Oikos 121: 1878–1888, 2012
doi: 10.1111/j.1600-0706.2012.20295.x

Subject Editor: Anna Traveset. Accepted 12 January 2012

Relationships among nectar-dwelling yeasts, flowers and ants: patterns and incidence on nectar traits

Clara de Vega and Carlos M. Herrera

C. de Vega (cvega@ebd.csic.es) and C. M. Herrera, Estación Biológica de Doñana, Consejo Superior de Investigaciones Científicas (CSIC), Avenida de Américo Vespucio s/n, ES-41092 Sevilla, Spain.

Nectar-dwelling yeasts are emerging as widely distributed organisms playing a potentially significant and barely unexplored ecological role in plant–pollinator mutualisms. Previous efforts at understanding nectar–pollinator–yeast interactions have focused on bee-pollinated plants, while the importance of nectarivorous ants as vectors for yeast dispersal remains unexplored so far. Here we assess the abundance and composition of the nectar fungal microbiota of the ant-pollinated plant *Cytinus hypocistis*, study whether yeast transmission is coupled with ant visitation, and discern whether ant-transported yeasts promote changes in nectar characteristics. Our results show that a high percentage of flowers (77%) and plants (94%) contained yeasts, with yeast cell density in nectar reaching up to 6.2×10^4 cells mm^{-3}, being the highest densities associated with the presence of the nectar-specialist yeast *Metschnikowia reukaufii*. The establishment of fungal microbiota in nectar required flower visitation by ants, with 70% of yeast species transported by them being also detected in nectar. Ant-vectored yeasts diminished the nutritional quality of nectar, with flowers exposed to pollinators and yeasts containing significantly lower nectar sugar concentration than virgin flowers (13.4% and 22.8%, respectively). Nectar of flowers that harbored *M. reukaufii* showed the lowest quality, with nectar concentration declining significantly with increasing yeast density. Additionally, yeasts modified patterns of interpopulation variation in nectar traits, homogenizing differences between populations in some nectar attributes. We show for the first time that the outcome of the tripartite pollinator–flower–yeast interaction is highly dependent on the identity and inherent properties of the participants, even to the extent of influencing the species composition of this ternary system, and can be mediated by ecological characteristics of plant populations. Through their influence on plant functional traits, yeasts have the potential to alter nectar consumption, pollinator foraging behavior and ultimately plant reproduction.

Plant–pollinator mutualism has historically been considered a binary system in which every partner benefits from the relationship: pollinators transport pollen and plants provide in return nutritional floral rewards. However, such representation is too simplistic as it ignores the influence of others organisms that may shape pollination systems, and leads to an oversimplification of the complex multitrophic interactions occurring in nature (Armbruster 1997, Bronstein 2001, Herrera et al. 2002). It is now well established that pollination services may be influenced by the direct and indirect effects of macroorganisms (e.g. nectar robbers, herbivores) and microorganisms (e.g. mycorrhizal fungi) on foraging patterns of pollinators, while exerting selective pressures on plant and floral traits over evolutionary time (Brody 1997, Irwin and Brody 1998, Strauss et al. 1999, Gange and Smith 2005, Cahill et al. 2008).

In the last few years it has been suggested that pollination services may be additionally affected by a group of microorganisms largely ignored by ecologists, the nectar-dwelling yeasts. Recent studies have demonstrated that nectar-dwelling yeasts, which are transported by floral visitors, consume nectar actively and can modify nectar characteristics by altering sugar profiles and diminishing sugar and amino acid concentration (Herrera et al. 2008, de Vega et al. 2009a, Peay et al. 2012). This is of a pivotal importance, since nectar chemistry affects the identity and foraging choices of pollinators (Baker and Baker 1983, Alm et al. 1990, Klinkhamer and de Jong 1990, Romeis and Wäckers 2000). Furthermore, yeasts may have potential positive or negative effects on plant reproduction and pollinator services in other ways. Many yeasts produce ethanol as the main fermentation product (Lin and Tanaka 2006), and the alcohol present in nectar could cause intoxication and alter pollinator behaviour (Ehlers and Olesen 1997, Wiens et al. 2008). Yeasts may inhibit pollen germination (Eisikowitch et al. 1990), but also their metabolic activity increase intrafloral temperature, which might be a metabolic reward offered by winter flowering plants (Norgate et al. 2010), ultimately benefiting plant reproduction via enhanced pollinator visitation (Herrera and Pozo 2010). This recent evidence has led nectar to be rediscovered as a habitat from a micro-ecological perspective, and a key element for structuring diverse and complex trophic webs where organisms from three kingdoms (Plantae, Animalia

and Fungi) interact in a highly dynamic way. Plant–pollinator–yeast system radically differs from other plant–pollinator–third organism systems in that in the first, pollinators are the causal agent for the appearance of the yeasts, while in other systems pollinators and third organisms appear independently.

The scarce information available to date concerning nectar–pollinator–yeast relationships has focused on bees and bee-pollinated plants (Kevan et al. 1988, Brysch-Herzberg 2004, Herrera et al. 2010, Pozo et al. 2011), and more recently on bird-pollinated plants (Belisle et al. 2012). No study has so far examined the possible relationships linking ants, ant-visited plants and their associated fungal microbes despite ants frequently feed on floral nectar in many plant communities including tropical forests (Haber et al. 1981, Rico-Gray et al. 1998), Mediterranean habitats (Herrera et al. 1984, Retana et al. 1987, García et al. 1995, Gómez et al. 1996, de Vega et al. 2009b) and alpine ecosystems (Galen 1983, Puterbaugh 1998). It remains therefore unknown whether ants and ant-visited flowers harbor ecologically significant yeast biota, and whether these induce changes in nectar chemistry. If demonstrated, it will provide new ecological insights into the factors determining pollination, particularly if changes in nectar characteristics are large enough as to influence subsequent foraging by ants or other pollinating visitors. From the ant's perspective, nectar concentration is crucial for foraging decisions, solutions with higher sugar concentration being consistently preferred over lower ones (Josens et al. 1998, Blüthgen and Fiedler 2004a). If nectarivorous ants are dispersal agents for nectar-dwelling yeasts, and such microbes diminish nectar energetic value, this would support the existence of an unexplored ecological feedback loop in ant–plant systems, which could be consequential for pollination services.

