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Micro-organisms behind the pollination scenes: microbial imprint on floral nectar sugar variation in a tropical plant community

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• *Background and Aims* Variation in the composition of floral nectar reflects intrinsic plant characteristics as well as the action of extrinsic factors. Micro-organisms, particularly yeasts, represent one extrinsic factor that inhabit the nectar of animal-pollinated flowers worldwide. In this study a 'microbial imprint hypothesis' is formulated and tested, in which it is proposed that natural community-wide variation in nectar sugar composition will partly depend on the presence of yeasts in flowers.
• *Methods* Occurrence and density of yeasts were studied microscopically in single-flower nectar samples of 22 animal-pollinated species from coastal xeric and sub-humid tropical habitats of the Yucatán Peninsula, Mexico. Nectar sugar concentration and composition were concurrently determined on the same samples using high-performance liquid chromatography (HPLC) methods.
• *Key Results* Microscopical examination of nectar samples revealed the presence of yeasts in nearly all plant species (21 out of 22 species) and in about half of the samples examined (51·8 % of total, all species combined). Plant species and individuals differed significantly in nectar sugar concentration and composition, and also in the incidence of nectar yeasts. After statistically controlling for differences between plant species and individuals, nectar yeasts still accounted for a significant fraction of community-wide variance in all nectar sugar parameters considered. Significant yeast × species interactions on sugar parameters revealed that plant species differed in the nectar sugar correlates of variation in yeast incidence.
• *Conclusions* The results support the hypothesis that nectar yeasts impose a detectable imprint on community-wide variation in nectar sugar composition and concentration. Since nectar sugar features influence pollinator attraction and plant reproduction, future nectar studies should control for yeast presence and examine the extent to which microbial signatures on nectar characteristics ultimately have some influence on pollination services in plant communities.

Key words: Floral nectar, nectar composition, nectar sugars, flower yeasts.

INTRODUCTION

The majority of angiosperms are pollinated by animals (Ollerton *et al.*, 2011), and nectar is the most common type of floral reward offered by plants to pollinators (Simpson and Neff, 1983) and a keystone resource exploited by an extraordinary variety of consumers in most terrestrial ecosystems (Nicolson, 2007; Wäckers *et al.*, 2007). Consequently, nectar secretion, availability, chemical composition and consumption by animals have long been a focus of research on plant reproduction (Percival, 1961; Baker and Baker, 1983; Hodges, 1995; Irwin and Adler, 2008; Willmer, 2011), animal ecology (Stiles, 1978; Pleasants, 1989; Perez and Waddington, 1996; Waddington, 2001; Wäckers *et al.*, 2007) and, more generally, the evolution of plant–animal mutualisms (Bronstein *et al.*, 2006; Herrera, 2009; Irwin *et al.*, 2010). One aspect that has traditionally attracted considerable attention from researchers is the variation in nectar features that commonly occurs at scales spanning from species and communities down to individuals, inflorescences and flowers, and how such variation relates to pollinator composition, behaviour, pollinating effectiveness and plant reproduction

(Rathcke, 1992; Lanza *et al.*, 1995; Galetto *et al.*, 1998; Galetto and Bernardello, 2003; Petanidou, 2005; Herrera *et al.*, 2006; Herrera, 2009; Schlumpberger *et al.*, 2009). Although natural variation in nectar traits reflects intrinsic plant features (Walker *et al.*, 1974; Klinkhamer *et al.*, 1999; Mitchell, 2004), it also depends on extrinsic abiotic and biotic factors unrelated to the plants, including ambient temperature, water and nutrient availability, soil fungal effects and contamination derived from the activity of floral visitors (Willmer, 1980; Gottsberger *et al.*, 1990; Villarreal and Freeman, 1990; Gardener and Gillman, 2001; Canto *et al.*, 2008; Baude *et al.*, 2011; Becklin *et al.*, 2011). Insofar as extrinsic determinants possess the capacity to blur, enhance or distort patterns of variation in nectar features originated by intrinsic plant characteristics, they can become influential modifiers of plant–pollinator interactions, a possibility which has only rarely been addressed so far (Corbet, 1978; Willmer, 1980; Davis, 1997; Canto *et al.*, 2007; Herrera *et al.*, 2008; Wiens *et al.*, 2008).

An extrinsic biotic factor potentially influencing nectar features are nectar-dwelling micro-organisms, particularly yeasts, which recent studies have shown to occur abundantly in

animal-pollinated flowers worldwide (Sandhu and Waraich, 1985; Brysch-Herzberg, 2004; Herrera *et al.*, 2009; de Vega *et al.*, 2009; Belisle *et al.*, 2012). Among other short-term effects (e.g. Wiens *et al.*, 2008; Herrera and Pozo, 2010), yeasts can substantially alter the composition and concentration of sugars and amino acids in floral nectar (Herrera *et al.*, 2008; de Vega *et al.*, 2009; Peay *et al.*, 2012; de Vega and Herrera, 2012). For example, in some species, nectar yeasts reduce the total sugar concentration and turn sucrose-dominated nectars into fructose-dominated nectars (Herrera *et al.*, 2008). Such metabolic effects, acting in combination with the patchy distribution of yeasts across conspecific flowers, inflorescences and individuals, can render yeasts a significant source of intraspecific variation in nectar composition (Herrera *et al.*, 2006, 2008, 2009; Pozo *et al.*, 2009; Belisle *et al.*, 2012). It remains unknown so far, however, whether yeast effects on intraspecific nectar variation are sufficiently widespread and quantitatively important in plant communities as to eventually alter patterns of nectar variation at the community level. In other words, it is not known whether community-wide nectar variations in natural plant communities bear some discernible 'yeast signature' in addition to the variation generated by individual- and species-specific differences. Determining if such a microbial signal exists in natural habitats and, if it does, elucidating its magnitude and biological significance, are important steps towards assessing the functionality of nectar micro-organisms as a third party in the mutualistic relationships linking plants and pollinators, an emerging tripartite relationship whose ecological and evolutionary significance is still far from being well established (Eisikowitch *et al.*, 1990; Herrera *et al.*, 2008; Wiens *et al.*, 2008; Herrera and Pozo, 2010; Belisle *et al.*, 2012; de Vega and Herrera, 2012). Of particular relevance in this context are the possible community-wide effects of yeasts on nectar sugar parameters, since sugars are the dominant chemical constituents of most nectars, provide the key energetic reward for attracting pollinators, and their variation may influence pollination success and seed production (Heinrich, 1975; Holtkamp *et al.*, 1992; Roldán-Serrano and Guerra-Sanz, 2005).

Yeasts can alter nectar sugar variation at the plant community level through one or more of the following mechanisms. Everything else being equal, the impact on nectar composition of the yeasts inhabiting a flower is expected to relate directly to their density (cells per nectar volume unit; Herrera *et al.*, 2008; de Vega *et al.*, 2009; Herrera and Pozo, 2010). Consequently, patchiness in the distribution of yeasts across flowers, inflorescences or individuals of a plant population will enhance intraspecific variance in nectar sugar composition and/or modify the apportionment of such variance among flowers, inflorescences or individuals (Canto *et al.*, 2007, 2011; Pozo *et al.*, 2009). In addition, nectar yeasts also possess the capacity to influence interspecific variation in nectar composition. This will happen, for example, if plant species in a community consistently differ in average density of yeasts in their floral nectars (Herrera *et al.*, 2009; de Vega *et al.*, 2009); if susceptibility to yeast-mediated nectar sugar alteration differs intrinsically between plant species; and/or if plant species consistently differ in the species composition of their nectar yeast communities, since physiological differences between yeast species are expected to translate into differential

metabolic capacities to alter the nectar environment (Goddard, 2008; Herrera *et al.*, 2010; Peay *et al.*, 2012; Pozo *et al.*, 2012).

