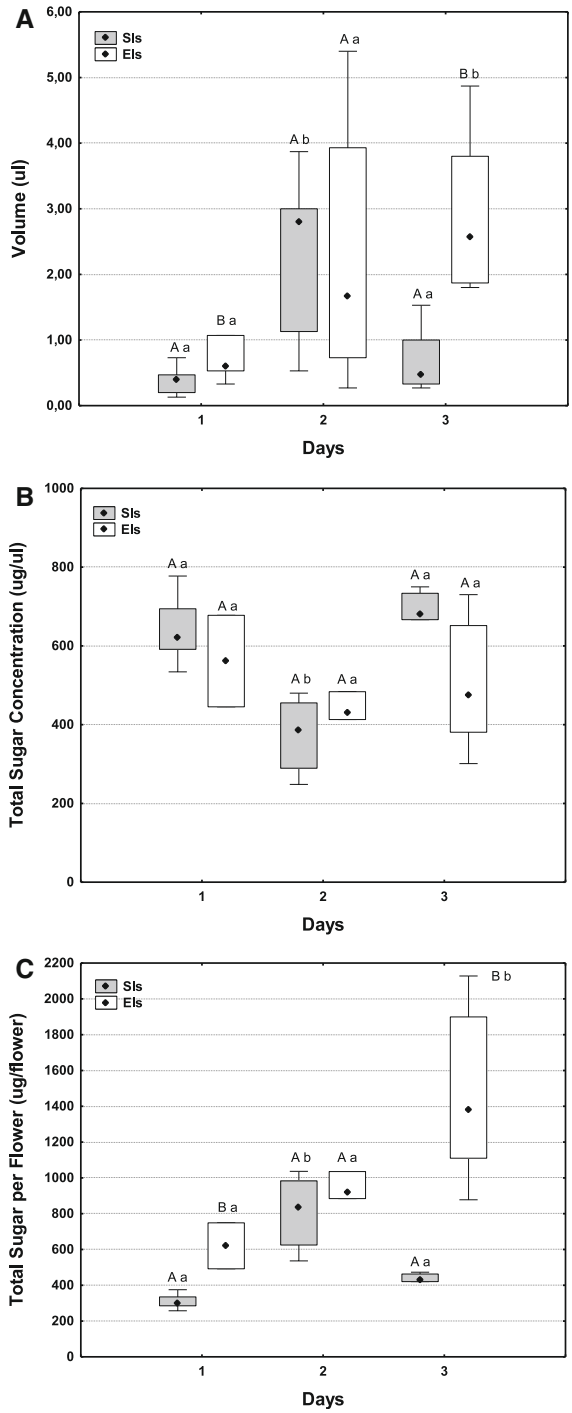

Fig. 1 Nectar volume (**a**), total sugar concentration (**b**) and total sugar per flower (**c**) in *B. purpurocaerulea* during flower anthesis (days 1–3) in shaded plants (SIs; *grey boxes*) and sun-exposed plants (EIs; *white boxes*). *Different letters* indicate statistically significant differences. *Capital letters* show differences between SIs and EIs within days, whereas *small letters* show differences between days within SIs or EIs

flowers on day 2 and days 1 and 3 (Fig. 1a). Flowers of EIs also showed variation in volume with flower age ($H_{2.39} = 9.55$; $p = 0.000$), but maximum production of nectar was on day 3 (Fig. 1a). Differences in nectar volume were only significant between days 1 and 3 (Fig. 1a). Flowers of EIs produced a greater overall volume of nectar than flowers of SIs ($Z = -4.20$; $p = 0.000$ Mann–Whitney U test). This difference was particularly evident on days 1 and 3 ($Z = -1.99$; $p = 0.046$ and $Z = -4.27$; $p = 0.000$, respectively).

In flowers of SIs, nectar concentration was affected by flower age ($F_{2.16} = 18.87$; $p = 0.000$, Fig. 1b). Significant differences were recorded between nectar concentrations in flowers on day 2 and days 1 and 3 (Fig. 1b). In flowers of EIs, concentration did not vary with flower age ($F_{2.16} = 0.68$; $p = 0.524$; Fig. 1b). After day 3, the flowers were devoid of nectar. There is no significant difference in overall nectar concentration between SIs and EIs (Fig. 1b).

Total sugars were affected by flower age in SIs ($F_{2.16} = 23.06$; $p = 0.000$) showing significant differences between day 2 and days 1 and 3 (Fig. 1c). Flower age also affected total sugars in EIs ($F_{2.16} = 6.54$; $p = 0.011$) with significant differences between day 3 and days 1 and 2 (Fig. 1c). Nectar sugars were higher on the whole in flowers of EIs than SIs ($t = -4.372$; $p = 0.000$) with significant differences between days 1 and 3 ($t = -5.11$; $p = 0.002$ and $t = -4.96$, $p = 0.005$, respectively).

