

Yeasts in floral nectar: a quantitative survey

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- **Background and Aims** One peculiarity of floral nectar that remains relatively unexplored from an ecological perspective is its role as a natural habitat for micro-organisms. This study assesses the frequency of occurrence and abundance of yeast cells in floral nectar of insect-pollinated plants from three contrasting plant communities on two continents. Possible correlations between interspecific differences in yeast incidence and pollinator composition are also explored.
- **Methods** The study was conducted at three widely separated areas, two in the Iberian Peninsula (Spain) and one in the Yucatán Peninsula (Mexico). Floral nectar samples from 130 species (37–63 species per region) in 44 families were examined microscopically for the presence of yeast cells. For one of the Spanish sites, the relationship across species between incidence of yeasts in nectar and the proportion of flowers visited by each of five major pollinator categories was also investigated.
- **Key Results** Yeasts occurred regularly in the floral nectar of many species, where they sometimes reached extraordinary densities (up to 4×10^5 cells mm^{-3}). Depending on the region, between 32 and 44% of all nectar samples contained yeasts. Yeast cell densities in the order of 10^4 cells mm^{-3} were commonplace, and densities $>10^5$ cells mm^{-3} were not rare. About one-fifth of species at each site had mean yeast cell densities $>10^4$ cells mm^{-3} . Across species, yeast frequency and abundance were directly correlated with the proportion of floral visits by bumble-bees, and inversely with the proportion of visits by solitary bees.
- **Conclusions** Incorporating nectar yeasts into the scenario of plant–pollinator interactions opens up a number of intriguing avenues for research. In addition, with yeasts being as ubiquitous and abundant in floral nectars as revealed by this study, and given their astounding metabolic versatility, studies focusing on nectar chemical features should carefully control for the presence of yeasts in nectar samples.

Key words: Bumble-bees, floral nectar, microbial communities, pollination, yeasts.

‘Il y a évidemment un intérêt très grand, dans une question encore peu connue, à recenser exactement et cela dans diverse régions, les organismes qu’on rencontre.’

Schoellhorn (1919, p. 155)

INTRODUCTION

Nectar is the most frequent form of floral reward that animal-pollinated plants provide for their mutualistic counterparts (Simpson and Neff, 1983). Consequently, pollination biologists have historically devoted vast amounts of effort to elucidate the physiology, chemical features, energetic and nutritional content, and spatial and temporal secretion patterns of nectar, as well as to assess the degree to which the extensive variation in these features occurring in nature bear some adaptive relationship to the characteristics of pollinators (e.g. Wykes, 1952; Percival, 1961; Baker and Baker, 1983, 1986; Feinsinger, 1983; Herrera, 1985; Elisens and Freeman, 1988; Petanidou *et al.*, 2000; De la Barrera and Nobel, 2004; Galetto and Bernardello, 2004; Nicolson and Thornburg, 2007). Despite the huge number of publications

on nectar properties appearing in the last few decades, there is still one peculiar feature of floral nectar that remains largely unexplored to date from an ecological perspective, namely its role as a natural habitat for many kinds of micro-organisms and, more specifically, yeasts.

That yeasts are frequent inhabitants of floral nectar was already familiar to microbiologists more than a century ago (Boutroux, 1884; Schuster and Úlehla, 1913; Grüss, 1917; Schoellhorn, 1919; Guilliermond, 1920; Hautmann, 1924; Jimbo, 1926; Nadson and Krassilnikov, 1927). More recently, pollination biologists have also been aware that a variety of micro-organisms, including yeasts, sometimes occur in floral nectar (Baker and Baker, 1975, 1983; Gilliam *et al.*, 1983; Kevan *et al.*, 1988; Eisikowitch *et al.*, 1990a, b; Kearns and Inouye, 1993; Ehlers and Olesen, 1997). Baker and Baker (1975, p. 104), for example, noted that ‘osmophilic yeasts and some bacteria can be found in some nectars that have been exposed for a period of time’, and Kevan *et al.* (1988, p. 26) stated that ‘the presence of yeasts in the nectar of flowers is well known’. This awareness also extended to the fact that nectar-inhabiting yeasts can modify nectar composition, as illustrated by Baker and Baker’s (1983, p. 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’.

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Despite this awareness, however, the primary literature on pollination biology contains few publications directly concerned with yeasts in floral nectar (Gilliam *et al.*, 1983; Kevan *et al.*, 1988; Eisikowitch *et al.*, 1990*a, b*; Lawton *et al.*, 1993), and these generally lack quantitative information on the frequency of occurrence (proportion of flowers whose nectar contains yeasts) or abundance (cell number per nectar volume unit) of yeasts in the floral nectar of wild plants. Quantitative microbiological studies on nectar yeast populations have also been scarce in recent decades (but see Sandhu and Waraich, 1985; Brysch-Herzberg, 2004). About a century after microbiologists first emphasized their interest in conducting quantitative surveys of yeasts in floral nectar, as summarized in Schoellhorn's (1919) statement at the beginning of this paper, and reported the results of a few such surveys (Schuster and Úlehla, 1913; Jimbo, 1926), little is still known about the abundance and distribution patterns across habitats and plant species of nectar yeasts in the wild. This gap in our knowledge is shown by the absence of these topics in recent comprehensive reviews addressing nectar and yeast ecology (Spencer and Spencer, 1997; Rosa and Péter, 2006; Nicolson *et al.*, 2007).