In this study, we test in a tropical plant community the preceding 'microbial imprint hypothesis' that nectar yeasts account in part for community-wide variation in nectar sugar characteristics. By concurrently evaluating the size of yeast populations, sugar composition and sugar concentration in single-flower nectar samples from many species and individuals of animal-pollinated plants, we will specifically address the following question. After accounting for individual- and species-specific effects on nectar sugar features, do yeasts explain an additional, statistically significant fraction of intra- and interspecific variance in nectar sugar composition and concentration? Our results substantiate this expectation. We show that patchiness in the distribution of nectar yeasts across plant species and individuals, in combination with differential propensity of plant species to harbour nectar yeasts and/or undergo nectar degradation by yeasts, generates a discernible microbial imprint on variation in nectar sugar composition and concentration at the plant community level.

MATERIALS AND METHODS

Study area and species sampled

Floral nectar was sampled for this study at several localities falling within a relatively limited area (approx. 430 km^2) in north-western Yucatán Peninsula, Mexico, which included the broad coastal dune strip at sea level between Chelem and Telchac towns, and adjacent dry deciduous forests at approx. 10 m elevation in the Cuxtal Ecological Reserve and between Dzibichaltun and Mérida. The coastal dune strip is a semi-xeric environment with 370 mm annual rainfall and 26 °C mean temperature on average. The vegetation is a low, open scrub dominated by xerophytes, halophyte herbs, thorny bushes, palms and 1–3 m treelets growing on sandy, nutrient-poor soils. Climate in the dry forest is tropical sub-humid, with 1077 mm of annual rainfall and mean temperature of 26 °C. The dry forest vegetation is made up of cacti, thorny shrubs and deciduous medium-height trees (3–8 m tall) growing on limestone bedrock soil with a thick litter layer (Chan Vermont *et al.*, 2002; Canto *et al.*, 2004).

On different dates between September 2008 and November 2009, a total of 527 floral nectar samples were collected from 22 plant species occurring in the study area and belonging to 11 different families (Table 1). Our species sampling was representative of plant communities in the study area, and included a diversity of life forms (cacti, herbs, shrubs, trees and vines) and pollinator types (social and solitary bees, beetles, moths, bats and hummingbirds; Table 1).

Field and laboratory methods

Flowering branches, inflorescences or single flowers of all species studied that had been previously exposed to natural visitation were collected in the field during their respective peak flowering seasons. Collected branches, inflorescences or flowers were carefully placed in glass jars in a portable cooler until taken indoors, and then kept at ambient

TABLE 1. *Plant species included in this study, main floral visitors, sample sizes and frequency (percentage of samples with yeasts), density (yeast cells mm^{-3}) and species composition of nectar-dwelling yeasts*

Family	Species (life habit)*	Main floral visitors[†]	Nectar samples studied[‡]	Percentage of samples with yeasts	Mean ± s.e. log$_{10}$ (yeast cells mm^{-3})	Yeast species identified in nectar samples[§]
Acanthaceae	*Bravaisia berlandieriana* (Nees) T. F. Daniel (S)	Be	23 (22)	8·7	0·19 ± 0·13	*Cryptococcus laurentii* (3), *Cryptococcus* aff. *taibaiensis* (1)
Agavaceae	*Agave angustifolia* Haw. (GH)	Ba, Be	32 (31)	28·1	0·52 ± 0·16	*Cryptococcus* sp. (1), *Clavispora lusitaniae* (3)
Apocynaceae	*Thevetia gaumeri* Hemsl. (T)	Be, Hu	22	59·1	1·57 ± 0·29	–
Bignoniaceae	*Tecoma stans* (L.) Juss. ex Kunth (T)	Be, Bt	20	40·0	1·00 ± 0·32	*Metschnikowia koreensis* (9), *Candida ipomoeae* (2)
Boraginaceae	*Cordia dodecandra* DC. (T)	Hu, Be	25	4·0	0·07 ± 0·07	–
	Cordia sebestena L. (S)	Hu	18	55·6	1·54 ± 0·36	–
Cactaceae	*Opuntia stricta* Haw. (C)	Be, Bt, Hu	29 (21)	72·4	2·59 ± 0·34	*Candida etchellsii* (1)
Convolvulaceae	*Ipomoea alba* L. (V)	Bt, Mo	23	13·0	0·23 ± 0·13	–
	Ipomoea crinicalyx S. Moore (V)	Be, Bt	25 (23)	84·0	2·96 ± 0·30	–
	Ipomoea hederifolia L. (V)	Hu	22	72·7	2·06 ± 0·31	*Cryptococcus laurentii* (1)
	Ipomoea nil (L.) Roth. (V)	Be, Bt	40	62·5	2·27 ± 0·31	*Ustilago sparsa* (1)
	Ipomoea triloba L. (V)	Be	19 (15)	73·7	2·78 ± 0·40	–
	Merremia aegyptia (L.) Urb. (V)	Be, Bt	37	54·1	1·51 ± 0·25	*Sympodiomycopsis paphiopedili* (1)
	Merremia dissecta (Jacq.) Hallier (V)	Be, Bt	21 (17)	100·0	3·77 ± 0·15	–
	Operculina pinnatifida (Kunth) O'Donell (V)	Be, Bt	18 (13)	100·0	3·50 ± 0·25	–
Fabaceae	*Centrosema schottii* (Millsp.) K. Schum. (V)	Be	18 (16)	0·0	0	–
	Lonchocarpus longistylus Pittier (T)	Be	24	20·8	0·60 ± 0·26	*Metschnikowia koreensis* (3)
	Piscidia piscipula (L.) Sarg. (T)	Be	27	55·6	1·42 ± 0·26	*Cryptococcus* aff. *taibaiensis* (3), *Cryptococcus liquefaciens* (6), *Sympodiomycopsis paphiopedili* (2)
Malvaceae	*Gossypium barbadense* L. (S)	Be	20	40·0	0·95 ± 0·30	–
	Malvaviscus arboreus Cav. (S)	Hu	24	70·8	2·03 ± 0·30	–
Passifloraceae	*Passiflora foetida* L. (V)	Be	16 (15)	50·0	1·06 ± 0·31	*Candida sorbosivorans* (3)
Polygonaceae	*Gymnopodium floribundum* Rolfe (S)	Be	24 (23)	70·8	1·41 ± 0·21	*Cryptococcus laurentii* (2)

* Life habit: C, cactus; GH, giant herb; S, shrub; T, tree or treelet; V, vine.

[†] Based on unpublished observations conducted on plants of the study area. Ba, bats; Be, bees; Bt, beetles; Hu, hummingbirds; Mo, moths.

[‡] When the number of nectar samples examined microscopically for yeast counts and analysed for sugar composition differed, the figure for the latter is shown in parentheses.

[§] Number of isolates of each species in parentheses. Dashes denote plant species for which yeast identifications were not undertaken.

temperature until extraction and microscopic examination of nectar samples, which was done within 3 h of collection (except for *Gymnopodium floribundum* and *Ipomoea alba* samples, which were examined within 12 h of collection). It was not possible to determine accurately the age of sampled flowers at the time of nectar collection. Except for *Agave angustifolia*, whose long-lived flowers last for up to 5 d, flower duration was much shorter and roughly similar for the rest of species, mostly ranging between 0·5 and 2 d. This makes us confident that flowers sampled for nectar had been previously exposed for roughly similar time periods to visitation and potential yeast colonization. For every plant species, separate nectar samples were obtained with calibrated microcapillaries from individual flowers (mean $\pm$ s.e. = 3·3 $\pm$ 0·1 flowers per plant) from different plants (mean = 7·2 $\pm$ 0·3 plants per species). Particular care was taken to collect only nectar samples from open, functional flowers that had been exposed to visitation prior to the time of collection and thus had had the opportunity of being colonized by yeasts. Although we did not attempt to identify the vectors bringing yeasts to the floral nectar of the plants included in this study, we assume that flower visitors were the main or sole vectors of nectar-dwelling yeasts as found by previous investigations conducted elsewhere (Brysch-Herzberg, 2004; Canto *et al.*, 2008; Belisle *et al.*, 2012; de Vega and Herrera, 2012).