Nectar composition

Total sugar concentrations of SIs and EIs were 563.92 ± 164.91 and 502.64 ± 60.50 mg ml^{-1}, respectively (mean ± SD). Individuals from both sites had very similar hexose-dominant sugar profiles (Table 1; overall ratio $S/G + F = 0.05$ and overall ratio $F/G = 0.97$). Relative percentages were also almost constant in the three stages in flowers of SIs and EIs. No significant differences were found between the two sites or between flower stages within sites.

Amino acid concentrations in flowers of SIs and EIs were 10.42 ± 1.50 and 9.60 ± 3.64 nmol μl^{-1}, respectively. The two sites showed similar rich amino acid profiles (Table 1) with protein amino acids constituting 89.94 % ± 0.98 (mean of the two sites) of the overall free amino acids profile and non-protein amino acids 10.07 % ± 0.98. In both sites, proline was the predominant protein amino acid, followed by alanine and serine. Thirteen other protein amino acids were detected in both sites (Table 1). β-Alanine was the most abundant non-protein amino acid, followed by GABA. AABA and ornithine were also detected.

When amino acids were grouped in classes (I–IV and 'Other amino acids'; Table 1), those of class III were the most abundant (SIs = 56.18 %; EIs = 64.70 %) followed by those of class I (SIs = 24.79 %; EIs = 18.20 %), 'Other amino acids' (SIs = 9.36 %; EIs = 10.76 %), class II (SIs = 7.00 %; EIs = 4.74 %) and class IV (SIs = 2.64 %; EIs = 1.63 %; Table 1). There were significant differences in relative percentages of class I and class III amino acids between the two sites (Table 1). Class I amino acids (serine, glycine, threonine, alanine, tyrosine) had higher relative percentages in flowers of SIs than EIs ($Z = 2.31$, $p = 0.020$, Mann–Whitney U test). On the contrary, class III (proline) had a higher relative percentage in flowers of EIs than SIs ($Z = -2.31$; $p = 0.020$).

Flower visitors

The following insects visited flowers of *B. purpurocaerulea*: the Diptera *Empis pennipes* L. 1758 (Fig. 2a), *Bombilyus major* L. 1758 (Fig. 2b) and *Bombylella atra* Scopoli 1763; the Hymenoptera *Bombus muscorum* L. 1761, *Anthophora* sp. Latreille 1803 and *Eucera* sp. L. 1758 (Fig. 2c); the Lepidoptera *Limenitis reducta* Staudinger 1901, *Anthocharis cardamines* L. 1758 (Fig. 2d), *Gonepteryx rhamni* L. 1758 (Fig. 2e), *Iphiclides podalirius* L. 1758 (Fig. 2f) and *Macroglossum stellatarum* L. 1758.

Empis pennipes made 559/583 visits (95.9 %) to SIs. *B. major* only made 11 visits (1.9 %) and the contribution of Hymenoptera and Lepidoptera was minor. *E. pennipes* made only 16/183 visits (8.7 %) to EIs, whereas *B. major* made 91 (49.7 %), Hymenoptera 64 (35.0 %) and Lepidoptera 12 (6.6 %). Compared to the relatively short visits of *B. major* (2–4 s per visits), *E. pennipes* spent much more time in foraging at single flowers (up to 2 min).

Discussion

Microclimate conditions affect nectar characteristics and pollinator assemblage in several species (Herrera 1995; Petanidou and Smets 1996; Nicolson and Nepi

Table 1 Nectar composition in flowers of *B. purpurocaerulea* in shaded plants (SIs) and sun-exposed plants (EIs; mean ± SD)

		Sugar profile			Amino acids Profile																	Total Concentrations	
		Major sugars			Class I					Class II				Class III		Class IV			Class Oth			[SGF]	[AAs]
		Suc	Glu	Fru	Ala	Gly	Ser	Thr*	Tyr	Arg*	Asp	Glu	His*	Lys*	Pro	Ile*	Leu*	Met*	Phe*	Val*	AABA	Bala	GABA	Orn	mg·ml^{-1}	nmol·µl^{-1}
SIs	%	5.08	48.13	46.79	10.91	2.22	10.07	1.27	0.32	0.93	1.78	2.62	1.21	0.46	56.18	0.53	0.53	0.13	0.47	0.98	0.23	4.91	3.82	0.41	563.92	10.42
	sd	±0.56	±0.48	±0.80	±2.94	±1.96	±5.12	±0.68	±0.21	±0.45	±0.88	±2.28	±0.78	±0.50	±5.69	±0.48	±0.43	±0.14	±0.34	±0.65	±0.14	±2.49	±1.60	±0.27	±16.91	±1.50
	Σ %$_{a/b}$	a	a	a			24.79$_a$					7.00$_a$			56.18$_a$			2.64$_a$					9.37$_a$			a	a
EIs	%	5.20	48.00	46.80	10.45	1.14	5.47	0.91	0.23	0.79	0.63	2.64	0.50	0.18	64.70	0.23	0.21	0.04	0.24	0.91	0.45	6.54	3.54	0.23	502.64	9.60
	sd	±0.26	±0.10	±0.28	±3.33	±0.51	±1.12	±0.27	±0.11	±0.26	±0.31	±2.22	±0.14	±0.04	±7.32	±0.10	±0.06	±0.05	±0.06	±1.42	±0.44	±4.69	±1.95	±0.10	±80.50	±3.64
	Σ %$_{a/b}$	a	a	a			18.20$_b$					4.74$_a$			64.70$_b$			1.63$_a$					10.76$_a$			a	a