Investigations on a few bumble-bee-pollinated plants from southeastern Spain have recently revealed that (a) yeasts are quite frequent and can reach extraordinarily high densities in the floral nectar of some species; (b) yeast populations degrade floral nectar by reducing the sugar concentration and drastically altering the sugar composition profile (proportions of sucrose, glucose and fructose), two effects that can imply a deterioration of the nectar's value from the viewpoint of pollinators; and (c) very small-scale patchiness in nectar yeast densities generates small-scale, intraspecific patchiness in nectar characteristics that can influence pollinator behaviour (Canto *et al.*, 2007, 2008; Herrera *et al.*, 2008). These findings suggest that nectar yeasts can be playing unrecognized roles in plant–pollinator interactions, yet an assessment of the generality of the results (a–c) above is needed. As an indispensable first step, and given the scarcity of quantitative data on the frequency and abundance of nectar yeasts in the wild noted above, here we present the results of a wide-ranging quantitative survey of the occurrence of yeast in floral nectar of insect-pollinated plants that attempts to test the generality of result (a) above. The main objective of this study was to determine, by means of microscopic examination of nectar samples, the frequency of occurrence (proportion of flowers whose nectar contained yeasts) and abundance (cell density in nectar) of yeast cells in the floral nectar of as many species as possible

from three contrasting plant communities on two continents. A secondary objective was to ascertain whether interspecific variation in yeast prevalence is related to differences in pollinator composition. This question was prompted by the recent experimental finding of Canto *et al.* (2008) that species of pollinators can differ drastically in their potential to induce nectar degradation of probed flowers through yeast inoculation.

MATERIALS AND METHODS

Study areas

This study was conducted at three widely separated areas, two located in the southern Iberian Peninsula (Spain) about 350 km apart, and one in northwestern Yucatán Peninsula, eastern Mexico. The areas differ greatly in biogeographical affinities and ecological characteristics, including type of vegetation, climate and elevation (Table 1). Lowland pine forests and mixed pine–oak montane woodlands were sampled in Spain (referred to hereafter as the 'Doñana' and 'Cazorla' areas, respectively). Coastal dune scrublands and neighbouring seasonally dry, deciduous tropical forest and thorny scrub were sampled in Mexico (collectively named 'Yucatán' hereafter).

Species samples

Floral nectar samples from 40, 63 and 37 species, belonging to 21, 23 and 21 families, were examined microscopically for yeast cells at the Doñana, Cazorla and Yucatán areas, respectively. In total, the survey included 44 families and 130 species (ten species were surveyed at both Cazorla and Doñana). The taxonomic distribution among families of species sampled is shown in the Appendix, and a complete list of the species surveyed is available online as Supplementary Data. Lamiaceae (14.6% of species, the three areas combined), Boraginaceae (9.2%), Fabaceae (6.9%), Caryophyllaceae (5.4%) and Convolvulaceae (5.4%) were the five families contributing most species to our sample.

Methods

Flowering branches, inflorescences or single flowers of as many nectar-producing species as possible were collected at each of the three study areas during March–August 2008, a period encompassing the peak flowering season at all sites. The only criteria used for including a species in the survey

TABLE 1. Summary of ecological characteristics of the three study areas

	Doñana, south-western Spain	Cazorla, south-eastern Spain	Yucatán, eastern Mexico
Habitat type	<i>Pinus pinea</i> woodlands with an understorey of sclerophyllous, evergreen scrubs	<i>Pinus nigra</i> – <i>Quercus ilex</i> mixed woodland with sparse understorey of deciduous treelets and shrubs	Xerophyte-dominated coastal scrubland and tropical deciduous forest
Climate	Hot Mediterranean	Cool Mediterranean	Seasonally dry tropical
Elevation	0–120 m	750–1600 m	3–10 m
Predominant soil type	Nutrient-poor acid sandy soils	Limestone-derived lithosols and clays	Sandy nutrient-poor soils (coastal strip) and limestone-derived soils (forest)
Mean annual rainfall	560 mm	790 mm	370 mm (coastal strip) to 1077 mm (forest)
Mean annual temperature	16 °C	13 °C	26 °C

were availability (at least 6–10 flowering, widely spaced individuals should be locally available, each bearing a minimum of 5–10 flowers) and that individual flowers lasted for >1 d and produced measurable amounts of nectar (>0.15 μL) within 12 h of collection.