The nectar in each sample was split into two sub-samples, which were used for concurrently characterizing nectar sugar composition and size of nectar-dwelling yeast communities. For chemical analyses, a known nectar volume (measured with calibrated micropipettes) was blotted onto a 10 $\times$ 2 mm Whatman 3MM paper wick. To avoid sample contamination, particular care was taken to avoid plant tissue damage (which could cause fluids other than nectar to leak into the nectar) and to ensure exclusive contact between the nectar and the wick. Immediately after nectar absorption, wicks were individually placed into clean, small paper envelopes. Until analysis, these were stored at ambient temperature in a sealed plastic box full of silica gel. Sugar composition was determined for $n = 498$ nectar samples using ion-exchange high-performance liquid chromatography (HPLC), following the analytical procedures and equipment described in detail by Herrera *et al.* (2006) and Canto *et al.* (2007). Only sucrose, glucose and fructose appeared regularly in the analyses. For each sample, proportions of individual sugars were obtained by integrating areas under chromatogram peaks. Separate estimates of glucose, fructose and sugar concentration on a per cent mass of solute to volume of solution basis (g solute per 100 mL solution) were obtained for each nectar sample. Total sugar concentration was computed for each sample by summing up partial figures for these three sugars. The second sub-sample consisted in most instances of the rest of nectar in the original sample. After measuring its volume with a calibrated micropipette, it was diluted up to 1–15 μL by addition of lactophenol cotton blue solution to facilitate microscopic examination. Yeast cell density (cells mm^{-3} of nectar volume) was then estimated directly under a microscope using a Neubauer chamber and standard cell counting procedures. A small proportion of samples had insufficient nectar to furnish the two sub-samples, hence sample sizes for yeast cell density

estimates and nectar composition differ slightly in some species (Table 1).

Rigorous identification of the micro-organisms present in all our nectar samples would have required extensive culturing, isolation and DNA sequencing (e.g. Brysch-Herzberg, 2004; Pozo *et al.*, 2011). Size and morphological features of cells and cell clusters observed in nectar, however, unequivocally characterized them as yeasts (i.e. fungal micro-organisms) in all instances, and this coarse level of taxonomic resolution was deemed sufficient for the purposes of this study. The validity of this approach was corroborated for a sub-set of nectar samples ($n = 21$, from 11 plant species), from which yeasts were isolated and identified by molecular means. Nectar sample aliquots were streaked individually onto Yeast Malt agar plates (1·0 % glucose, 0·5 % peptone, 0·3 % malt extract, 0·3 % yeast extract, 2·0 % agar) with 0·01 % chloramphenicol, and incubated at 25 °C. A total of 42 isolates were obtained from the resulting colonies following standard morphological criteria described in Yarrow (1998). For each isolate, the D1/D2 domain of the 26S sub-unit ribosomal DNA, the gene most commonly used for yeast identification, was two-way sequenced following methods in Kurtzman and Robnett (1998) and Lachance *et al.* (1999). A consensus sequence was assembled for each isolate using Geneious Pro 5·5 bioinformatics software (Biomatters Ltd, Auckland, New Zealand). Nucleotide collection databases at GenBank were queried with the Basic Local Alignment Search Tool (BLAST; Altschul *et al.*, 1997) to look for named yeast species with DNA sequences matching those obtained for our isolates. All sequences queried yielded highly significant alignments with named yeast accessions in GenBank databases, generally at very high levels of sequence coverage and pairwise identity (98–100 %).

Data analysis

The significance of intra- and interspecific variation in the incidence of nectar yeasts (cell density and per sample probability of yeast occurrence) was tested by fitting generalized linear mixed models to the data using procedure GLIMMIX in the SAS statistical package (SAS Institute, 2008). Binomial error distribution and logit link function were used for yeast presence data, and normal errors and identity link function for log-transformed yeast cell density data. Plant species and individuals (nested within species) were treated in these models as random effects. Their respective variance components were estimated by residual pseudolikelihood, and statistical significance tested by comparison with zero using Z-value approximate asymptotic tests, where the Z-value was computed by dividing the variance component estimate by its standard error (Littell *et al.*, 1996).

The hypothesis that community-wide variation in nectar sugar parameters (concentration of individual sugars, total sugar concentration and relative proportions of individual sugars) was significantly influenced by nectar yeasts was tested by fitting linear mixed models to individual flower data using the procedure MIXED in SAS using restricted maximum likelihood. A model was fitted for each nectar sugar parameter, with plant species and individuals treated as random effects, and yeast incidence and its interaction

with plant species as fixed effects. Separate analyses were carried out using either cell density (log-transformed) or presence–absence in nectar samples as descriptors of yeast incidence. These analyses made it possible to determine whether, after statistically accounting for species and individual specific differences in nectar sugar composition, there were still some additional measurable effects of yeast cell density or yeast presence on nectar sugar features, and whether such effects remained consistent across plant species. Statistical significance of variance components associated with random effects (plant species and individuals) was tested by comparing them with zero using approximate Z-value tests.

RESULTS

Identity, frequency and density of yeasts in nectar

On the basis of morphological criteria, microscopic examination revealed only the presence of yeasts in nectar samples. A total of 11 fungal species were identified in the 42 isolates recovered from a sub-set of the nectar samples examined (Table 1). Except for a single smut fungus (*Ustilago sparsa*, Ustilaginales), all species were ascomycetous (21 isolates; five species of *Candida*, *Clavispora* and *Metschnikowia*, Saccharomycetales) and basidiomycetous yeasts (17 isolates; four species of *Cryptococcus*, Tremellales) or yeast-like fungi (three isolates of *Sympodiomycopsis paphiopedili*, Microstromatales; Table 1). Although these identifications correspond to only a sub-set of the nectar samples studied, they support our conclusion based on morphological criteria that micro-organisms recorded during microscopic examinations of nectar samples were exclusively or predominantly ascomycetous and basidiomycetous yeasts and yeast-like fungi.

Yeasts were present in the nectar of nearly all plant species (21 out of 22 species) (Table 1) and in about half of all the samples examined (51·8 % of the total, all species combined). Plant species were significantly heterogeneous in per-sample probability of yeast ocurrence (range = 0–100 % of samples;

Z-value = 2·48, P = 0·0066) and cell density (range = 3–26 225 cells mm^{-3}; Z-value = 2·94, P = 0·0016) (Table 1). In addition to broad interspecific variation in yeast frequency and density in nectar samples, individual plants of the same species were also heterogeneous with regard to the incidence of yeasts in nectar, as denoted by the statistically significant between-individual variance components in per-sample probability of yeast occurrence (Z-value = 1·83, P = 0·034) and yeast cell density (Z-value = 2·69, P = 0·0035).

Variation in nectar sugar features

Sugar-related nectar features varied extensively across individual flowers in the sample of species and individuals studied. For all plant species combined (n = 498 samples), single-flower nectar samples exhibited moderate variation in the concentrations of glucose and fructose, and extensive variation in the concentration of sucrose and total sugars (Fig. 1). There was also considerable variation across flowers in relative proportions of glucose, fructose and sucrose in nectar, with almost any possible major combination of sugars occurring in the nectar samples studied (Fig. 2). Nectar samples included fructose- and sucrose-dominated nectars, as well as nectars containing virtually only sucrose or only fructose. Glucose-dominated nectar was the only major sugar combination that remained unrecorded in our sample (Fig. 2).