Cytinus hypocistis (Cytinaceae) is an ideal plant for exploring the interactions between ants, flowers and nectar dwelling yeasts, and the outcome of these interactions in terms of nectar characteristics. Ants are by far the dominant pollinators and nectar consumers in *C. hypocistis*, with as many as ten different diurnal and nocturnal ant species visiting their flowers (de Vega et al. 2009b). Our general goals in this study are to assess whether ants are dispersal agents of yeasts to floral nectar in natural populations, and, if they are, whether ant-transported yeasts promote changes in nectar characteristics of visited flowers and if such changes depend on the species involved. This is the first study to link the effects of different yeast species to interpopulation variation in nectar quality as mediated through the activity of different pollinators and plant characteristics. Four questions are specifically addressed: 1) What is the frequency of occurrence and abundance of yeasts in nectar? 2) What is the species composition of the nectar microbial community and that carried by ant body surfaces? 3) Is yeast transmission coupled with ant visitation? 4) Does the metabolic activity of yeasts change nectar characteristics?

Material and methods

Study species

Cytinus hypocistis is a perennial parasitic plant with a vegetative body reduced to an endophytic system that parasitizes roots of Cistaceae species (de Vega et al. 2007). This parasitic plant shows remarkable specialization at the host level, and can be separated clearly into genetically distinct races that infest different host species (de Vega et al. 2008). Only in spring are the plants visible, with inflorescences bursting through the host root tissues. Inflorescences appear at ground level in clusters of 1–22 on the same host root, which can be either ramets of the same individual or genetically different individuals (de Vega 2007). Each inflorescence has around six basal female flowers and a similar number of distal male flowers. Flowers last for six days on average, and female and male flowers produce similar amounts of nectar, with a daily nectar production of ~1.5 µl (de Vega 2007). Plants exhibit high fruit set and seed production under natural conditions, with fruits containing thousands of dust-like seeds (de Vega et al. 2009b, 2011).

The flowers of *C. hypocistis* are mainly visited by ants, which are much more abundant than flying visitors and account for 97% of total floral visits (de Vega et al. 2009b). Exclusion experiments demonstrate that ants are the most important pollinators in *C. hypocistis*, yielding a fruit set close to 80% when other potential visitors are excluded (de Vega et al. 2009b). Among the most abundant daytime ant species visiting *Cytinus* flowers are *Pheidole pallidula* (35.3% of total floral visits), *Plagiolepis pygmaea* (19.4%), *Crematogaster auberti* (13.4%), *C. scutellaris* (2.7%) and *Aphaenogaster senilis* (1.5%) (for further details see de Vega et al. 2009b). Additionally, during nightime the ant *Camponotus pilicornis* frequently visits *Cytinus* flowers (0.3% of total visits). Flying visitors are scarce, and mainly forage in *C. hypocistis* inflorescences without ants, being the most abundant the fly *Oplisa aterrima* (Rhinophoridae; 1.3% of overall floral visits); occasionally female solitary bees (*Lasioglossum* sp., Halictidae) are observed (de Vega et al. 2009b). All ant species forage for nectar, *Lasioglossum* foraged for both pollen and nectar, and *O. aterrima* for nectar and secretions from tepal glandular trichomes. Ants spend a long time foraging at each flower, which increases the probability to contact the reproductive organs of *C. hypocistis*, promoting pollination, and potentially the transport yeast from flower to flower.

Study area

This study was carried out in six natural populations involving three genetic races of *C. hypocistis* parasitizing three Cistaceae host species: two populations parasitizing *Cistus ladanifer* (race Cl; populations Cl1 and ClN hereafter), two populations on *Cistus salviifolius* (race Cs; populations Cs1 and CsN) and two populations on *Halimium halimifolium* (race Hh; populations HhN and Hh2). We selected three *C. hypocistis* races on three host species because different ecological characteristic of their associated soils and plant communities could potentially lead to contrasting plant–ant–yeasts systems. *Cytinus hypocistis* race Cl occurred on clay soils, and vegetation consisted of mixed woodland of *Pinus pinea* and *Quercus suber*, where the understory was clear, with widely scattered herbs and shrubs. Populations of *C. hypocistis* races Cs and Hh occurred on sandy soils; the vegetation consisted for the first race of a dense *Q. suber* forest, and for the second of woodland of

P. pinea, and both supported a fairly rich understory flora. The studied populations were separated by 0.3–2.5 km. Study populations were located in the surroundings of Doñana National Park (Huelva province, southwestern Spain; 37°18′N, 6°25′W, 80–90 m a.s.l.). Climatic conditions were similar in the six populations.

Sampling design and laboratory methods

After two days of natural exposure to pollinators, randomly selected inflorescences were bagged in the field for 24 h to exclude pollinators and allow for nectar accumulation. We only selected inflorescences that had at least four female and four male flowers, and only one inflorescence per individual host plant was selected. Inflorescences were cut and carried in a cooler to the lab where nectar sampling was done within the few hours after collection. Nectar samples from two female and two male flowers per inflorescence were collected on different plants in each population (mean ± SE = 8.7 ± 2.3 inflorescences/population and 32.0 ± 8.1 flowers/population). The nectar from each individual flower was split into three subsamples, which were used for determining the size of yeast communities, characterizing the diversity and composition of yeast communities, and conducting nectar chemical analysis.

Yeast incidence and cell density was determined microscopically on aliquots of nectar samples, following methods previously described (Herrera et al. 2009). Briefly, an aliquot of nectar (mean 0.4 ± 0.03 µl; range 0.13–1.3 µl) was extracted with a sterile micropipette, its volume determined by the length of the column, and then diluted by adding a known volume of 15% lactophenol cotton blue solution to facilitate microscopical observations. Yeast concentration (cells mm^{-3}) was estimated under a light microscope using a Neubauer improved cell counting chamber. In total, 192 nectar samples from 52 inflorescences were examined microscopically.