Collected branches, inflorescences or flowers were carefully placed in glass jars in a portable cooler until taken indoors, and then kept at ambient temperature until extraction and microscopic examination of nectar samples, which was generally done within 12 h of collection. For every species included in the survey, separate nectar samples were obtained from individual flowers (mean \pm s.d. = 2.4 ± 0.9 flowers per plant) from different individual plants (mean = 7.9 ± 2.8 plants per species) using calibrated microcapillaries. Between 11 and 44 nectar samples (mean = 19.5 ± 5.2 samples per species) were microscopically examined per species ($n = 2733$ samples in total, all species and areas combined). Particular care was taken to examine only nectar samples from flowers that were already open, and thus had been exposed to pollinator visitation, at the time of collection in the field. The mean duration of flowers of the species included in the survey mostly falls between 3 and 5 d (range = 2–20 d), hence the <12 h period elapsing between flower collection and microscopic examination was shorter than the interval of time nectar is naturally available in the field for yeast multiplication within single flowers. In addition, some of the highest yeast cell densities were found in species with very long-lived flowers, lasting for >2 weeks (Herrera *et al.*, 2008). These observations make us confident that the high cell densities found in this study are biologically realistic figures, rather than artefacts arising from yeasts being allowed more time for multiplication than they ordinarily have in the field.

The volume of nectar extracted from each flower (usually <1 μL) was calculated by the length of the column within a calibrated micropipette, and then diluted up to 5–8 μL by addition of 25–60% (depending on the observer's preferences) lactophenol cotton blue solution to facilitate microscopic examination. Yeast cell density (cells mm^{-3} of nectar) was then estimated directly for each nectar sample under a microscope at $\times 400$ using a Neubauer chamber and standard cell counting methods. A rigorous identification of the micro-organisms present in all nectar samples would have required culturing and isolation (e.g. Brysch-Herzberg, 2004), which was impractical given the large number of yeast-containing samples examined in this study. Micro-organisms present in nectar samples were identified as yeasts from consideration of size, arrangement and diagnostic morphological features of cells, such as the presence of budding cells and large vacuoles containing highly refractive corpuscles. This coarse level of taxonomic resolution was sufficient for the purposes of this study, and we are confident that microbes being reported on in this study are yeasts in all cases. This was corroborated for many of the yeast-containing nectar samples examined from Cazorla. Drops from a total of 170 nectar samples from 22 different plant species were streaked onto YM + chloramphenicol agar plates, isolates were obtained from the resulting colonies, and the D1/D2 domain of the large sub-unit rDNA was partially sequenced following methods in Kurtzman and Robnett (1998) and Lachance *et al.* (1999). The identity of species involved was

obtained by BLAST-querying the GenBank database with the sequences obtained. These identifications revealed the presence of species of *Metschnikowia*, *Candida*, *Cryptococcus*, *Rhodotorula*, *Sporobolomyces* and *Aureobasidium* in the floral nectar samples from Cazorla examined microscopically (M. I. Pozo *et al.*, unpubl. res.), and will be reported elsewhere as part of other studies.

The possible correlations of pollinator type and yeast incidence in floral nectar were explored in a sub-set of the species sampled. Detailed information on the composition of the pollinator assemblages of 22 species from Cazorla was obtained from a large unpublished database of plant–pollinator interactions in the region (C. M. Herrera, unpubl. res.). This database contains pollinator composition data obtained during 2003–2008 by directly carrying out a census of pollinator visitation following the methods described by Herrera *et al.* (2001) and Herrera (2005). Pollinator censuses were conducted in the same general area from which flowers were sampled for this study. For the purpose of the analyses presented here, pollinator composition was assessed for every species by using the proportions of flower visits contributed by each of the following five major pollinator categories: bumble-bees (*Bombus*), solitary bees (mostly species of Halictidae, *Andrena* and *Anthophora*), Lepidoptera, Coleoptera and Diptera. Relationships between yeast incidence in nectar and pollinator composition were explored by correlating the frequency of occurrence and density estimates of yeast cells in nectar, on the one hand, with the proportion of flower visits accounted for by each major pollinator category on the other. To account for the possible influence of phylogenetic correlations present in the data, analyses of phylogenetically independent contrasts were conducted in addition to those based on directly correlating the actual values for each species [PIC (phylogenetically independent contrast) and TIP analyses, respectively; e.g. Garland *et al.*, 1992; Ricklefs and Starck, 1996]. For the PIC analyses a phylogeny of the 22 species involved was constructed using the Phylomatic web tool available at <http://www.phylodiversity.net/phyloomatic/phyloomatic.html> (last accessed 9 September 2008). Estimates of family divergence times were used as branch lengths in the phylogenetic tree, and were obtained by running the BLADJ application with plant family ages from Wikström *et al.* (2001). Computations involving PICs and significance tests were done with the PDAP:PDTree module of Mesquite (Maddison and Maddison, 2008; Midford *et al.*, 2008).