Variation between species, and between individuals within species, accounted for significant portions of the variance in sugar composition occurring in the nectar samples analysed, thus denoting a role for species- and individual-specific features as determinants of community-wide nectar variation. Regardless of the sugar composition parameter under consideration, variance components associated with species and conspecific individuals were always significantly greater than
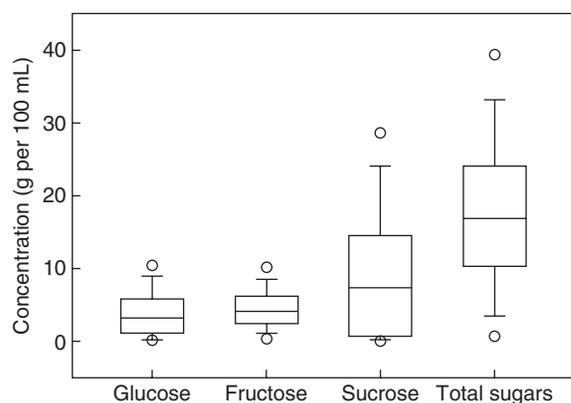

FIG. 1. Concentration of individual sugars (glucose, fructose and sucrose) and all sugars combined in the set of single-flower nectar samples from 22 plant species analysed for this study. Concentrations are expressed on a weight-to-volume basis. In each boxplot, the lower and upper boundaries of the box indicate the 25th and 75th percentiles, the line within the box marks the median, whiskers indicate the 10th and 90th percentiles, and circles denote the 5th and 95th percentiles of distributions.
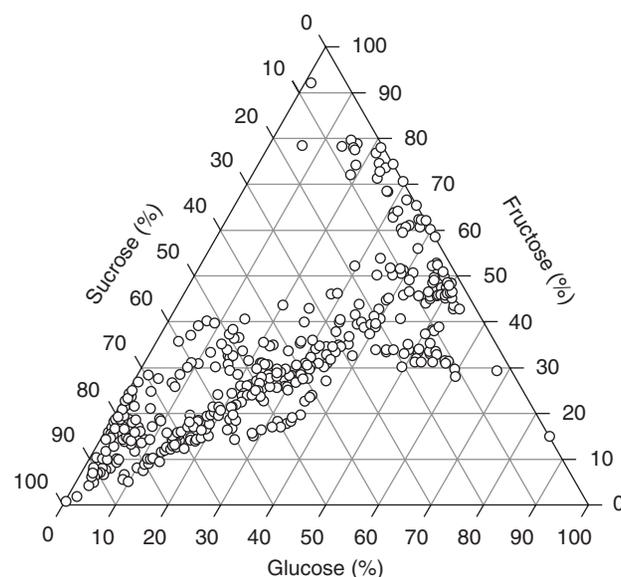

FIG. 2. Ternary diagram showing the distribution of nectar samples from all plant species combined (n = 498) over the plane defined by the proportions of glucose, fructose and sucrose relative to total sugars. Each point depicts the sugar composition of the nectar from a single flower.

TABLE 2. *Analysis of inter- and intraspecific variance components of sugar composition parameters in the set of nectar samples studied, obtained by fitting linear mixed models to individual flower data (n = 498), with species and individuals (nested within species) as random effects and log-yeast cell density and its interaction with species as fixed effects (see Table 3 for the results of fixed effects from the same analyses)*

	Between species			Individuals within species		
Nectar sugar composition parameter	Variance estimate ± s.e.	Z-value	P-value	Variance estimate ± s.e.	Z-value	P-value
Concentration*						
Fructose	4·20 ± 1·53	2·75	0·0029	0·93 ± 0·26	3·58	0·0002
Glucose	7·72 ± 2·64	2·93	0·0017	1·14 ± 0·29	3·90	<0·0001
Sucrose	89·94 ± 29·37	3·06	0·0011	6·66 ± 1·62	4·11	<0·0001
All sugars	87·30 ± 30·64	2·85	0·0022	10·20 ± 2·92	3·49	0·0002
Percentage of total sugars						
Fructose	195·04 ± 63·67	3·06	0·0011	21·90 ± 4·50	4·87	<0·0001
Glucose	286·07 ± 92·35	3·10	0·0010	15·16 ± 3·50	4·33	<0·0001
Sucrose	797·30 ± 255·33	3·12	0·0009	50·43 ± 11·03	4·57	<0·0001

* Grams of solute per 100 mL of solution.

TABLE 3. *Significance of effects of yeast cell density (log-transformed) and its interaction with plant species on sugar composition parameters in the set of single-flower nectar samples studied, after statistically accounting for intrinsic differences between plant species and individuals*

	Yeast cell density			Yeast cell density × plant species		
Nectar sugar composition parameter	F	d.f.	P-value	F	d.f.	P-value
Concentration*						
Fructose	5·96	1, 320	0·015	3·99	20, 320	<0·0001
Glucose	3·67	1, 320	0·056	4·30	20, 320	<0·0001
Sucrose	8·27	1, 320	0·0043	2·89	20, 320	<0·0001
All sugars	11·87	1, 320	0·0006	3·38	20, 320	<0·0001
Percentage of total sugar						
Fructose	7·48	1, 320	0·0068	2·18	20, 320	0·0027
Glucose	2·89	1, 320	0·090	1·49	20, 320	0·0835
Sucrose	0·37	1, 320	0·54	1·32	20, 320	0·16

Analyses involved fitting linear mixed models to individual flower data (n = 498), with log-yeast cell density and its interaction with species as fixed effects and species and individuals as random effects (see Table 2 for results corresponding to random effects).

* Grams of solute per 100 mL of solution.

zero (Table 2). In absolute terms, interspecific variance estimates were about one order of magnitude greater than intraspecific estimates for all compositional parameters considered (Table 2).

Yeasts and nectar sugar variation

Results of fitting linear mixed models to the data revealed that, after controlling for differences between species and individuals in mean nectar sugar parameters (random effects in models, Table 2), yeasts had statistically significant effects on the concentration of individual sugars, total sugar concentration and relative proportions of individual sugars, irrespective of whether yeast cell density (log-transformed; Table 3) or just presence–absence coded as a categorical, binary variable

(Supplementary Data Table S1) was used as the descriptor of yeast incidence. In addition, there were highly significant yeast × species interaction effects on the concentration of individual sugars, total sugar concentration and relative proportions of the different sugars, again irrespective of whether yeast cell density or presence–absence was used in the analyses to describe yeast incidence (Table 3 and Supplementary Data Table S1).

The significant yeast × species interaction effects denote that sugar changes associated with the density or just the presence of yeasts in nectar did not remain consistent across plant species. Interspecific heterogeneity in the shape of the relationship linking nectar sugar composition and yeast cell density is illustrated in Fig. 3 for four representative plants in our sample. In this group of species, *Merremia aegyptia* was characterized by highly significant, steep declines in nectar fructose, glucose and sucrose content with increasing (log) yeast cell density. In contrast, and over similar ranges of yeast densities, the corresponding relationships were shallower and barely significant in *Cordia sebestena*, and mostly flat and statistically nonsignificant in *Malvaviscus arboreus* and *Ipomoea hederifolia* (Fig. 3). Interspecific heterogeneity in the relationships between concentrations of individual sugars and yeast presence was also apparent in intraspecific, paired plots of sugar concentrations for samples with and without yeasts in the set of 19 species that exhibited between-sample variation in yeast occurrence (Supplementary Data Fig. S1).