The peculiarities of *Cytinus* inflorescences, which are formed underground and remain at ground level during anthesis, may facilitate direct soil-borne yeast contamination independent of insect visitation, a possibility which was tested by pollinator-exclusion experiments. Prior to opening, 10 floral buds (five female and five male) per population were protected to exclude pollinator visits. Floral buds were individually bagged with nylon mesh (200 µm mesh) netting to exclude ants but allow microorganisms access. A small plastic tube was inserted at the base of each flower, and used to fasten the bag to the flower. Due to the small number of flowers per inflorescence (ca six flowers of each sex), and given that bags could prevent ants from visiting flowers of the same inflorescence, nectar analyses of control and visited flowers were conducted on different plants. Nectar samples from these control flowers were examined microscopically as described above.

To obtain yeast isolates 0.5 µl of each nectar sample, was streaked with a sterile loop onto YM agar plates (2.0% agar, 1.0% glucose, 0.5% peptone, 0.3% malt extract, 0.3% yeast extract, 0.01% chloramphenicol, pH = 6.0), and incubated at room temperature. Yeasts were isolated only from nectar samples where their presence had been previously observed under the microscope. Some flowers did not harbour yeasts in nectar, and others secreted a small quantity of nectar and could not be used for yeast isolation and identification. In total we processed nectar samples from 92 flowers, and yeast colonies appeared in 88% of them (81 flowers from 42 plants). For each nectar sample, distinct yeast isolates were obtained from the colonies following standard methods described in Yarrow (1998). To assess the reliability of microscopic observations we used as control 10 female and 10 male flowers with 'clean nectar' (i.e. in which yeast were not detected under a microscope). No yeast colonies developed when nectar from these flowers was streaked on plates.

Ants were collected in *C. hypocistis* populations Cl1, Cs1, and Hh2. All ant species were not observed in all studied populations. During the study year *Pheidole pallidula* and *Aphaenogaster senilis* appeared in all populations. *Plagiolepis pygmaea* only appeared in Cl1, ClN, Cs1, CsN; *Crematogaster auberti* appeared in Cl1, Cs1, CsN, HhN, and Hh2; *Crematogaster scutellaris* in Cl1, Cs1, Hh2, and *Camponotus pilicornis* in Cl1, ClN, Cs1.

For isolation of yeast from ant body surfaces we selected six ant species previously described as pollinators of *C. hypocistis* flowers (de Vega et al. 2009b): *Aphaenogaster senilis* (n = 27 individuals), *Camponotus pilicornis* (n = 13), *Crematogaster auberti* (n = 36), *Crematogaster scutellaris* (n = 6), *Pheidole pallidula* (n = 35) and *Plagiolepis pygmaea* (n = 10). In total, 127 ants were collected in the field as they approached (but before entering) *C. hypocistis* flowers using sterile forceps and immediately placed in sterile vials. Ants were then aseptically transferred to YM agar plates and allowed to walk for one hour, after which they were removed and the plates incubated at room temperature. Individual colony forming units of each type were then transferred to new plates to obtain pure cultures.

For every yeast isolates obtained from nectar and ant samples, the divergent D1/D2 domains were amplified by PCR using the primers NL–1 and NL–4 (Kurtzman and Robnett 1998) and sequenced on an automatic sequencer. Forward and reverse sequences were assembled and edited using Sequencher 4.9 (Gene Codes, Ann Arbor, MI). See Supplementary material for further details on genetic analyses.

The third aliquot of nectar from each flower (mean ± SE 0.54 ± 0.03 µl; range 0.2–3 µl, n = 171) was extracted, its volume determined as explained above, and then blotted onto separate 10 × 2 mm sterile filter-paper wicks, which were allowed to dry and then individually stored in sterile envelopes. Wicks were stored in plastic bags containing silica gel and kept at room temperature until chemical analysis. This subset included all nectar samples previously studied, excluding only those where nectar production was insufficient to allow reliable quantification.

To compare nectar characteristics of visited flowers versus control flowers, nectar chemical analyses were additionally conducted on samples from bagged flowers. Nectar chemical analyses of control and visited flowers were conducted on different plants as explained above. Nectar samples from two female and two male flowers per plant (mean ± SE 1.62 ± 0.17 µl; range 0.22–8 µl, n = 109) were collected on different plants in each population. In total we analyzed nectar of 59 female and 50 male control

flowers in the six populations. Nectar sugar concentration of open and bagged flowers (n = 280) was analyzed by high performance liquid chromatography. Analytical procedures were as previously outlined (Canto et al. 2007). See Supplementary material for further details on nectar analyses.

Statistical analyses

Statistical analyses were conducted using the SAS statistical program (SAS Inst.). We evaluated the potential effect of race and sex on the probability of yeast incidence (with logit link function and binomial distribution) and on yeast density in nectar (with identity link function and lognormal distribution) by fitting generalized linear mixed models using the GLIMMIX procedure. Similar analyses were conducted to test for differences in nectar concentration between bagged and exposed flowers, and between plants containing different yeast species (with identity link function and gaussian distribution). Data on nectar concentration were square-root transformed. Races and sex were treated as fixed effect, and populations as random effects in the models. To avoid pseudoreplication due to the sampling of individual flowers from a given individual plant, the identity of each plant was included as a random effect in the model to capture the variance across experimental blocks which correct for intrinsic correlations and adjust the degrees of freedom accordingly (Hurlbert 1984).

Differences in the frequency of yeast genera between nectar and ant samples were examined using contingency tables and χ^2-tests. Correlations between cell density and total sugar concentration in nectar samples were estimated by Spearman's rank correlation test with the CORR procedure. All means and associated standard errors reported are model-corrected means calculated with the LSMEANS statement. Total sugar concentration is expressed as percentage w/v (g solute per 100 ml solution).