RESULTS

Frequency of occurrence

Yeasts occurred very frequently in floral nectar at all areas, as revealed by the high proportion of nectar samples that contained them: 31.8% (Doñana, $n = 740$ flowers), 42.3% (Cazorla, $n = 1318$ flowers) and 54.4% (Yucatán, $n = 675$ flowers). Differences among areas in yeast incidence were statistically significant ($\chi^2 = 73.9$, d.f. = 2, $P < 0.0001$), and denote a trend towards highest incidence in the tropical dry forest, lowest in the sclerophyllous Mediterranean scrubland and intermediate at the montane Mediterranean forest.

When plant species rather than individual nectar samples are treated as the units for analyses, mean (\pm s.e.; this notation will

be used hereafter) per-species incidence of yeasts was lowest in Doñana (29.3 ± 5.4 % of samples, $n = 40$ species), intermediate in Cazorla (40.8 ± 4.4 %, $n = 63$ species) and highest in Yucatán (54.3 ± 5.1 %, $n = 40$ species), and regional differences were statistically significant ($\chi^2 = 10.9$, d.f. = 2, $P = 0.004$, Kruskal–Wallis analysis of variance). There was, however, extensive interspecific variation in yeast incidence within all areas. The proportion of nectar samples containing yeasts encompassed the whole 0–100 % range at all sites, and at each area there was a continuum in yeast incidence running from species where yeasts were never recorded (15, 15 and two species in Doñana, Cazorla and Yucatán, respectively) to species where all nectar samples examined contained some yeasts (one, three and five species, respectively; Fig. 1).

Cell density

Yeast cells reached extraordinarily high densities in some nectar samples, as denoted by the highest concentrations

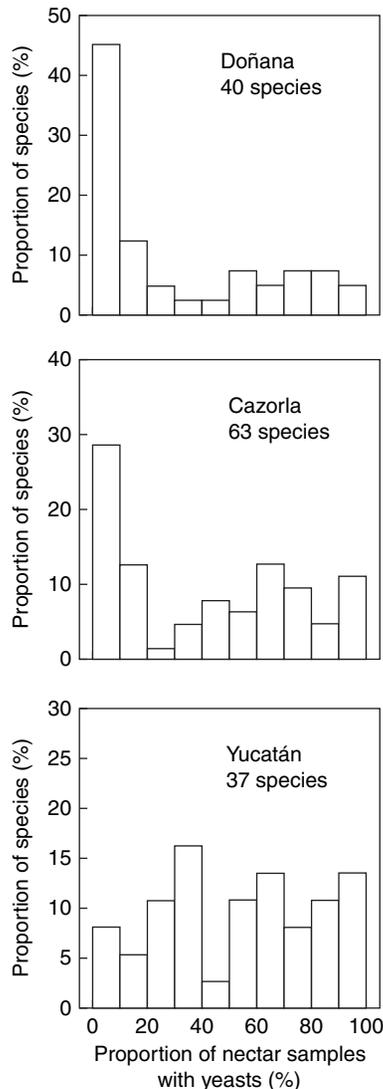


FIG. 1. Frequency distributions of the proportion of nectar samples from a given plant species that contained yeasts, for each of the three areas studied.

recorded at the three areas: 394 800, 370 895 and 412 036 cells mm^{-3} in Doñana, Cazorla and Yucatán, respectively. The similarity of these figures at the three areas suggests that densities of approx. 4×10^5 cells mm^{-3} are probably near an absolute ceiling for yeast cell density in floral nectar under natural conditions. Similarity among areas also extended to the range of variation of yeast cell densities in individual nectar samples. Considering only samples with yeasts, the interquartile ranges of cell density in individual samples were 1980–21 120 cells mm^{-3} in Doñana ($n = 235$ samples), 512–8801 cells mm^{-3} in Cazorla ($n = 558$ samples) and 175–6304 cells mm^{-3} in Yucatán ($n = 367$ samples).

There was broad interspecific variation within each area in mean yeast cell densities for individual plant species. Nearly all of this variation occurred among species within areas, and very little among areas (Fig. 2). The range of variation of species means spanned nearly six orders of magnitude within every area, and there were several species at every site with average yeast cell densities close to 10^5 cells mm^{-3} . In contrast, differences among areas in the average value of species means were negligible (filled circles in Fig. 2).

Pollinator composition and yeast incidence

A summary of data for the 22 Cazorla species with information simultaneously available on yeast incidence and pollinator composition is presented in Table 2. The broad ranges of variation in frequency of occurrence (0–91 %) and mean density (0–21 114 cells mm^{-3}) of yeast cells in this sub-set of species are representative of the whole sample of species surveyed in this study (Figs 1 and 2). A broad spectrum of pollination modes is also represented in this sub-sample, as denoted by interspecific differences in the proportion of flower visits contributed by bumble-bees (range = 0–97 %), solitary bees (0–100 %), Lepidoptera (0–90 %), Coleoptera (0–26 %) and Diptera (0–86 %).