DISCUSSION

Inter- and intraspecific sources of community-wide nectar sugar variation

A quintessential feature of floral nectar is the substantial variability in solute composition and concentration that takes place between species, individuals, flowers and even different nectaries or moments in the life of single flowers (Rathcke, 1992; Lanza *et al.*, 1995; Davis *et al.*, 1998; Pierre *et al.*, 1999; Galetto and Bernardello, 2003; Nepi *et al.*, 2003; Petanidou, 2005; Herrera *et al.*, 2006; Canto *et al.*, 2011). Sugars are both the dominant chemical constituents of most
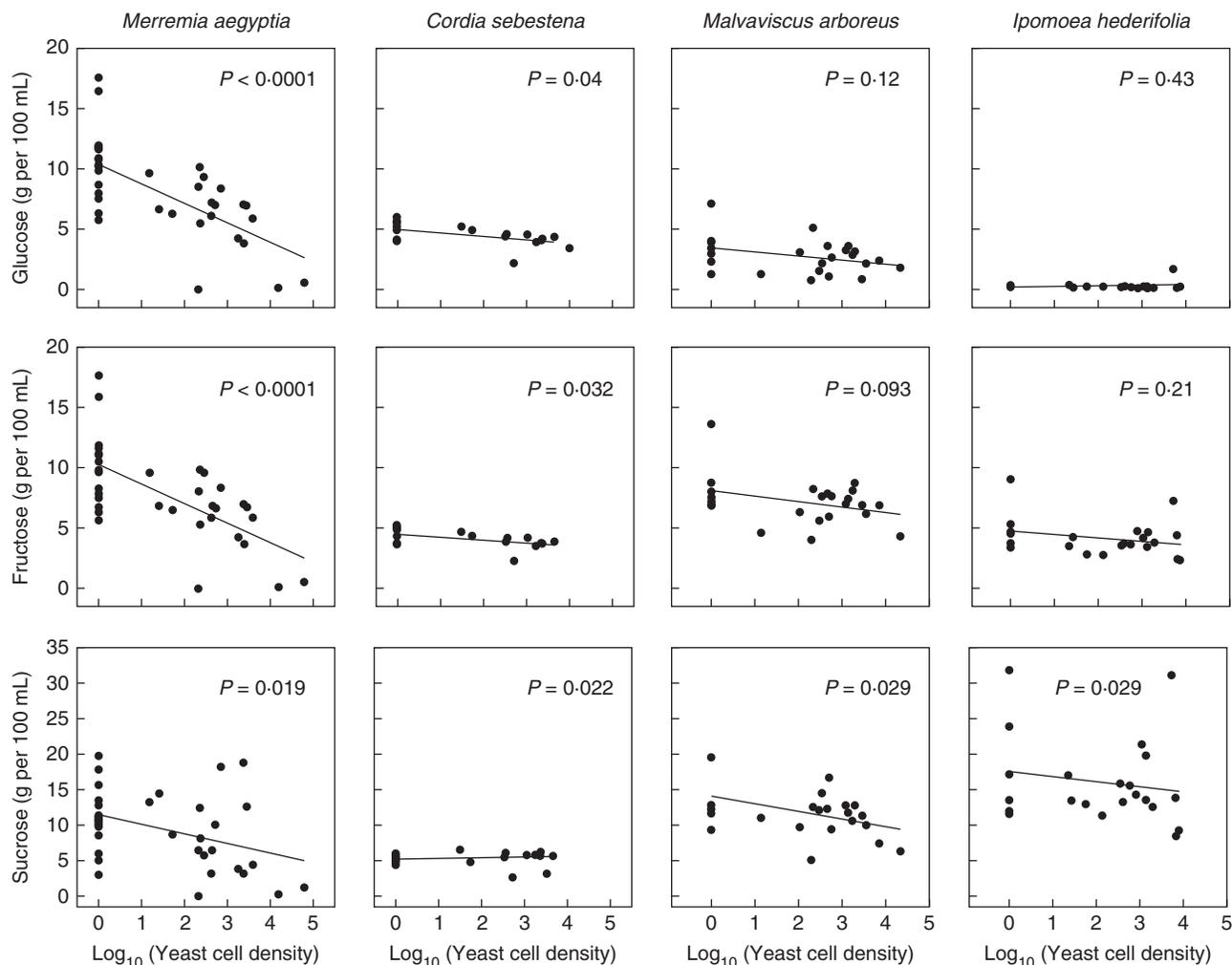
FIG. 3. Relationships between the concentration of glucose, fructose and sucrose (expressed as grams of solute per 100 mL of solution) and yeast cell density in single-flower nectar samples of four representative plant species, illustrating the interspecific differences in regression slopes that underly the significant species × yeast density effects on nectar sugar composition (Table 3). Lines are least-squares fitted regressions, and *P*-values denote their respective statistical significance levels.

floral nectars and the key energetic reward for attracting polli-nators (Heinrich, 1975), and their variation has been subjected to considerable scrutiny following the pioneering works of, among others, Wykes (1952*a*, *b*, 1953) and Percival (1961). Pollinators differ widely in energetic requirements, sugar pre-ferences and harvesting capacities of sugar-dominated nectar (Wykes, 1952*a*; Heinrich, 1975; Kim *et al.*, 2011), hence vari-ation in nectar sugar composition and concentration can influ-ence both pollinator attraction and plant reproductive success (Harder, 1986; Holtkamp *et al.*, 1992; Roldán-Serrano and Guerra-Sanz, 2005; Herrera, 2009; Baude *et al.*, 2011). Motivated by this reasoning, many studies have looked for associations across species between pollinator type and nectar sugar composition, and, when found, have interpreted their findings as evidence of pollinator partitioning mediated by variation in nectar sugar features (Baker and Baker, 1983; Baker *et al.*, 1998; Dupont *et al.*, 2004; Petanidou, 2005). Perhaps as a consequence of the frequent success of this re-search programme, interspecific variation has come to be

considered the major or sole source of variation in nectar sugar features in plant communities (see references in Herrera *et al.*, 2006). A number of studies, however, have documented substantial floral nectar sugar variation between conspecific individuals, flowers of the same plant and even nectaries of the same flower (Freeman, 1986; Freeman and Wilken, 1987; Lanza *et al.*, 1995; Herrera *et al.*, 2006; Canto *et al.*, 2007, 2011), which indicates that if adequately sampled, intraspecific variation may emerge as an important source of community-wide variance in nectar sugar character-istics. This possibility was confirmed by our analyses of single-flower nectar samples from different species, individuals of the same species and flowers of the same individuals.

In addition to the well-known interspecific component, community-wide variance in nectar sugar characteristics in the set of tropical species studied here also reflected extensive intraspecific variation, which was the combined consequence of (1) differences among conspecific plants, as denoted by the statistical significance of the between-individual variance

components; and (2) variation between flowers of the same plant (results not shown), as reported for some non-tropical species as well (Freeman and Wilken, 1987; Herrera *et al.*, 2006; Canto *et al.*, 2007, 2011). Since an accurate knowledge of the proportions of community-wide variance in nectar composition due to variation among species, individuals within species and within individuals is crucial to understand how pollinator-mediated selection acts on nectar traits (Herrera, 2009), our results emphasize the importance of adequately sampling all biologically relevant levels of variation, including intraspecific ones, when studying natural variation in nectar sugar characteristics (Herrera *et al.*, 2006).

Nectar yeasts

Yeasts were quite frequent and reached high densities in floral nectar in our Yucatán study area, as also found in other plant communities from different continents (Sandhu and Waraich, 1985; Brysch-Herzberg, 2004; Herrera *et al.*, 2009; de Vega *et al.*, 2009; Belisle *et al.*, 2012). The nectar of all but one of the 22 plant species studied, and more than half of samples examined, had yeasts. Although yeasts from only a sub-set of samples were identified to species, the predominance of basidiomycetous and ascomycetous species from the genera *Cryptococcus*, *Metschnikowia* and *Candida* agreed closely with results from previous studies of nectar-dwelling yeasts (Brysch-Herzberg, 2004; Pozo *et al.*, 2011; Belisle *et al.*, 2012; de Vega and Herrera, 2012). As in earlier investigations, species belonging to the *Metschnikowia* clade (*Metschnikowia*, *Clavispora* and *Candida ipomoeae*) also accounted here for a substantial proportion of total yeast isolates, which further confirms in a Neotropical context the central role of this group of ascomycetous yeasts in nectar-dwelling communities worldwide.