Sixteen samples per site were pooled for sugar analysis, whereas eight samples per site were pooled for amino acid analysis. The analyses were conducted on nectar samples from flowers on days 1, 2 and 3 of anthesis. Amino acids are grouped in five classes, four of which are those reported by Nicolson and Thornburg (2007) and the last is a new class "Others" containing non protein amino acids. Σ %$_{a/b}$ = sum of compounds within a class of amino acids, letters a or b indicate significant differences between the two sites according to the Mann–Whitney *U* test; total [SGF] = sum of sucrose, glucose and fructose concentrations (mg ml^{-1}); total [AAs] = sum of amino acid concentrations (nmol µl^{-1}). Essential amino acids are marked by asterisk (Shoonhoven et al. 2006)

2005; Petanidou 2007). Herrera (1995) reported that for *Lavandula latifolia* differences in pollinator composition depended significantly only on the sunlight regime associated with each plant's location in the habitat and not with the species' intrinsic characteristics (flower morphology, nectar standing crop, size of floral display). In *Buglossoides purpurocaerulea*, microclimate conditions (i.e. sun exposure) influence several aspects of nectar dynamics (volume of nectar, duration of secretion and reabsorption) as well as nectar chemistry and relative abundance of the main pollinators.

Nectar productivity

Nectar secretion starts on day 1 of flower anthesis, not in buds as in other species of this family (Pacini and Nepi 2007; Nocentini et al. 2012). Nectar production therefore proceeds in synchrony with maturation of sexual organs. At the end of pollen presentation and stigma receptivity, the reproductive function of flowers and their attractiveness also cease. Thus on day 3, uncollected nectar is resorbed, as indicated by the decrease in nectar volume and total sugars at this stage in SIs (Fig. 1a, c) and by the total absence of nectar in flowers on day 4 in EIs. Nectar production is an expensive adaptation that attracts pollinators (Nepi and Stpiczynska 2008 and references therein), thus recovery of part of the energy invested in nectar production is a useful strategy, especially in hot dry Mediterranean habitats where water is a precious resource. Nectar resorption in senescent flowers has been well documented in various species (Búrquez and Corbet 1991; Koopowitz and Marchant 1998; Luyt and Johnson 2002; Stpiczyńska 2003a, b), including the Lithospermeae tribe (Nocentini et al. 2012), and may reduce damage to reproductive organs caused by visits after pollination has taken place (Búrquez and Corbet 1991).

Nectar production varies with environmental parameters. When we compared the results of the two sites of the studied population we found diversity in nectar production related to the sun exposure. Nectar volumes and total sugar per flower were significantly higher in flowers of EIs than SIs. The influence of environmental parameters on nectar properties has already been described and the effects of temperature, relative humidity and solar irradiance on nectar production have been documented in several