Interspecific variation in yeast incidence was significantly related to quantitative differences in pollinator composition. The TIP analyses revealed significant positive correlations across species between percentage flower visits contributed by

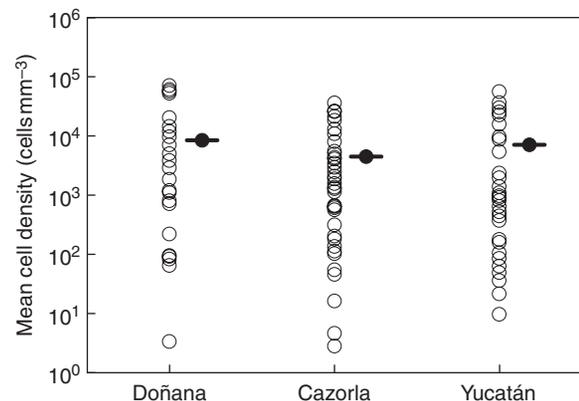


FIG. 2. Mean yeast cell density in floral nectar samples at the three study areas. Each symbol (open circles) corresponds to a different species (see Fig. 1 for the number of species per area). Filled circles denote the average of species means for each site. Samples with and without yeasts were included in the computations of means.

TABLE 2. Yeast incidence and pollinator composition for the 22 Cazorla species included in the analysis of the relationship between pollination mode and presence of yeasts

Species	Yeast incidence		Pollinator composition (% flower visits)				
	Frequency of occurrence* (% samples with yeasts)	Mean cell density† (cells mm ⁻³)	Bumble-bees	Solitary bees	Lepidoptera	Coleoptera	Diptera
<i>Anthericum liliago</i>	0.0	0	19.3	40.9	12.2	26.4	1.2
<i>Anthyllis vulneraria</i>	43.5	1141	0.0	100.0	0.0	0.0	0.0
<i>Aquilegia cazorlensis</i>	62.9	11127	77.0	4.6	18.4	0.0	0.0
<i>Aquilegia vulgaris</i>	60.0	4356	89.7	10.3	0.0	0.0	0.0
<i>Asphodelus albus</i>	2.7	0.1	51.3	44.3	1.9	0.3	2.2
<i>Cleonia lusitanica</i>	0.0	0	4.5	75.7	13.4	0.0	6.4
<i>Digitalis obscura</i>	90.9	3402	94.0	6.0	0.0	0.0	0.0
<i>Echium flavum</i>	5.0	5	43.8	47.0	9.2	0.0	0.0
<i>Erinacea anthyllis</i>	40.0	2948	33.5	59.3	7.2	0.0	0.0
<i>Gladiolus illyricus</i>	69.7	5048	0.0	65.0	33.8	0.0	1.2
<i>Helleborus foetidus</i>	90.0	21114	91.7	8.3	0.0	0.0	0.0
<i>Lavandula latifolia</i>	13.0	186	30.0	31.3	17.5	0.0	21.3
<i>Linaria aeruginea</i>	20.0	4062	0.0	76.9	0.0	23.1	0.0
<i>Lonicera etrusca</i>	13.2	316	16.1	2.6	81.4	0.0	0.0
<i>Marrubium supinum</i>	88.9	584	75.9	16.8	6.6	0.0	0.8
<i>Phlomis lychnitis</i>	45.0	137	96.9	3.1	0.0	0.0	0.0
<i>Rosmarinus officinalis</i>	61.1	1291	35.9	63.7	0.0	0.0	0.4
<i>Silene lasiostyla</i>	10.0	3	0.0	3.5	10.0	0.0	86.5
<i>Thymus mastichina</i>	0.0	0	38.9	44.8	12.6	1.1	2.6
<i>Thymus orospedanus</i>	0.0	0	11.9	42.0	8.6	0.0	37.5
<i>Vicia villosa</i>	52.6	2225	46.0	43.9	9.8	0.0	0.3
<i>Viola cazorlensis</i>	0.0	0	0.0	0.0	89.7	0.0	10.3

* Proportion of nectar samples containing yeasts.

† Species mean of yeast cell density (cells mm⁻³) per individual nectar sample.

TABLE 3. Product–moment correlation coefficients between yeast incidence in floral nectar, as measured by frequency of occurrence and mean cell density in samples, and the proportion of flower visits contributed by each major pollinator group in a sub-sample of 22 species from the Cazorla area

Yeast incidence	Pollinators	TIP analysis		PIC analysis	
		<i>r</i>	<i>P</i> -value	<i>r</i>	<i>P</i> -value
Frequency of occurrence*	Bumble-bees	0.631	0.0017	0.709	0.0002
	Solitary bees	−0.194	0.39	−0.608	0.003
	Lepidoptera	−0.313	0.16	−0.178	0.43
	Coleoptera	−0.261	0.24	−0.228	0.31
	Diptera	−0.339	0.12	−0.219	0.33
Cell density†	Bumble-bees	0.600	0.003	0.675	0.0006
	Solitary bees	−0.169	0.45	−0.570	0.006
	Lepidoptera	−0.286	0.19	−0.153	0.50
	Coleoptera	−0.246	0.26	−0.233	0.30
	Diptera	−0.360	0.10	−0.243	0.28

Significant correlations are shown in bold.