In the tropical plant community studied, yeast density and frequency of occurrence in nectar samples differed significantly between plant species and between individuals of the same species, as found by previous studies on multispecies plant assemblages or single species populations (Brysch-Herzberg, 2004; Herrera *et al.*, 2009; de Vega *et al.*, 2009; Belisle *et al.*, 2012; de Vega and Herrera, 2012). This emerging consensus of interspecifically and individually variable yeast incidence in floral nectar offers new insight for formulating predictions in the light of the 'antimicrobial hypothesis' (AMH hereafter), which explains toxic substances frequently present in nectar as defensive adaptations of plants against microbes that spoil nectar or are pathogens to the plant (Adler, 2000; Thornburg *et al.*, 2003; Carter and Thornburg, 2004; Sasu *et al.*, 2010). The AMH essentially represents a transposition to the intrafloral realm of the well-established theory accounting for plant chemical defences against herbivores and pathogens (Strauss and Zangerl, 2002). The success of this theory owed no small part to the recognition of the ecological and evolutionary significance of interspecific and individual variation in resistance to herbivores and pathogens (e.g. different authors in Fritz and Simms, 1992). We suggest that, by the same token, scrutinizing correlates of interspecific and individual differences in nectar yeast incidence may contribute to evaluate the merits of the AMH and identify possible coevolutionary links between plants and

nectarivorous microbes. In particular, we predict: (1) there should be an inverse relationship across species between yeast incidence and presence in nectar of substances with fungicidal action; (2) among species producing fungicidal substances in nectar, there should be an inverse relationship across individuals between yeast incidence and concentration in nectar of fungicidal substances; and (3) the two preceding relationships should appear tightest when species of generalist yeasts that exploit a variety of microhabitats (e.g. nectar, petals, phylloplane, or soil) predominate in nectar, and loosest when the dominance corresponds to yeasts specializing on floral nectar (Pozo *et al.*, 2012), as these latter should have evolved tolerance to antifungal substances in nectar (Herrera *et al.*, 2008) just like specialist herbivores tolerate allelochemicals of their host plants better than generalist ones (Bowers and Putick, 1988; Berenbaum, 1990; Agrawal, 2000). Verification of these predictions would lend support to the AMH and open new avenues for research on the complex coevolutionary relationships between plants and fungi.

Community-wide microbial imprint on nectar sugars

The results of this study have shown that, after statistically controlling for differences between plant species and individuals, nectar yeasts accounted for an additional, statistically significant fraction of community-wide variation in all nectar sugar parameters considered. Since the metabolic activity of nectar yeasts can substantially alter the composition of floral nectar (Herrera *et al.*, 2008; de Vega *et al.*, 2009; Peay *et al.*, 2012; de Vega and Herrera, 2012), we interpret the correlational results of this study as denoting a causal influence of nectar yeasts on community-wide nectar sugar characteristics and thus supporting the microbial imprint hypothesis.

Our results are also consistent with some of the proximate mechanisms advanced in the Introduction as potential causes of a microbial imprint on community-wide nectar variation, namely patchiness in yeast distribution across species and individuals, and interspecific differences in nectar susceptibility to alteration by yeasts. The first mechanism, whereby interspecific and intraspecific variance in nectar features is amplified beyond that due exclusively to intrinsic differences between species and individuals, was already suggested by field experiments showing that intraspecific variance in nectar sugar features increased considerably among flowers exposed to yeast colonization (Canto *et al.*, 2007, 2008, 2011). The second mechanism has been proposed here for the first time, and the significant yeast incidence × plant species interaction effects on nectar sugar parameters clearly support it. Such interactions are most likely to be due to several factors, including variation among plants species in concentration or effectiveness of antifungal defences in nectar, and in composition of their associated nectar yeast communities, perhaps as a consequence of differences in pollinator type. Our identifications of yeast isolates suggest that the composition of nectar yeast assemblages varies among plant species (see also Pozo *et al.*, 2012). Yeast species often differ drastically in overall physiological features and metabolic abilities (Middelhoven and Kurtzman, 2003; Goddard, 2008; Peay *et al.*, 2012; Pozo *et al.*, 2012), hence variation across plant species in composition of nectar yeast communities could account for the

interspecific variation in the nature of yeast effects on nectar that ultimately contributes to the microbial signature on community-wide nectar features. Further studies simultaneously examining nectar yeast community composition and impact on nectar features are needed to substantiate this interpretation. Of particular interest would also be to elucidate whether some predictable association exists between pollinator type and nectar yeast community composition, as such a relationship could disclose some unanticipated, indirect causal pathways between pollinator type and nectar features in natural communities.

Concluding remarks

Microbiologists and pollination biologists alike have long been aware of the fact that a variety of micro-organisms populate floral nectar (see review in Herrera *et al.*, 2009). Furthermore, early students of nectar chemistry already acknowledged that nectar-inhabiting microbes can modify nectar composition, as exemplified by Baker and Baker's (1983; pp. 120–121) explicit warning that nectar samples for sugar analyses should not be allowed to stand in the liquid condition because such samples may be significantly degraded by micro-organisms, 'especially yeasts'. Nevertheless, the frequency and extent of modifications of nectar features induced by nectar yeasts in natural habitats remained unexplored until quite recently, when a few studies focusing on the nectar of individual plant species confirmed the capacity of yeasts to alter nectar characteristics in intraspecific contexts (Canto *et al.*, 2007, 2011; Herrera *et al.*, 2008). We have here extended these findings one step further by showing that nectar yeasts can impose a detectable signature on community-wide variation in nectar sugar features. Given the well-known effects on pollinators of variation in nectar rewards (Rathcke, 1992; Waddington, 2001; Herrera, 2009), the significant role of yeasts as nectar modifiers at the plant community level could eventually influence pollinator behaviour, plant–pollinator interactions and plant reproduction. Future studies should examine whether the microbial effects on nectar features have some influence on pollinator service and, ultimately, plant reproduction. In addition, pervasive microbial signatures on nectar sugar variation in natural habitats would call for more careful interpretations of nectar variation. This particularly applies to the assumption traditionally implicit in many nectar studies (but see, for example, Willmer, 1980; Gottsberger *et al.*, 1990; for some divergent views) that, in plant communities, variations in nectar features mostly or exclusively reflect inherent plant properties.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxford-journals.org and consist of the following. Table S1: significance of effects on sugar composition parameters of yeast occurrence in nectar and the yeast × plant species interaction. Figure S1: intraspecific variation in average concentration of individual sugars in nectar samples with and without yeasts for those species that exhibited intraspecific, between-sample variation in yeast occurrence.

LITERATURE CITED

Adler LS. 2000. The ecological significance of toxic nectar. *Oikos* **91**: 409–420.

Agrawal AA. 2000. Specificity of induced resistance in wild radish: causes and consequences for two specialist and two generalist caterpillars. *Oikos* **89**: 493–500.

Altschul SF, Madden TL, Schäffer AA, *et al*. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**: 3389–3402.

Baker HG, Baker I. 1983. Floral nectar sugar constituents in relation to pollinator type. In: Jones CE, Little RJ. eds. *Handbook of experimental pollination biology.* New York: Van Nostrand Reinhold, 117–141.

Baker HG, Baker I, Hodges SA. 1998. Sugar composition of nectars and fruits consumed by birds and bats in the tropics and subtropics. *Biotropica* **30**: 559–586.