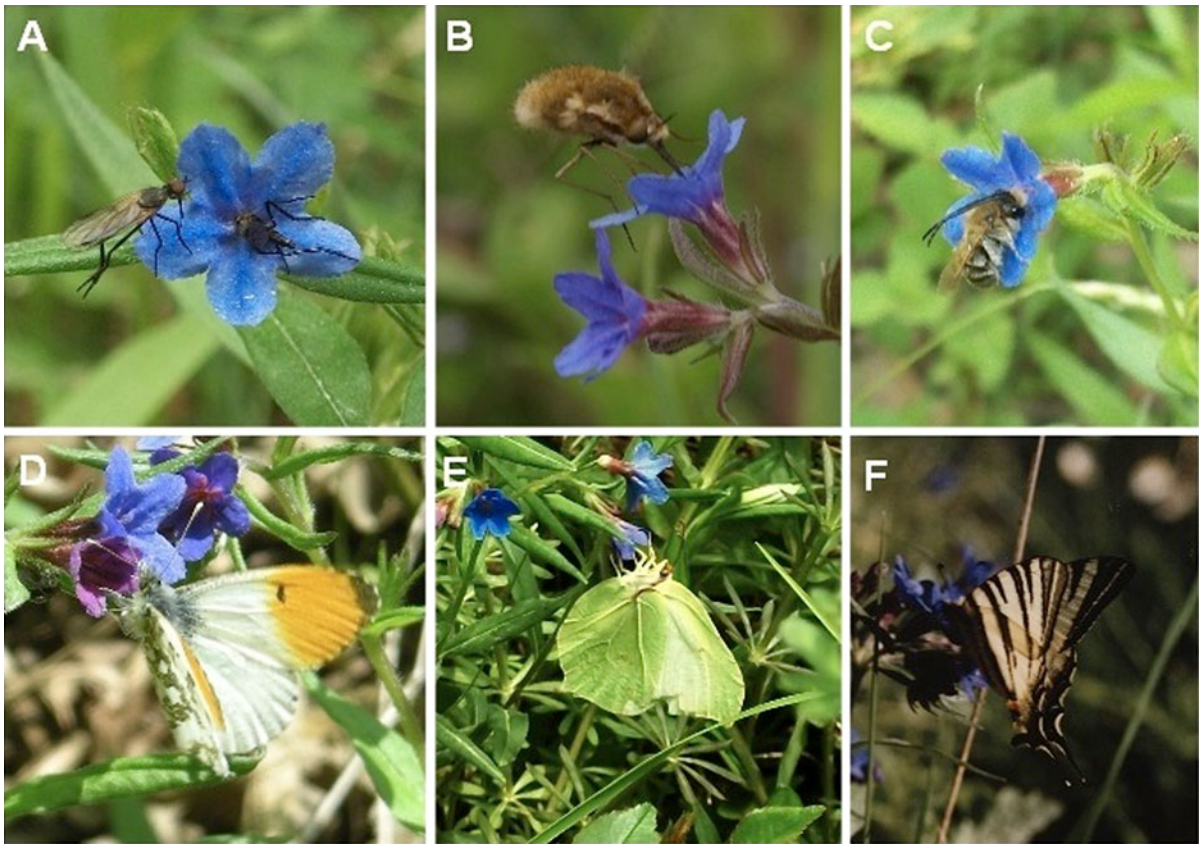

Fig. 2 Insect visitors of *B. purpurocaerulea* recorded during the study. **a** *Empis pennipes* L. 1758 (Diptera-Empididae). **b** *Bombylius major* L. 1758 (Diptera–Bombyliidae). **c** *Eucera* sp. L. 1758 (Hymenoptera–Apidae). **d** *Anthocharis cardamines* L. 1758 (Lepidoptera–Pieridae). **e** *Gonepteryx rhamni* L.1758 (Lepidoptera–Pieridae). **f** *Iphiclides podalirius* L. 1758 (Lepidoptera–Papilionidae)

other species (Hocking 1968; Heinrich and Raven 1972; Pacini and Nepi 2007; Petanidou 2007). Petanidou and Smets (1996) found that low light intensity was the most significant limiting factor for nectar secretion by the Mediterranean species *Thymus capitatus* (Lamiaceae) and that flowers in the sun secreted more nectar of a higher concentration than flowers growing mostly in the shade (Petanidou 2007). Under well-watered conditions increased light intensity influences photosynthesis, increasing carbon fixation. As nectar consists largely of simple sugars—the primary product of photosynthesis, photosynthetic performance seems to be determinant for nectar production (Southwick 1984; Pyke 1991) and a natural or artificial decrease in photosynthesis reduces nectar production (Búrquez and Corbet 1998 and references therein). Differences in nectar production due to different sun exposure are reported for other Mediterranean species, individuals exposed to sun always having higher nectar production (Gyan and Woodel 1987; Barbi 2004), in line with the present results.

In flowers of SIs, volume and total sugar per flower reached a maximum on day 2 and reabsorption occurred on day 3, whereas in EIs they reached maximum values on day 3 and nectar resorption followed on day 4. This difference may be correlated with greater availability of products of photosynthesis, delaying the end of nectar production and the beginning of its resorption. Although we measured a higher mean relative humidity in the shade than in the sun (48.8 and 39.9 %, respectively), this had no effect on overall nectar concentration that was not statistically different in the two sites along the 3 days (Fig. 1b). Actually, the lower concentration and higher volume registered on day 2 in SIs can be accounted for by a

higher relative humidity registered in that day (55.4 % compared to 46.2 and 36.8 % in day 1 and day 3, respectively).

Nectar sugar composition

The results of this study clearly demonstrate that nectar production dynamics are influenced by the environment, while nectar composition is relatively constant and independent of environmental parameters. The constancy of nectar composition revealed in this study could be attributed also to the exclusion of insect visits by bagging flowers. Pollinators are recognized to be the vectors of yeasts that contaminate the nectar affecting its concentration and composition (Herrera et al. 2008, 2009).

According to Petanidou (2005), species with sucrose-dominant nectar are more frequent in the Mediterranean region, where prolonged arid conditions may occur in late spring and summer. As hexoses are derived from the hydrolysis of sucrose, hexose-dominant nectar has higher osmolarity than sucrose-dominant nectar for a given sugar concentration and therefore requires a greater amount of water for its production (Corbet et al.1979; Nicolson 2007a). Thus, hexose-dominant nectar is less suitable for the conditions of low water availability so common in the Mediterranean region. Interestingly, most species of the Boraginaceae, which show maximum biodiversity in the Mediterranean basin, feature a sucrose-dominant nectar profile (Nepi et al. 2010). By contrast *B. purpurocaerulea* (at all flower stages) has a hexose-dominant nectar sugar profile with relative percentages of fructose and glucose both of about 46–48 %. This diversity could be related to the usual habitat of this plant, forest edges, which are less arid than the open sunny spaces where most species of Boraginaceae usually grow.