* Proportion of samples of a given species containing yeasts.

† Species mean of log₁₀-transformed cell density estimates (cells mm⁻³) per sample.

bumble-bees on one hand, and both the frequency of occurrence and mean density of yeast cells in nectar samples (Table 3). The PIC analyses corroborated this direct relationship between yeast incidence and the proportional importance of bumble-bees as pollinators, and also revealed significant negative correlations between yeast incidence and the proportion of flower visits contributed by solitary bees (Table 3). Neither the TIP nor the PIC analyses revealed any significant correlation between yeast

incidence and the proportional importance of Lepidoptera, Coleoptera or Diptera as pollinators.

DISCUSSION

The main results of this study are that, irrespective of continent or habitat type, yeasts occur regularly in the floral nectar of many species, and that they frequently reach extraordinarily

high densities. Since the presence of yeasts in nectar has been long known to microbiologists and pollination biologists alike, as noted in the Introduction (see references there), the first part of our results serves to corroborate quantitatively the observations reported by previous studies and to extend them to a broader geographical context. Our results for yeast cell densities, in contrast, seem to be the first comprehensive estimates to date of yeast cell densities in nectar of wild plants, and reveal that microbial communities capable of reaching such high densities most probably are more consequential than hitherto recognized and deserve more attention from pollination biologists than they have received so far, as discussed below.

The high frequencies of occurrence of yeasts in floral nectar found in this investigation are similar to those reported in some previous quantitative microbiological studies on yeast incidence in nectar samples. Jimbo (1926) assessed the frequency of occurrence of yeasts in floral nectar of 23 species from 14 families at several Japanese sites. For all species combined, the frequency of occurrence of yeasts in nectar samples (44 %, $n = 273$ flowers) was close to the 42.3 % found in this study for Cazorla. For individual species, frequencies of occurrence in Jimbo's study ranged between 0 and 100 %, and the mean (47.0 %) was close to the figures found in this study for Cazorla (40.5 %) and Yucatán (54.3 %). For nine cultivated species in northwestern India, Sandhu and Waraich (1985) reported frequencies of occurrence of yeasts in floral nectar ranging between 39.5 and 92.1 % (mean = 67.8 %). In a central European location, Brysch-Herzberg (2004) found that 72 % of nectar samples from *Helleborus foetidus* contained yeasts. This species was included in our survey for Cazorla, where 90 % of nectar samples contained yeasts (Table 2; see also Herrera *et al.*, 2008). These quantitative estimates, along with qualitative or anecdotal reports of yeast presence in floral nectar worldwide (Schuster and Úlehla, 1913; Schoellhorn, 1919; Nadson and Krassilnikov, 1927; Eisikowitch *et al.*, 1990a; Lachance *et al.*, 2001; Brysch-Herzberg, 2004; Mushtaq *et al.*, 2006, 2007), clearly support the conclusion that yeasts are regular inhabitants of the floral nectar of many animal-pollinated plants irrespective of continent or habitat type.

Our survey has revealed that yeast cell densities in the order of 10^3 – 10^4 cells mm^{-3} are commonplace in nectar samples, and that densities $>10^5$ cells mm^{-3} are not unusual (see also Herrera *et al.*, 2008). In the only other study known to us directly assessing yeast cell abundance in nectar by counting under the microscope, Brysch-Herzberg (2004) found densities of up to 16 000 cells mm^{-3} in floral nectar of *Digitalis purpurea*, a value roughly equivalent to the maximum density found in Cazorla for the congeneric *Digitalis obscura* (32 100 cells mm^{-3}). Further comparative data are not available, since systematic cell counts of nectar yeasts under the microscope rarely have been undertaken despite the simplicity of the method. Assessments of yeast cell abundance based on counting colonies in culture media can only be used for comparative purposes, as they underestimate cell density and are poorly correlated with direct cell counts under the microscope (Brysch-Herzberg, 2004; A. Canto, unpubl. res.). Our survey was sufficiently encompassing, however, to support the conclusion that floral nectars with very dense yeast populations are probably the rule, rather than the exception, in a non-trivial proportion of the animal-pollinated plants at any habitat type. If

species with mean yeast cell densities $>10^4$ cells mm^{-3} are arbitrarily designated as 'heavily yeast-loaded', then our species sets from Doñana, Cazorla and Yucatán contain eight (20 % of total), ten (16 %) and seven (19 %) of such species, respectively (Fig. 2). Heavily yeast-loaded species are taxonomically quite diverse, belonging to a number of phylogenetically disparate families such as Amaryllidaceae (*Pancratium maritimum*), Bignoniaceae (*Tecoma stans*), Bromeliaceae (*Tillandsia dasyliirifolia*), Boraginaceae (*Anchusa calcarea*), Iridaceae (*Iris xiphium*), Malvaceae (*Malvaviscus arboreus*), Primulaceae (*Primula vulgaris*) or Ranunculaceae (*Helleborus foetidus*), which are widely scattered over the angiosperm phylogenetic tree (Stevens, 2008).