Baude M, Leloup J, Suchail S, *et al*. 2011. Litter inputs and plant interactions affect nectar sugar content. *Journal of Ecology* **99**: 828–837.

Becklin KM, Gamez G, Uelk B, Raguso RA, Galen C. 2011. Soil fungal effects on floral signals, rewards, and aboveground interactions in an alpine pollination web. *American Journal of Botany* **98**: 1299–1308.

Belisle M, Peay KG, Fukami T. 2012. Flowers as islands: spatial distribution of nectar-inhabiting microfungi among plants of *Mimulus aurantiacus*, a hummingbird-pollinated shrub. *Microbial Ecology* **63**: 711–718.

Berenbaum MR. 1990. Evolution of specialization in insect–umbellifer associations. *Annual Review of Entomology* **35**: 319–343.

Bowers MD, Puttick GM. 1988. Response of generalist and specialist insects to qualitative allelochemical variation. *Journal of Chemical Ecology* **14**: 319–334.

Bronstein JL, Alarcón R, Geber M. 2006. The evolution of plant–insect mutualisms. *New Phytologist* **172**: 412–428.

Brysch-Herzberg M. 2004. Ecology of yeasts in plant–bumblebee mutualism in Central Europe. *FEMS Microbiology Ecology* **50**: 87–100.

Canto A, Parra-Tabla V, García-Franco JG. 2004. Variations in leaf production and floral display of *Anthurium schlechtendalii* (Araceae) in response to herbivory and environment. *Functional Ecology* **18**: 692–699.

Canto A, Pérez R, Medrano M, Castellanos MC, Herrera CM. 2007. Intraplant variation in nectar sugar composition in two *Aquilegia* species (Ranunculaceae): contrasting patterns under field and greenhouse conditions. *Annals of Botany* **99**: 653–660.

Canto A, Herrera CM, Medrano M, Pérez R, García IM. 2008. Pollinator foraging modifies nectar sugar composition in *Helleborus foetidus* (Ranunculaceae): an experimental test. *American Journal of Botany* **95**: 315–320.

Canto A, Herrera CM, García IM, Pérez R, Vaz M. 2011. Intraplant variation in nectar traits in *Helleborus foetidus* (Ranunculaceae) as related to floral phase, environmental conditions and pollinator exposure. *Flora* **206**: 668–675.

Carter C, Thornburg RW. 2004. Is the nectar redox cycle a floral defense against microbial attack? *Trends in Plant Science* **9**: 320–324.

Chan Vermont C, Rico-Gray V, Flores JS. 2002. Guía ilustrada de la flora costera representativa de la Península de Yucatán. *Etnoflora Yucatanense* **19**: 1–133.

Corbet SA. 1978. Bee visits and the nectar of *Echium vulgare* L. and *Sinapis alba* L. *Ecological Entomology* **3**: 25–37.

Davis AR. 1997. Influence of floral visitation on nectar-sugar composition and nectary surface changes in *Eucalyptus*. *Apidologie* **28**: 27–42.

Davis AR, Pylatuik JD, Paradis JC, Low NH. 1998. Nectar-carbohydrate production and composition vary in relation to nectary anatomy and location within individual flowers of several species of Brassicaceae. *Planta* **205**: 305–318.

Dupont YL, Hansen DM, Rasmussen JT, Olesen JM. 2004. Evolutionary changes in nectar sugar composition associated with switches between bird and insect pollination: the Canarian bird-flower element revisited. *Functional Ecology* **18**: 670–676.

Eisikowitch D, Kevan PG, Lachance MA. 1990. The nectar-inhabiting yeasts and their effect on pollen germination in common milkweed, *Asclepias syriaca* L. *Israel Journal of Botany* **39**: 217–225.

Freeman CD. 1986. Nectar-sugar composition in an individual of *Ruellia peninsularis* (Acanthaceae). *Madroño* **33**: 300–302.

Freeman CE, Wilken DH. 1987. Variation in nectar sugar composition at the intraplant level in *Ipomopsis longiflora* (Polemoniaceae). *American Journal of Botany* **74**: 1681–1689.

Fritz RS, Simms EL. eds**.** **1992.** *Plant resistance to herbivores and pathogens: ecology, evolution, and genetics*. Chicago, IL: University of Chicago Press.

Galetto L, Bernardello G. 2003. Nectar sugar composition in angiosperms from Chaco and Patagonia (Argentina): an animal visitor's matter? *Plant Systematics and Evolution* **238**: 69–86.

Galetto L, Bernardello G, Sosa CA. 1998. The relationship between floral nectar composition and visitors in *Lycium* (Solanaceae) from Argentina and Chile: what does it reflect? *Flora* **193**: 303–314.

Gardener MC, Gillman MP. 2001. The effects of soil fertilizer on amino acids in the floral nectar of corncockle, *Agrostemma githago* (Caryophyllaceae). *Oikos* **92**: 101–106.

Goddard MR. 2008. Quantifying the complexities of *Saccharomyces cerevisiae*'s ecosystem engineering via fermentation. *Ecology* **89**: 2077–2082.

Gottsberger G, Arnold T, Linskens HF. 1990. Variation in floral nectar amino acids with aging of flowers, pollen contamination, and flower damage. *Israel Journal of Botany* **39**: 167–176.

Harder LD. 1986. Effects of nectar concentration and flower depth on flower handling efficiency of bumble bees. *Oecologia* **69**: 309–315.

Heinrich B. 1975. Energetics of pollination. *Annual Review of Ecology and Systematics* **6**: 139–170.

Herrera CM. 2009. *Multiplicity in unity. Plant subindividual variation and interactions with animals*. Chicago, IL: University of Chicago Press.

Herrera CM, Pozo MI. 2010. Nectar yeasts warm the flowers of a winter-blooming plant. *Proceedings of the Royal Society B: Biological Sciences* **277**: 1827–1834.

Herrera CM, Pérez R, Alonso C. 2006. Extreme intraplant variation in nectar sugar composition in an insect-pollinated perennial herb. *American Journal of Botany* **93**: 575–581.

Herrera CM, García IM, Pérez R. 2008. Invisible floral larcenies: microbial communities degrade floral nectar of bumblebee-pollinated plants. *Ecology* **89**: 2369–2376.

Herrera CM, de Vega C, Canto A, Pozo MI. 2009. Yeasts in floral nectar: a quantitative survey. *Annals of Botany* **103**: 1415–1423.

Herrera CM, Canto A, Pozo MI, Bazaga P. 2010. Inhospitable sweetness: nectar filtering of pollinator-borne inocula leads to impoverished, phylogenetically clustered yeast communities. *Proceedings of the Royal Society B: Biological Sciences* **277**: 747–754.

Hodges SA. 1995. The influence of nectar production on hawkmoth behavior, self pollination, and seed production in *Mirabilis multiflora* (Nyctaginaceae). *American Journal of Botany* **82**: 197–204.

Holtkamp RH, Morthorpe KJ, Clift AD. 1992. Influence of nectar volume and sugar content on seed set in lucerne. *Australian Journal of Experimental Agriculture* **32**: 713–716.

Irwin RE, Adler LS. 2008. Nectar secondary compounds affect self-pollen transfer: implications for female and male reproduction. *Ecology* **89**: 2207–2217.

Irwin RE, Bronstein JL, Manson JS, Richardson L. 2010. Nectar robbing: ecological and evolutionary perspectives. *Annual Review of Ecology, Evolution, and Systematics* **41**: 271–292.

Kim W, Gilet T, Bush JWM. 2011. Optimal concentrations in nectar feeding. *Proceedings of the National Academy of Sciences, USA* **108**: 16618–16621.

Klinkhamer PGL, van der Veen-van Wijk CAM. 1999. Genetic variation in floral traits of *Echium vulgare*. *Oikos* **85**: 515–522.