For diversity analyses, sequences were compared with the GenBank database using the Basic local alignment search tool (BLAST). Most sequences matched to known yeast species at very high levels of sequence identity (99–100%). Nine percent of the sequences exhibited lower similarity to their closest related species (pairwise identity $\leq$96%) and following Kurtzman and Robnett (1998) were tentatively considered to belong to undescribed taxa. Potential biases in richness estimates caused by the presence of undescribed species were evaluated by considering operational taxonomic units (OTUs) at a 0.03 cutoff, defined on the basis of nucleotide dissimilarity between sequences (Hughes et al. 2001, Fierer et al. 2007). Yeast isolates from nectar and ants were classed into OTUs to provide input for ecological comparisons using MOTHUR (Schloss et al. 2009). See Supplementary material for further details on OTUs analysis.

Although the initial sampling effort was comparable for flowers and ant samples (n = 192 and n = 127 respectively), the small quantity of nectar in some samples along with the absence of yeasts or the contamination by filamentous fungi from ant body surfaces in others, finally led to unbalanced sample sizes. Variable sample size may bias species richness and diversity comparisons, thus we standardized the species counts using rarefaction methods to assess how well our sampling effort covers this diversity (Gotelli and Colwell 2001). Taxa accumulation curves for nectar and ant samples were calculated with 1000 replicates and sampling without replacement. Sample-based Mao Tau method was used to plot the rarefaction curves. Yeast species richness expected in nectar and ant samples was estimated by using the incidence-based estimator Chao2 (Chao 1987), and the incidence-based coverage estimator ICE (Lee and Chao 1994). Both estimators calculate total species richness, giving therefore richness values that are larger than the observed richness, and are particularly suitable for relatively small sample sizes (Colwell and Coddington 1994). The number of singletons was additionally calculated. We report also rarefied values at the smallest common sample size of 26 sequences for the Chao2 estimator, and Shannon and Simpson biodiversity indices. For nectar samples, this value is reported as an average of the random selection of 26 sequences from the pool of 111 sequences, calculated using 1000 runs. All the preceding diversity-related computations were performed using the EstimateS ver. 8.00 software (RK Colwell, <http://purl.oclc.org/estimates>). EstimateS calculates the reciprocal form (1/D) of Simpson diversity index. Here we provide Simpson's index of diversity (1-D).

Results

Frequency and abundance of yeasts

Bagged flowers unvisited by insects harbour no yeasts, indicating that vectors are necessary for fungal infection of nectar. In contrast, yeasts appeared very frequently in *Cytinus hypocistis* nectar of flowers exposed to pollinators (77.1% of flowers and 94.2% of plants; Table 1). When yeast cells occurred in a flower of a given plant, there was a high probability of finding them also in another flower of the same plant (90.4%), and in all analysed flowers of the same plant (59.2%). Yeasts occurred in all populations, and their probability of occurrence did not differ significantly between *C. hypocistis* races ($F_{2,137} = 1.16$, $p = 0.32$) or flower sex ($F_{1,137} = 0.09$, $p = 0.76$). The interaction race × sex was not significant ($F_{2,137} = 1.26$, $p = 0.28$).

Estimated yeast cell density per nectar sample ranged between 45 and 6.2×10^4 yeast cells mm^{-3} (mean ± SE = $4.1 \times 10^3 \pm 5.9 \times 10^2$ cells mm^{-3}), with the interquartile ranges ranging from 6.8×10^2 to 3.6×10^3 cells mm^{-3} (n = 149). When the nectar-specialist yeast *Metschnikowia*

Table 1. Percentage of flowers containing yeasts and mean yeast cell density in nectar samples from three races of *Cytinus hypocistis*. Abbreviations: Cl, race parasitizing *Cistus ladanifer*; Cs, on *Cistus salviifolious*; Hh, on *Halimium halimifolium*.

Race	Population	Yeast incidence (n)	Mean cell density (cells mm^{-3}) ± SE (n)
Cl	Cl1	86 (44)	$3.6 \times 10^3 \pm 1.2 \times 10^3$ (38)
	ClN	70 (30)	$1.7 \times 10^3 \pm 1.6 \times 10^3$ (21)
Cs	Cs1	82 (39)	$6 \times 10^3 \pm 1.3 \times 10^3$ (32)
	CsN	87 (31)	$3.4 \times 10^3 \pm 1.4 \times 10^3$ (27)
Hh	HhN	50 (24)	$6 \times 10^3 \pm 2.1 \times 10^3$ (12)
	Hh2	79 (24)	$5 \times 10^3 \pm 1.7 \times 10^3$ (19)

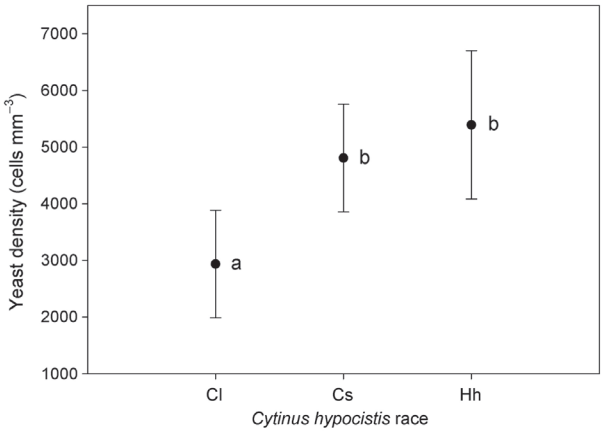

Figure 1. Yeast cell density in nectar samples from three host races of *Cytinus hypocistis* (populations belonging to the same race combined). Dots represent mean values, and vertical segments denote ± 1 SE. Abbreviations: Cl, race parasitizing *Cistus ladanifer*; Cs, on *Cistus salviifolious*; Hh, on *Halimium halimifolium*. Means sharing a letter do not differ significantly ($p > 0.05$).

reukaufii was present in nectar samples, yeast density increased to their maximum values, and the interquartile range of cell density in individual samples ranged from 1.1×10^3 to 7×10^3 cells mm^{-3} ($n = 21$). Yeast density did not vary with flower sex ($F_{1,96} = 0.71$, $p = 0.40$). However, we found a significant race effect ($F_{2,96} = 3.24$, $p = 0.04$), with the race Cl harbouring significantly lower yeast densities than races Cs and Hh (Fig. 1). The interaction term race × sex was not significant ($F_{2,96} = 1.26$, $p = 0.29$).