Congeneric species such as *B. calabra*, which grows in the pinewoods and dry forests of the Sila mountains (southern Italy), also has hexose-dominant nectar, whereas *B. arvensis*, a common species in uncultivated and barren pastures, has a sucrose-rich nectar (Nocentini unpublished data). The type of habitat (shaded woods or forests and sunny arid pastures), that most probably affect also the type of insect communities, seems to be determinant for nectar sugar composition.

Nectar amino acid composition

Amino acids were documented in the first chemical studies of nectar (Ziegler 1956) and are present in considerable variety in both floral and extrafloral nectar (Petanidou et al. 2006; Nicolson and Thornburg 2007; Shenoy et al. 2012; González-Teuber and Heil 2009a; Mathur et al. 2013). Amino acid complement was found to be related to visitors' preferences in floral and extrafloral nectars (González-Teuber and Heil 2009b).

Twenty amino acids were detected in the floral nectar of *B. purpurocaerulea*: 16 protein and four non-protein amino acids. The quantitative ratio of protein to non-protein amino acids was about 9:1. Nine of the protein amino acids are essential (Table 1) for plant-feeding insects (Shoonhoven et al. 2006). They were found in relatively low concentrations in the nectar of *B. purpurocaerulea* as well as in 32 species of southern African plants pollinated by passerine birds (Nicolson 2007b) and enhance the nutritional value of nectar of this species. On the other hand, large amounts of seven non-essential amino acids were detected, proline being the most abundant in both sites. Proline is a common component of floral nectar, as pointed out by Baker and Baker (1983b), who detected it in 87 % of 395 species. This amino acid is a special compound for insects because it contributes to a preferred taste (Alm et al. 1990; Bertazzini et al. 2010) and stimulates the salt cell, a labellar chemoreceptor, promoting feeding behaviour (Hansen et al. 1998). Proline is quickly metabolised by bees by oxidative degradation to produce great amounts of ATP, playing a key role in the lift phase from flowers and in the initial phase of flight (Carter et al. 2006). In contrast proline is not particularly abundant in extrafloral nectar (Inouye and Inouye 1980; Ruffner and Clark 1986; Pate et al. 1985; González-Teuber and Heil 2009a; Shenoy et al. 2012; Mathur et al. 2013) that interacts mainly with non flying insects (ants).

Serine is another abundant amino acid in the nectar of *B. purpurocaerulea* (Table 1). Bertazzini et al. (2010) performed dual-choice feeding tests using artificial nectars and found that forager honeybees were attracted by proline-rich nectar and avoided serine-rich nectar. Serine seems to repel bees, although it is assigned to class I, i.e. amino acids with no effect on labellar chemoreceptors of flies (Nicolson and Thornburg 2007 and references therein).

Interestingly, although we are aware of the limited number of samples (see also Gardener and Gillman 2001), the only significant differences in amino acid composition in our two sites of *B. purpurocaerulea* concerned proline (class III) and serine (class I; see Table 1). The nectar of flowers of the EIs plants, visited more by Hymenoptera, was richer in proline and poorer in serine.

Some non-toxic non-protein amino acids, including β-alanine, ornithine, homoserine and GABA, are known to accumulate in nectar and are sometimes predominant (Nepi et al. 2012; Kaczorowski et al. 2005). In *B. purpurocaerulea*, β-alanine and GABA were the most abundant non-protein amino acids and accounted for about 10 % of total amino acids. Several functions have been recognized for GABA in both animals and plants (Stevenson 1999; Chevrot et al. 2006; Vranova et al. 2011), nonetheless its function in nectar remain unclear.

Visiting insects

Buglossoides purpurocaerulea flowers are erect and have a relatively deep corolla tube. Thus access to nectar, located in the deepest part of the tube at the base of the ovary, is limited. Moreover, restriction of the funnel opening by five plicae of petals further protects the nectar. Protected nectar at the base of deep corolla tubes is typical of plants pollinated by specialists (Faegri and Van der Pijl 1979) and reduces the spectrum of potential feeders to those with long mouthparts or small body size.

Beside morphological constrains, the guild of foragers may be related also to nectar sugar composition. In fact, species sharing the same type of pollinator show similar nectar chemical profiles (Baker and Baker 1983b; Nicolson and Thornburg 2007). Mediterranean Boraginaceae generally have sucrose-dominant sugar profile and are visited mainly by Hymenoptera Apoidea that usually prefer this type of nectar. *B. purpurocaerulea* is an exception to this because it has a hexose-dominant nectar. The only documented cases of hexose-dominant nectar in the Boraginaceae are *Echium wildpretii* and *Echium decaisnei*, both endemic to the Canary Islands (Dupont et al. 2004). In *Echium wildpretii*, hexose-dominant nectar is a floral character that evolved as a rapid adaptation to bird pollination (Valido et al. 2002; Dupont et al. 2004). *B. purpurocaerulea* typically grows at forest edges and therefore interacts with different insects from those of the open sunny environments where Boraginaceae normally grow. This may have shaped a nectar chemical composition in line with the preferences of insect communities more typical of shaded habitats, particularly Diptera, which are known to prefer hexose-dominant nectar. Diptera (*E. pennipes* and *B. major*) were common visitors to *B. purpurocaerulea*, although Hymenoptera were also observed in EIs. It is evident that chemical composition of floral as well as extrafloral nectar can switch in response to the preferences of foragers and may affect their feeding behaviour (González-Teuber and Heil 2009b; Wilder and Eubanks 2010; Bixenmann et al. 2011; Shenoy et al. 2012; Mathur et al. 2013).