For three of the species included in our survey for Cazorla (*H. foetidus*, *Aquilegia vulgaris* and *A. cazorlensis*), Herrera *et al.* (2008) showed that yeast populations alter important characteristics of nectar, including total sugar concentration, relative proportions of constituent sugars (sucrose, glucose and fructose) and the sucrose:hexose ratio. The magnitude of nectar degradation was directly related to yeast cell densities, with densities $>10^3$ cells mm^{-3} such as those commonly found in the present survey being associated with extensive nectar degradation, sometimes entailing the virtual disappearance of sugars. Given the astounding metabolic differences existing among yeast species (Barnett *et al.*, 2000), the nature and magnitude of the effects of yeast populations on nectar chemical features will be contingent on the species composition of yeast communities, which can vary considerably among plant species (e.g. Mushtaq *et al.*, 2006, 2007; M. I. Pozo *et al.*, unpubl. res.). Keeping this important caveat in mind, and on the basis of the high cell densities found in the present survey, we suggest that extensive degradation induced by yeasts similar to that reported by Herrera *et al.* (2008) is expected to occur regularly in the floral nectar of many species in the field. This would mean that an undetermined number of published chemical analyses of floral nectar based on field-collected samples from flowers that had been previously exposed to pollinators, and thus susceptible to yeast colonization and growth, could reflect the consequences of the yeasts' metabolic activity as much or more than the intrinsic properties of plants, as traditionally implied. Consistent with this suggestion is the frequent observation of very unequal proportions of glucose and fructose in published reports of nectar composition (e.g. Baker *et al.*, 1998; Galetto and Bernardello, 2003), which could denote a sort of 'chemical signature' of yeast metabolism rather than an inherent feature of the plants themselves (Canto *et al.*, 2008; Herrera *et al.*, 2008).

Broad interspecific differences in yeast frequency and abundance were found at the three areas surveyed. Brysch-Herzberg (2004) found significant differences among plant families in yeast incidence. He was unable to find any clear relationship linking such variation with nectar properties such as pH, sugar concentration and the sucrose:hexose ratio, and concluded that nectar chemical and physical properties most probably do not have a significant impact on the abundance of nectar yeasts. Information on nectar characteristics is not available for the species included in our survey, thus the role of variation in species- or family-specific nectar attributes in determining the size of yeast populations cannot be explored

for our data set. The results of this study show instead that interspecific variation in nectar yeast incidence is correlated with differences in pollinator composition, a finding that provides an interesting and unexplored connection between pollination ecology and floral nectar microbiology. In the 22 species from Cazorla with quantitative information on pollinator composition, yeast frequency and abundance were significantly related to differences in the relative importance of solitary bees vs. bumble-bees in the pollinator assemblage. Across species, yeast incidence increased significantly with increasing proportions of floral visits by bumble-bees and decreasing proportions of visits by solitary bees. Although the results of the TIP and PIC analyses were qualitatively similar, the inverse relationship between yeast incidence and proportion of solitary bees only became apparent in the PIC analysis, which points to some underlying phylogenetic correlation(s) in our data worthy of further study.

The significant relationships found in Cazorla between yeast incidence and relative importance of bumble-bees and solitary bees as pollinators are consistent with the results of an experimental study by Canto *et al.* (2008) on the main pollinators of *Helleborus foetidus* in the same region. Their experiments involved assaying the capacity of the main bee pollinators of *H. foetidus* to modify the sugar composition of natural and artificial nectar through mimicking single-nectary visits by wild-caught individuals. The bee taxa assayed differed widely in the subsequent effects of experimentally probing nectar with their mouthparts. Nectar probing by *Andrena*, *Anthophora* and *Lasioglossum* had no subsequent effects on nectar sugar composition, while probing by *Bombus terrestris* and *B. pratorum* induced extensive reduction in the percentage of sucrose and a marked increase in the percentage of fructose as a consequence of nectar contamination by yeasts. These two groups of bees correspond to two pollinator categories recognized in this study, and their differential effects on nectar in the experiments of Canto *et al.* (2008) match the sign of their respective correlations with yeast incidence found here. In the particular context of the Cazorla region, the correlation found here between incidence of yeasts and pollination by bumble-bees might therefore have a causal basis, with bumble-bee pollinators being much more frequent yeast vectors to nectar than solitary bees. The regular association of bumble-bees with the yeasts *Metschnikowia reukaufii* and *M. gruessii* (Brysch-Herzberg, 2004), two of the most abundant species in nectar in Cazorla (M. I. Pozo *et al.*, unpubl. res.), is also consistent with a causal relationship between the proportion of bumble-bee visits to flowers and yeast incidence. Other plausible, mutually non-exclusive hypotheses may, however, be envisaged that could contribute to explain the observed association between yeasts and bumble-bee pollination. For example, such an association could arise as a consequence of bumble-bees preferentially foraging on plants characterized by their dense nectar yeast populations, or yeasts and bees just preferring flowers with similar nectar characteristics. Further studies are clearly needed before firm conclusions can be drawn.