Ants acted as dispersal agents of yeasts into *C. hypocistis* nectar, with yeasts colonies arising in 38.6% of plates ($n = 127$) where ants were left to walk (Fig. 2). Nearly two thirds (69.4%) of ant-visited plates which produced yeasts also contained filamentous fungi. Twenty-six yeast isolates from 22 individual ants could be obtained for DNA sequencing. This small number was partially due to rapid contamination of cultivars with filamentous fungi present on ant body surfaces (mainly *Penicillium* spp.), hampering yeast growth and precluding isolation even after successive replating efforts. Interestingly, contamination by filamentous fungi was exceptional in isolates from nectar. Yeasts were recovered from all ant species assayed except for *C. pilicornis*, where plates showed no sign of microbial occurrence ($n = 13$) (Fig. 2). On the opposite extreme, plates where *C. auberti* were left to walk showed the highest yeast densities, with more than five hundred yeast colonies per plate.

Yeast species composition

A total of 111 yeast isolates from 81 nectar samples ($n = 42$ inflorescences) were identified on the basis of their DNA sequences, with samples belonging to Ascomycota (63%) nearly doubling in frequency those of Basidiomycota (37%). Eighteen species from eight genera were identified (Table 2). The species most frequently isolated were *Rhodotorula nothofagi*, *Metschnikowia reukaufii* and *Aureobasidium pullulans*, accounting altogether for 69.4% of isolates. Only *R. nothofagi* occurred in nectar samples from all populations. The nectar-specialist *M. reukaufii* was not found in race Cl (Table 2). Nectar samples were characterized everywhere by low yeast species richness, with 69.1% of samples harbouring single species, 24.7% two species, and 6.2% three species. The most species-rich nectar yeast community occurred in samples from race Cl, from which 12 species were isolated (Table 2).

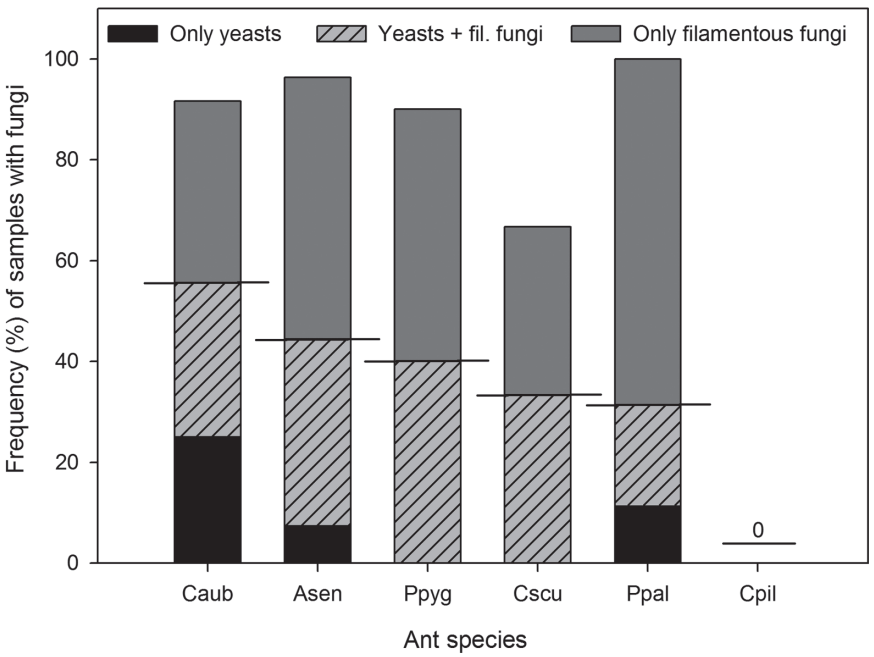

Figure 2. Proportion of ant-walked plates that yielded only yeasts, yeasts plus filamentous fungi, and only filamentous fungi, for the different ant species visiting *Cytinus hypocistis* flowers. Abbreviations: Asen, *Aphaenogaster senilis*, Caub, *Crematogaster auberti*, Cpil, *Camponotus pilicornis*, Cscu, *Crematogaster scutellaris*, Ppal, *Pheidole pallidula*, Ppyg, *Plagiolepis pygmaea*. In each bar, the horizontal segment denotes the cumulative proportion of ant-walked plates yielding yeasts.

Table 2. Ascomycetes and Basidiomycetes isolated from nectar (Ne) and ants (A), number of isolates (n), sampled populations, and ant species in which yeast species were found.

Species	n	Source (Ne/A)	*Cytinus* population	Ant species
Ascomycetes				
Aureobasidium pullulans∗	27	Ne/A	Cl1, ClN, Cs1, CsN, Hh2	*A. senilis*, *C. auberti*, *P. pallidula*, *P. pygmaea*
Candida melibiosica	2	Ne	Cl1, CsN	
Candida railenensis	7	Ne/A	ClN, HhN, Hh2	*A. senilis*, *P. pallidula*
Metschnikowia gruessii	3	Ne	CsN	
Metschnikowia reukaufii	24	Ne/A	Cs1, CsN, Hh2, HhN	*C. auberti*, *P. pygmaea*
Metschnikowia sp.†	7	Ne	Cl1, Cs1, CsN	
Debaryomyces maramus	2	Ne	Cs1	
Debaryomyces hansenii	3	A		*A. senilis*, *C. auberti*
Debaryomyces polymorphus	5	Ne/A	ClN	*C. auberti*
Dothichiza pithyophila	2	Ne	Cl1	
Pringsheimia smilacis	1	Ne	Cl1	
Basidiomycetes				
Cryptococcus carnescens	1	Ne	HhN	
Cryptococcus macerans	1	Ne	CsN	
Cryptococcus magnus	2	A		*P. pallidula*
Cryptococcus sp.	4	Ne/A	ClN	*C. auberti*, *C. scutellaris*
Cryptococcus phenolicus	3	A		*C. auberti*
Rhodotorula fujisanensis	2	Ne	Cs1	
Rhodotorula graminis	1	Ne	Cl1	
Rhodotorula nothofagi	38	Ne/A	Cl1, ClN, Cs1, CsN, Hh2, HhN	*A. senilis*, *C. auberti*, *P. pallidula*, *P. pygmaea*
Rhodotorula sp.	1	Ne	Cl1	
Sporobolomyces roseus	1	Ne	ClN	