It seems that the sucrose-dominant nectar is a basal condition in the Lithospermeae and the hexose prevalence in nectar is a derived condition that evolved independently in unrelated taxa mainly in response to adaptation to non hymenopteran pollen vectors. Nicolson and Van Wyk (1998) reported the opposite condition in Proteaceae species where hexose-rich nectar is the basal condition with repeated increases in sucrose concentration in unrelated groups but they conclude that pollinator type is not a primary selective force behind switches in nectar sugar composition. Broadening the study of chemical composition and flower visitors in other species of the tribe Lithospermeae may reveal further cases of hexose-rich nectars and will contribute to understanding how plant–insects relationships have been shaped.

Acknowledgments The authors are grateful to Federico Selvi (University of Florence, Department of Plant Biology) for his valuable comments and suggestions in the planning phase of the study, and to Małgorzata Stpiczyńska (Botanical Gardens, University of Warsaw) for improving an early version of the manuscript. Special thanks also go to Pietro Paolo Fanciulli (University of Siena, Department of Life Sciences), who helped identify the insect taxa. We acknowledge Martin Heil (CINVESTAV, Department of Genetic Engineering) and an anonymous reviewer for their constructive comments. This research was funded by PRIN 2008 (Italian Ministry for University and Research).

References

Alm J, Ohnmeiss TE, Lanza J, Vriesenga L (1990) Preference of cabbage white butterflies and honey bees for nectar that contains amino acids. Oecologia 84:53–57

Baker HG, Baker I (1982) Chemical constituents of nectar in relation to pollination biology: mechanisms and phylogeny. In: Nitecki MH (ed) Biochemical aspects of evolutionary biology. University of Chicago Press, Chicago, pp 131–171

Baker HG, Baker I (1983a) Floral nectar sugar constituents in relation to pollinator type. In: Jones CE, Little RJ (eds) Handbook of experimental pollination biology. Scientific and Academic, New York, pp 117–141

Baker HG, Baker I (1983b) A brief historical review of the chemistry of floral nectar. In: Bentley B, Elias T (eds) The biology of nectaries. Columbia University Press, New York, pp 126–151

Barbi S (2004) Reproductive biology of *Daphne sericea* Vahl (Thymelaeaceae), a vulnerable species of Mediterranean vegetation. MSc thesis, University of Siena, Siena

Bernardello G (2007) A systematic survey of floral nectaries. In: Nicolson SW, Nepi M, Pacini E (eds) Nectaries and nectar. Springer, Dordrecht, pp 19–128

Bertazzini M, Medrzycki P, Bortolotti L, Maistrello L, Forlani G (2010) Amino acid content and nectar choice by forager honeybees (*Apis mellifera* L.). Amino Acids 39:315–318

Beutler R (1953) Nectar-factors affecting the quantity and composition of nectar: external influences. Bee World 34:128–136

Bixenmann RJ, Coley PD, Kursar TA (2011) Is extrafloral nectar production induced by herbivores or ants in a tropical facultative ant-plant mutualism? Oecologia 165:417–425

Búrquez A, Corbet SA (1991) Do flowers reabsorb nectar? Funct Ecol 5:369–379

Búrquez A, Corbet SA (1998) Dynamics of production and exploitation of nectar: lessons from *Impatiens glandulifera* Royle. In: Bahadur B (ed) Nectary biology. Dattsons, Nagpur, pp 130–152

Carter C, Sharoni S, Yehonatan L, Palmer RG, Thornburg R (2006) A novel role for proline in plant floral nectars. Naturwissenschaften 93:72–79

Chevrot R, Rosen R, Haudecoeur E, Cirou A, Shelp BJ, Ron E, Faure D (2006) GABA controls the level of quorum-sensing signal in *Agrobacterium tumefacien*s. Proc Natl Acad Sci USA 103:7460–7464

Cohen SA, Micheaud D (1993) Synthesis of a fluorescent derivatizing reagent, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate, and its application for the analysis of hydrolysate amino acids via high-performance liquid chromatography. Anal Biochem 211:279–287

Corbet SA (1990) Pollination and the weather. Israel J Bot 39:13–30

Corbet SA, Wilmer PG, Beament JWL, Unwin DM, Prys-Jones O (1979) Post-secretory determinants of sugar concentration in nectar. Plant Cell Environ 2:293–303