Kurtzman CP, Robnett CJ. 1998. Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Antonie Van Leeuwenhoek* **73**: 331–371.

Lachance MA, Bowles JM, Starmer WT, Barker JSF. 1999. *Kodamaea kakaduensis* and *Candida tolerans*, two new ascomycetous yeast species from Australian *Hibiscus* flowers. *Canadian Journal of Microbiology* **45**: 172–177.

Lanza J, Smith GC, Sack S, Cash A. 1995. Variation in nectar volume and composition of *Impatiens capensis* at the individual, plant, and population levels. *Oecologia* **102**: 113–119.

Littell RC, Milliken GA, Stroup WW, Wolfinger RD. 1996. *SAS system for mixed models*. Cary, NC: SAS Institute.

Middelhoven WJ, Kurtzman CP. 2003. Relation between phylogeny and physiology in some ascomycetous yeasts. *Antonie Van Leeuwenhoek* **83**: 69–74.

Mitchell RJ. 2004. Heritability of nectar traits: why do we know so little? *Ecology* **85**: 1527–1533.

Nepi M, Pacini E, Nencini C, Collavoli E, Franchi GG. 2003. Variability of nectar production and composition in *Linaria vulgaris* (L.) Mill. (Scrophulariaceae). *Plant Systematics and Evolution* **238**: 109–118.

Nicolson SW. 2007. Nectar consumers. In: Nicolson SW, Nepi M, Pacini E. eds. *Nectaries and nectar*. Dordrecht, The Netherlands: Springer, 289–342.

Ollerton J, Winfree R, Tarrant S. 2011. How many flowering plants are pollinated by animals? *Oikos* **120**: 321–326.

Peay KG, Belisle M, Fukami T. 2012. Phylogenetic relatedness predicts priority effects in nectar yeast communities. *Proceedings of the Royal Society B: Biological Sciences* **279**: 749–758.

Percival MS. 1961. Types of nectar in angiosperms. *New Phytologist* **60**: 235–281.

Perez SM, Waddington KD. 1996. Carpenter bee (*Xylocopa micans*) risk indifference and a review of nectarivore risk-sensitivity studies. *American Zoologist* **36**: 435–446.

Petanidou T. 2005. Sugars in Mediterranean floral nectars: an ecological and evolutionary approach. *Journal of Chemical Ecology* **31**: 1065–1088.

Pierre J, Mesquida J, Marilleau R, Pham-Delegue MH, Renard M. 1999. Nectar secretion in winter oilseed rape, *Brassica napus* – quantitative and qualitative variability among 71 genotypes. *Plant Breeding* **118**: 471–476.

Pleasants JM. 1989. Optimal foraging by nectarivores: a test of the marginal-value theorem. *American Naturalist* **134**: 51–71.

Pozo MI, de Vega C, Canto A, Herrera CM. 2009. Presence of yeasts in floral nectar is consistent with the hypothesis of microbial-mediated signaling in plant–pollinator interactions. *Plant Signaling and Behavior* **4**: 1102–1104.

Pozo MI, Herrera CM, Bazaga P. 2011. Species richness of yeast communities in floral nectar of southern Spanish plants. *Microbial Ecology* **61**: 82–91.

Pozo MI, Lachance MA, Herrera CM. 2012. Nectar yeasts of two southern Spanish plants: the roles of immigration and physiological traits in community assembly. *FEMS Microbiology Ecology* **80**: 281–293.

Rathcke BJ. 1992. Nectar distributions, pollinator behavior, and plant reproductive success. In: Hunter MD, Ohgushi T, Price PW. eds. *Effects of resource distribution on animal–plant interactions*. New York: Academic Press, 113–138.

Roldán-Serrano AS, Guerra-Sanz JM. 2005. Reward attractions of zucchini flowers (*Cucurbita pepo* L.) to bumblebees (*Bombus terrestris* L.). *European Journal of Horticultural Science* **70**: 23–28.

Sandhu DK, Waraich MK. 1985. Yeasts associated with pollinating bees and flower nectar. *Microbial Ecology* **11**: 51–58.

SAS Institute. 2008. *SAS for Windows (version 9·2)*. Cary, NC: SAS Institute.

Sasu MA, Wall KL, Stephenson AG. 2010. Antimicrobial nectar inhibits a floral transmitted pathogen of a wild *Cucurbita pepo* (Cucurbitaceae). *American Journal of Botany* **97**: 1025–1030.

Schlumpberger BO, Cocucci AA, More M, Sersic AN, Raguso RA. 2009. Extreme variation in floral characters and its consequences for pollinator attraction among populations of an Andean cactus. *Annals of Botany* **103**: 1489–1500.

Simpson BB, Neff JL. 1983. Evolution and diversity of floral rewards. In: Jones CE, Little RJ. eds. *Handbook of experimental pollination biology*. New York: Van Nostrand Reinhold, 142–159.

Stiles FG. 1978. Ecological and evolutionary implications of bird pollination. *American Zoologist* **18**: 715–727.

Strauss SY, Zangerl AR. 2002. Plant–insect interactions in terrestrial ecosystems. In: **Herrera CM, Pellmyr O**. eds. *Plant–animal interactions: an evolutionary approach*. Oxford: Blackwell, 77–106.

Thornburg RW, Carter C, Powell A, Mittler R, Rizhsky L, Horner HT. 2003. A major function of the tobacco floral nectary is defense against microbial attack. *Plant Systematics and Evolution* **238**: 211–218.

de Vega C, Herrera CM. 2012. Relationships among nectar-dwelling yeasts, flowers and ants: patterns and incidence on nectar traits. *Oikos*, in press. http://dx.doi.org/10.1111/j.1600-0706.2012.20295.x.

de Vega C, Herrera CM, Johnson SD. 2009. Yeasts in floral nectar of some South African plants: quantification and associations with pollinator type. *South African Journal of Botany* **75**: 798–806.

Villarreal AG, Freeman CE. 1990. Effects of temperature and water stress on some floral nectar characteristics in *Ipomopsis longiflora* (Polemoniaceae) under controlled conditions. *Botanical Gazette* **151**: 5–9.

Wäckers FL, Romeis J, van Rijn P. 2007. Nectar and pollen feeding by insect herbivores and implications for multitrophic interactions. *Annual Review of Entomology* **52**: 301–323.

Waddington KD. 2001. Subjective evaluation and choice behavior by nectar- and pollen-collecting bees. In: Chittka L, Thomson JD. eds. *Cognitive ecology of pollination*. Cambridge, UK: Cambridge University Press, 41–60.

Walker AK, Barnes DK, Furgala B. 1974. Genetic and environmental effects on quantity and quality of alfalfa nectar. *Crop Science* **14**: 235–238.

Wiens F, Zitzmann A, Lachance MA, *et al*. 2008. Chronic intake of fermented floral nectar by wild treeshrews. *Proceedings of the National Academy of Sciences, USA* **105**: 10426–10431

Willmer P. 2011. *Pollination and floral ecology*. Princeton, NJ: Princeton University Press.

Willmer PG. 1980. The effects of insect visitors on nectar constituents in temperate plants. *Oecologia* **47**: 270–277.

Wykes GR. 1952*a*. The preferences of honeybees for solutions of various sugars which occur in nectar. *Journal of Experimental Biology* **29**: 511–519.

Wykes GR. 1952*b*. An investigation of the sugars present in the nectar of flowers of various species. *New Phytologist* **51**: 210–215.

Wykes GR. 1953. The sugar content of nectars. *Biochemical Journal* **53**: 294–296.

Yarrow D. 1998. Methods for the isolation, maintenance and identification of yeasts. In: Kurtzman CP, Fell JW. eds. *The yeasts, a taxonomic study*. Amsterdam: Elsevier, 77–100.