∗this yeast named here as *Aureobasidium pullulans* included var. *namibiae* (Zalar et al. 2008, CBS 147.97), recognized by rDNA D1/D2 sequencing and clearly different colony morphology.
†this yeast named here as *Metschnikowia* sp. represents a new species falling in the phylogenetic cluster of the *M. fructicola*/*M. sinensis*/*M. andauensis*/*M. pulcherrima* as revealed by the D1/D2 domain sequences (A. Lachance pers. comm.).

Ant-associated yeast biota included nine species in the Ascomycota (55.6% of samples) and Basidiomycota (44.5%; Table 2). The species most frequently isolated from ants were *R. nothofagi*, followed by *A. pullulans*, *Cryptococcus phenolicus*, and *Debaryomyces hansenii* (Fig. 3). The last two species appeared exclusively on ants.

Six yeast species were shared by ants and nectar: *Aureobasidium pullulans*, *Candida railenensis*, *Cryptococcus* sp., *Debaryomyces polymorphus*, *Metschnikowia reukaufii* and *Rhodotorula nothofagi* (Table 2, Fig. 3). Seventy-eight of total isolates recovered from nectar corresponded to yeast species isolated from ant samples. Most of yeasts

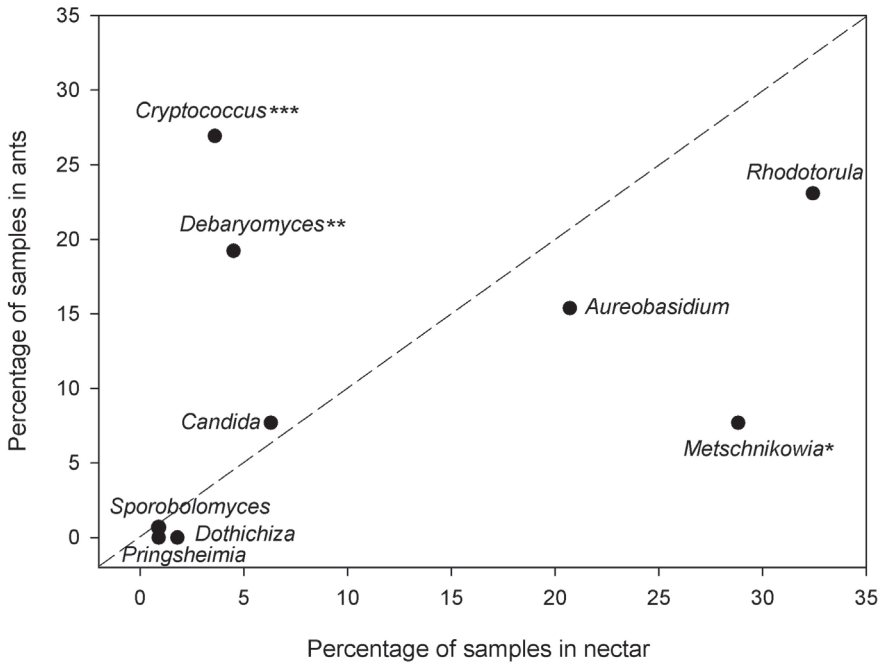

Figure 3. Comparative frequency of occurrence of Ascomycetes and Basidiomycetes genera in samples from *Cytinus hypocistis* nectar and ant samples. Symbols indicate statistically significant differences: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Points falling above the dashed line ($y = x$) denote genera occurring more frequently on ants than in nectar, and vice versa.

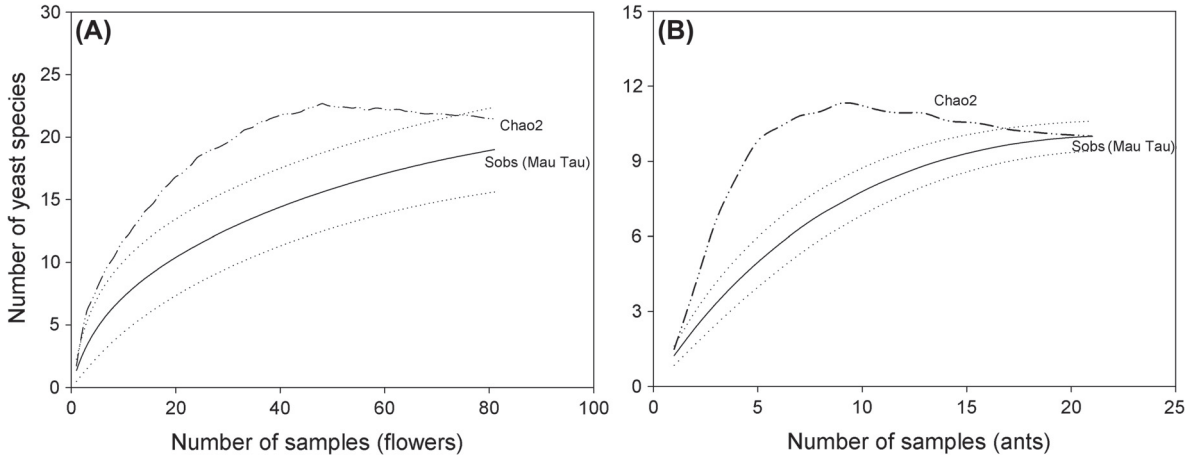

Figure 4. Sample-based rarefaction curves (black solid line, Mao Tau function, and 95% confidence intervals, dotted lines) and species richness estimated by the Chao2 non-parametric richness estimator (dash-dotted lines) for nectar (A) and ant samples (B). Plotted values for Chao2 are means of 1000 randomizations. Note that y-axes are not in the same scale.

exclusively isolated in nectar were rare, some being isolated only once, and thus its biological importance seems of little ecological interest.