Cruden RW, Hermann SM, Peterson S (1983) Patterns of nectar production and plant-pollinator coevolution. In: Bentley B, Elias T (eds) The biology of nectaries. Columbia University Press, New York, pp 80–125

Dafni A, Motte-Maues M (1998) A rapid and simple procedure to determine stigma receptivity. Sex Plant Reprod 11:177–180

Dupont YL, Hansen DM, Rasmussen JT, Olesen J (2004) Evolutionary changes in nectar sugar composition associated with switches between bird and insect pollination: the Canarian bird-flower element revisited. Funct Ecol 18:670–676

Faegri K, van der Pijl L (1979) The principles of pollination ecology, 3rd rev edn. Pergamon Press, Oxford

Gardener MC, Gillman MP (2001) Analysing variability in nectar amino acids: composition is less variable than concentration. J Chem Ecol 27:2545–2558

Gardener MC, Gillman MP (2002) The taste of nectar: a neglected area of pollination ecology. Oikos 98:552–557

González-Teuber M, Heil M (2009a) The role of extrafloral nectar amino acids for the preferences of facultative and obligate ant mutualists. J Chem Ecol 35:459–468

González-Teuber M, Heil M (2009b) Nectar chemistry is tailored for both attraction of mutualists and protection from exploiters. Plant Signal Behav 4:809–813

Gyan KY, Woodel SRJ (1987) Nectar production, sugar content, amino acids and potassium in *Prunus spinosa* L., *Crataegus monogyna* Jacq. and *Rubus fruticosus* L. at Wytham, Oxfordshire. Funct Ecol 1:251–259

Hansen K, Wacht S, Seebauer H, Schnuch M (1998) New aspects of chemoreception in flies. Ann N Y Acad Sci 855:143–147

Heil M (2011) Nectar: generation, regulation and ecological functions. Trends Plant Sci 16:191–200

Heinrich B, Raven PA (1972) Energetics and pollination ecology. Science 176:597–602

Herrera CM (1995) Microclimate and individual variation in pollinators: flowering plants are more than their flowers. Ecology 76:1516–1524

Herrera CM, Garcìa IM, Pérez R (2008) Invisible floral larcenies: microbial communities degrade floral nectar of bumble bee-pollinated plants. Ecology 89:2369–2376

Herrera CM, de Vega C, Canto A, Pozo MI (2009) Yeasts in floral nectar: a quantitative survey. Ann Bot Lond 103:1415–1423

Hocking B (1968) Insect-flower association in the high Arctic with special reference to nectar. Oikos 19:359–388

Huber H (1956) Die Abhängigkeit der Nektarsekretion von Temperatur, Luft- und Bodenfeuchtigkeit. Planta 48:47–98

Inouye DW, Inouye RS (1980) The amino acids of extrafloral nectar from *Helianthella quinquenervis* (Asteraceae). Am J Bot 67:1394–1396

Intoppa F, Piazza MG, Bolchi Serini G (1997) Elementi morfologici per una chiave dei sottogeneri di *Bombus Latreille* s. l. e *Psithyrus Lepeletier* s. l. presenti in Italia (Hymenoptera Apidae). Boll Zool Agr Bachic 29:1–43

Jakobsen HB, Kristjánsson K (1994) Influence of temperature and floret age on nectar secretion in *Trifolium repens* L. Ann Bot 74:327–334

Kaczorowski RL, Gardener MC, Holtsford TP (2005) Nectar traits in *Nicotiana* section *Alatae* (Solanaceae) in relation to floral traits, pollinators, and mating system. Am J Bot 92:1270–1283

Koopowitz H, Marchant TA (1998) Postpollination nectar resorption in the African epiphyte *Aerangis verdickii* (Orchidaceae). Am J Bot 85:508–512

Luyt R, Johnson SD (2002) Postpollination nectar resorption and its implications for fruit quality in an epiphytic orchidaceae. Biotropica 34:442–446

Mathur V, Wagenaar R, Caissard JC, Reddy AS, Vet LEM, Cortesero AM, Van Dam NM (2013) A novel indirect

defence in Brassicaceae: structure and function of extrafloral nectaries in *Brassica juncea*. Plant Cell Environ 36:528–541

Nepi M, Stpiczynska M (2008) The complexity of nectar: secretion and resorption dynamically regulate nectar features. Naturwissenshaften 95:177–184

Nepi M, Selvi F, Pacini E (2010) Variation in nectar-sugar profile of *Anchusa* and allied genera (Boraginaceae). Bot J Linn Soc 162:616–627

Nepi M, Cresti L, Guarnieri M, Pacini E (2011) Dynamics of nectar production and nectar homeostasis in male flowers of *Cucurbita pepo*. Int J Plant Sci 172:183–190

Nepi M, Soligo C, Nocentini D, Abate M, Guarnieri M, Cai G, Bini L, Puglia M, Bianchi L, Pacini E (2012) Amino acids and protein profile in floral nectar: much more than a simple reward. Flora 207:475–481