CONCLUDING REMARKS

Understanding the roles of ‘hidden players’, particularly microbial communities, in the assembly and functioning of

ecological communities has been considered a key element for research agendas at the ‘frontiers of ecology’ (Thompson *et al.*, 2001). Although studies on the influence of microbial communities on the interaction between plants and their animal pollinators are still in its infancy, there is already some evidence that micro-organisms can act as important hidden players in these ecological interactions, and influence them in previously unsuspected ways (e.g. Gange and Smith, 2005; Wolfe *et al.*, 2005; Cahill *et al.*, 2008). The results of the present study, along with those of other recent investigations (Raguso, 2004; Goodrich *et al.*, 2006; Canto *et al.*, 2008; Herrera *et al.*, 2008), allow us to predict that incorporating nectar yeasts into the scenario of plant–pollinator interactions will open up a number of novel avenues for research in the field. These include, for example, elucidating whether poisonous substances often present in nectar have been evolutionarily targeted as defences against generalized yeasts, and thus conform to the antimicrobial hypothesis of secondary compounds in nectar (Lawton *et al.*, 1993; Adler, 2000); investigating the effects of nectar modifications induced by yeasts (reduction in sugar concentration, alteration of sugar profiles, addition of yeast catabolites, emission of volatiles) on pollinator foraging and ultimately on pollination success, pollen flow and plant fitness; studying the relationships between spatial variation in yeast abundance and intraspecific patchiness in nectar characteristics at the within- and among-plant levels that could affect pollinator foraging and pollen flow (Herrera *et al.*, 2006; Canto *et al.*, 2007); and searching for predictable associations at the plant community level between pollinator composition of individual plant species and the frequency, abundance and/or species composition of its nectar yeast communities, as well as unravelling the proximate biological mechanisms underlying such associations. On a less positive note, however, nectar yeasts’ entry into the plant–pollinator scenario also raises some concerns in relation to the implicit assumption traditionally underlying studies on nectar chemical features, namely that measured nectar features mostly or exclusively reflect inherent plant properties (but see Willmer, 1980; Gottsberger *et al.*, 1984, 1989). With yeasts being as frequent and abundant in floral nectars as revealed by this and other studies, and given their anabolic and catabolic versatility, future studies focusing on nectar chemical features as mediating factors in plant–pollinator interactions should carefully control for the presence of yeasts in nectar samples.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxford-journals.org/ and give a list of the 130 plant species included in the survey of yeasts in floral nectar.

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(available online) for the complete list of species included in the survey.

Family	Study area			Total species
	Doñana	Cazorla	Yucatán	
Acanthaceae	0	0	4	4
Agavaceae	0	1	1	2
Aizoaceae	0	0	1	1
Alliaceae	1	2	0	3
Amaryllidaceae	2	0	1	3
Apocynaceae	0	0	3	3
Asclepiadaceae	0	0	4	4
Asphodelaceae	1	1	0	2
Asteraceae	0	2	0	2
Bignoniaceae	0	0	1	1
Boraginaceae	5	6	2	12*
Brassicaceae	1	1	0	2
Bromeliaceae	0	0	1	1
Campanulaceae	1	0	0	1
Caprifoliaceae	1	3	0	3*
Caryophyllaceae	4	4	0	7*
Convolvulaceae	0	1	6	7
Cucurbitaceae	0	0	1	1
Cytinaceae	1	0	0	1
Dipsacaceae	2	0	0	2
Euphorbiaceae	0	0	1	1
Fabaceae	2	6	2	9*
Hyacinthaceae	1	2	0	2*
Iridaceae	2	4	0	4*
Lamiaceae	8	12	1	19*
Lythraceae	1	1	0	1*
Malvaceae	0	0	2	2
Nyctaginaceae	0	0	1	1
Oleaceae	0	1	0	1
Orchidaceae	1	2	0	3
Orobanchaceae	2	1	0	3
Passifloraceae	0	0	1	1
Plantaginaceae	1	5	0	6
Plumbaginaceae	1	0	0	1
Polygalaceae	0	1	0	1
Primulaceae	0	2	0	2
Ranunculaceae	1	3	0	4
Rubiaceae	0	0	1	1
Ruscaceae	0	1	0	1
Solanaceae	0	0	1	1
Theophrastaceae	0	0	1	1
Thymelaeaceae	1	0	0	1
Verbenaceae	0	0	1	1
Violaceae	0	1	0	1
Total	40	63	37	130*

APPENDIX

Distribution among families of the 130 angiosperm species whose floral nectar was examined microscopically in this study for the presence of yeasts. Familial classification follows APG (2003). The total number of species for a family is sometimes smaller than the sum of species at all sites (marked with asterisks) because ten species were surveyed in both Doñana and Cazorla. See Supplementary Data