Yeast genera could be divided into three groups: group I, with yeasts occurring significantly more often on ant body surfaces, including *Cryptococcus* ($\chi^2 = 15.51$, $p = 0.0001$) and *Debaryomyces* ($\chi^2 = 6.75$, $p = 0.0094$); group II, represented by the genus *Metschnikowia* occurring more frequently in nectar samples, ($\chi^2 = 5.04$, $p = 0.025$); and group III, comprising yeasts in which no significant bias towards nectar or ant body surfaces was found (Fig. 3).

Delimitation of operational taxonomic units (OTUs) and species richness

The MOTHUR analysis assigned the 111 yeast sequences recovered from nectar samples to 16 OTUs, supporting the number of species shown above. The only exceptions were the sequences of *Debaryomyces polymorphus* + *D. maramus* that were combined into a single OTU, and the same occurred with *A. pullulans* sp. + *A. pullulans* var. *namibiae*. A total of eight OTUs were recognized from ant isolates, being combined *Debaryomyces hansenii* and *D. polymorphus* into a single OTU. Since richness estimates were closely similar using named species and OTUS, and given that the species differ in colony morphology, physiology, and rDNA sequences, we will consider hereafter only the results for named species.

The species accumulation curve for nectar samples approached a plateau (Fig. 4A). The comparison of the accumulation curves for observed species and the Chao2 and ICE estimators (final value ± SD = 21.5 ± 2.9 and 24.7 ± 0.01, respectively) indicate that we recovered ca 77–87% of the species present in floral nectar, sufficient to provide reliable estimates of the expected total species richness. Similar results were found when plants rather than individual flowers were used as sampling units (results not shown).

The Chao2 estimator reached a plateau for the ant isolates, thus suggesting that our sampling provided reliable estimates of the expected total species richness carried by ants (Fig. 4B). Due to the low final sample number, yeast isolates from all ant species were pooled for the analyses.

The number of species predicted by the Chao2 estimator using rarefied values at the lowest common sample size of 26 sequences was higher for nectar (17 ± 6.4 species) than for ant samples (10 ± 0.1 species). Shannon and Simpson's index of diversity indices showed comparable diversity for rarefied nectar (2 and 0.9, respectively) and ant samples (2.2 and 0.9, respectively).

Nectar chemical analyses

Nectar sugar concentration differed widely between bagged and exposed flowers (all races combined; $F_{1,196} = 26.6$, $p < 0.0001$), with bagged flowers containing on average higher nectar sugar concentration (22.8% ± 2.9%, mean ± SE) than flowers exposed to pollinators (13.4% ± 2.8%) (Fig. 5). When analyses were conducted separately for each race of *C. hypocistis*, differences hold for races Cs ($F_{1,71} = 7.4$, $p = 0.008$) and Hh ($F_{1,51} = 112.1$, $p < 0.0001$) but not for race Cl ($F_{1,73} = 0.2$, $p = 0.65$) (Fig. 5).

When analyzing exclusively nectar of virgin flowers differences among races in nectar concentration were statistically significant ($F_{2,76} = 17$, $p < 0.0001$). Flowers of race Cl showed the lowest nectar concentration (11.2% ± 2.4%), followed by flowers of the race Cs (19.6% ± 2.0%) and finally Hh (38.5% ± 1.9%) (Fig. 5). Differences in nectar concentration between races disappear when analyzing pollinator-exposed flowers ($F_{2,120} = 0.7$, $p = 0.50$), with all races homogeneously showing less sugar-concentrated nectar (11.5% ± 1.3%, 12.1% ± 1.3%, 14.1 ± 1.9% for races Cl, Cs, and Hh, respectively).

Nectar of exposed flowers that harbored the nectar-specialist *M. reukaufii* showed significantly lower sugar concentration (9.2% ± 2.7%) than those containing other yeast species (15.0% ± 1.8%) ($F_{1,29} = 5.7$, $p = 0.02$). Moreover, variation in nectar sugar concentration was correlated with variation in yeast cell density only when *M. reukaufii* appeared in the samples, with sugar concentration declining

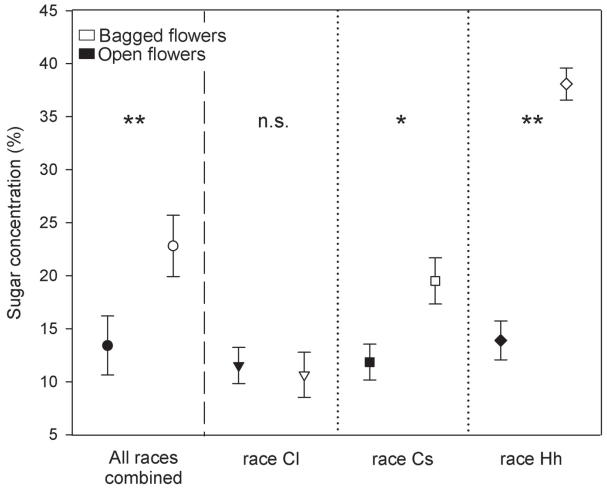

Figure 5. Mean nectar sugar concentration in bagged flowers of *Cytinus hypocistis* (white symbols) and flowers exposed to pollinators (black symbols). Symbols represent mean values and vertical bars represent one standard error. Symbols indicate statistically significant differences: *, $p < 0.01$; **, $p < 0.0001$; n.s. = non-significant differences ($p > 0.05$).