Nicolson SW (2007a) Nectar consumers. In: Nicolson SW, Nepi M, Pacini E (eds) Nectaries and nectar. Springer, Dordrecht, pp 289–342

Nicolson SW (2007b) Amino acid concentrations in the nectars of southern African bird-pollinated flowers, especially aloe and *Erythrina*. J Chem Ecol 33:1707–1720

Nicolson SW, Nepi M (2005) Dilute nectar in dry atmospheres: nectar secretion patterns in *Aloe castanea* (Asphodelaceae). Int J Plant Sci 166:227–233

Nicolson SW, Thornburg RW (2007) Nectar chemistry. In: Nicolson SW, Nepi M, Pacini E (eds) Nectaries and nectar. Springer, Dordrecht, pp 215–264

Nicolson SW, Van Wyk BE (1998) Nectar sugars in Proteaceae: patterns and processes. Aust J Bot 46:489–504

Nocentini D, Pacini E, Guarnieri M, Nepi M (2012) Flower morphology, nectar traits and pollinators of *Cerinthe major* (Boraginaceae–Lithospermae). Flora 207:186–196

Pacini E, Nepi M (2007) Nectar production and presentation. In: Nicolson SW, Nepi M, Pacini E (eds) Nectaries and nectar. Springer, Dordrecht, pp 167–214

Pacini E, Nepi M, Vesprini JL (2003) Nectar biodiversity: a short review. Plant Syst Evol 238:7–21

Pagliano G (1994) Catalogo degli imenotteri italiani. IV (Apoidea: Colletidae, Andrenidae, Megachilidae, Anthophoridae, Apidae). Mem Soc Entomol Ital 72:331–467

Pate JS, Peoples MB, Storer PJ, Atkins CA (1985) The extrafloral nectaries of cowpea (*Vigna Unguiculata* (L.) Walp.) II. Nectar composition, origin of nectar solutes, and nectary functioning. Planta 166:28–38

Pérez-Bañón C (2000) Biology of the syrphids (Diptera: Syrphidae) of the insular ecosystems in the community of Valencia: aspects of the insect–plant relationship. PhD thesis, University of Alicante, Alicante

Petanidou T (2005) Sugars in Mediterranean floral nectars: an ecological and evolutionary approach. J Chem Ecol 31: 1065–1088

Petanidou T (2007) Floral nectars in Mediterranean habitats. In: Nicolson SW, Nepi M, Pacini E (eds) Nectaries and nectar. Springer, Dordrecht, pp 167–214

Petanidou T, Smets E (1996) Does temperature stress induce nectar secretion in Mediterranean plants? New Phytol 133:513–518

Petanidou T, van Laere A, Ellis WN, Smets E (2006) What shapes amino acid and sugar composition in Mediterranean floral nectars? Oikos 115:155–169

Prŷs-Jones OR, Corbet SA (2011) Bumblebees, 3rd edn. Pelagic Publishing, Exeter

Pyke G (1991) What does it cost plant to produce floral nectar? Nature 350:58–59

Ruffner GA, Clark WD (1986) Extrafloral nectar of *Ferocactus acanthodes* (Cactaceae): composition and its importance to ants. Am J Bot 73:185–189

Shenoy M, Radhika V, Satish S, Borges RM (2012) Composition of extrafloral nectar influences interactions between the myrmecophyte *Humboldtia brunonis* and its ant associates. J Chem Ecol 38:88–99

Shoonhoven LM, van Loon JJA, Dicke M (2006) Insect–plant biology, 2nd edn. Oxford University Press, New York

Southwick EE (1984) Photosynthate allocation to floral nectar: a neglected energy investment. Ecology 65:1775–1779

Stevenson PA (1999) Colocalisation of taurine- with transmitter-immunoreactivities in the nervous system of the migratory locust. J Comp Neurol 404:86–96

Stpiczyńska M (2003a) Nectar resorption in the spur of *Platanthera chlorantha* Custer (Rchb.) Orchidaceae: structural and microautoradiographic study. Plant Syst Evol 238:119–126

Stpiczyńska M (2003b) Incorporation of [3H]sucrose after the resorption of nectar from the spur of *Platanthera chlorantha* (Custer) Rchb. Can J Bot 81:927–932

Tolman T, Lewington R (2008) Butterflies of Britain and Europe. Harper Collins, London

Valido A, Dupont YL, Hansen DM (2002) Native birds and insects, and introduced honey bees visiting *Echium wildpretii* (Boraginaceae) in the Canary Islands. Acta Oecol 23:413–419

Vranova V, Rejsek K, Skene KR, Formanek P (2011) Non-protein amino acids: plant, soil and ecosystem interactions. Plant Soil 342:31–48

Wilder SM, Eubanks MD (2010) Extrafloral nectar content alters foraging preferences of a predatory ant. Biol Lett 6:177–179

Ziegler H (1956) Untersuchungen über die Leitung und Sekretion der Assimilate. Planta 47:447–500