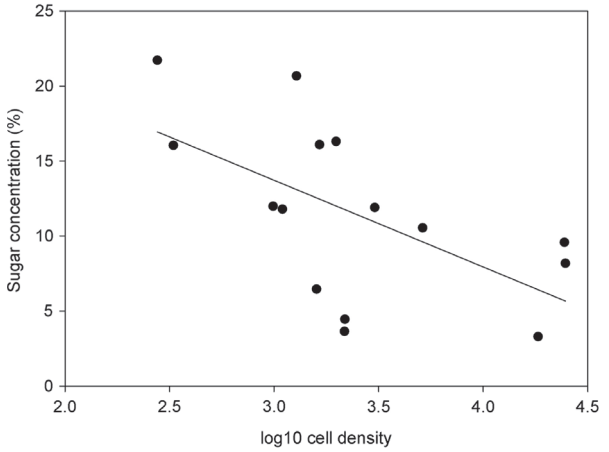

Figure 6. Relationship between total sugar concentration and yeast cell density in nectar samples of *Cytinus hypocistis* containing the nectar-dwelling yeast *Metschnikowia reukaufii* ($r = -0.629$, $p = 0.01$).

significantly with increasing yeast density ($r_s = -0.629$, $p = 0.01$, $n = 15$) (Fig. 6).

Discussion

This study has revealed that establishment of yeast biota in *C. hypocistis* nectar requires ant visits to flowers. By doing so ants contribute to create an unexplored feedback loop in ant–plant interactions. Nectarivorous ants are the dispersal agents for nectar-dwelling yeasts that consume nectar actively, thus competing through exploitation of the shared food source. Further, by diminishing the nutritional quality of nectar, ant-vectored yeasts may be affecting the foraging behavior of the ants themselves and the outcome of the ant–plant interaction, since ants generally tend to prefer higher sugar concentrations (Josens et al. 1998, Blüthgen and Fiedler 2004a, b). An early colonization of nectar by soil-borne yeast would be theoretically possible through accidental direct soil contamination before first floral visits, given that *C. hypocistis* flowers remain at ground level and yeasts are common soil inhabitants (Botha 2006). However, flowers excluded from pollinators harboured no yeasts, thus stressing the definite role of ants as dispersal agents for yeasts.

The proportion of colonized flowers and the species diversity of nectar dwelling yeasts in *C. hypocistis* nectar is similar to those previously found predominantly in bee-pollinated (Brysch-Herzberg 2004, Herrera et al. 2009, Pozo et al. 2011) and bird-pollinated plants (Belisle et al. 2011). Likewise, the observed number of yeast species per flower was also low, with most nectar samples having only one yeast species as previously reported (Pozo et al. 2011, Belisle et al. 2012). A high proportion of the yeast species transported by ants were found also in *C. hypocistis* nectar, ascomycetous yeasts being the most abundant in nectar. Nectar microbiota contains less basidiomycetous yeasts because nectar is not generally their optimal physiological niche due mainly to the osmotic stress conditions (Lachance 2006). In fact, *Cytinus* nectar can reach sugar concentrations of more than 50% in the middle of the day when temperatures are highest (de Vega 2007). High recovery of the basidiomycetous *Rhodotorula nothofagi* in nectar of all *C. hypocistis* races is attributable to its unusual osmotolerance (Kurtzman and Fell 1998). In contrast to the dominance of Ascomycetes in *C. hypocistis* nectar, the yeast assemblage carried by ants contained Basidiomycetes and Ascomycetes. In soil, ascomycetous yeasts are a minority, and basidiomycetous yeasts of the genera *Rhodotorula* and *Cryptococcus* are particularly abundant (Botha 2006). The similarity between the assemblages of yeasts found in soil and ant body surfaces is likely the result of direct contamination from soil while walking. However, ascomycetous and basidiomycetous yeasts can appear in close association with different ant species, and both positive and negative effects have been suggested (Ba and Phillips 1996, Little and Currie 2008). The biological significance of yeasts for our studied ant species remains unknown.

Extending traditional scenarios of plant–pollinator studies to include yeasts as an additional trophic level, as done here, has provided unexpected results. *Cytinus hypocistis* flowers under dual pollinator and yeast attendance exhibit lower nectar concentration, likely due to the metabolic activity of yeasts, which is consistent with findings of a previous study on bee pollinated plants (Herrera et al. 2008). Our results extent, however, these earlier observations. We show that the outcome of the interaction may depend on the identity of the pollinator (with some species being ineffective dispersal agents of yeasts), on infraspecific variations of nectar properties that are inherent to the plants species, and on the physiological characteristics and metabolic activity of the yeast species involved. Not all ants are efficient dispersal agents for yeasts. Four ant species, *A. senilis*, *C. auberti*, *P. pallidula* and *P. pygmaea* clearly favoured dispersal of yeasts, with seventy percent of yeast species found on these ants appearing in *Cytinus* nectar. In contrast, it remains unknown why *C. pilicornis* did not carry

viable yeasts. In spite of yeasts were indistinctly recovered from nectar in all study sites, *C. hypocistis* plants of race Cl did not exhibit lower nectar sugar concentration when yeasts were present. However, plants of race Cs and Hh showed a drastic reduction in nectar sugar concentration when exposed to yeasts. We discuss below the importance of contrasting yeast biota, plant population properties or specific nectar characteristics on the differential patterns observed.

Flowers of the race Cs and Hh showed the highest yeast densities, mainly attributed to the presence of the nectar specialist *M. reukaufii* which occur at high densities in the nectar of a wide array of plants (Brysch-Herzberg 2004). Flowers of *C. hypocistis* containing *M. reukaufii* exhibit significantly lower nectar sugar concentration than flowers containing other yeast species, and the higher the density of *M. reukaufii* the lower the sugar concentration. In contrast, *M. reukaufii* was absent in nectar of flowers of race Cl, which harbored the lowest yeast densities. Differences in the composition of the surrounding plant community might partially explain such absence. Populations of the races Cs and Hh are surrounded by a rich bee-pollinated plant community where *M. reukaufii* occur abundantly in nectar, and ants were frequently observed foraging for nectar in such species (de Vega unpubl.). While foraging for nectar on these plants, ants may facilitate the spread of *M. reukaufii* to *C. hypocistis* flowers in their continuous visits throughout the day. In contrast, populations of the race Cl has have species-poor surroundings due to the monospecific nature of its host stands related to the copious release of allelopathic compounds by *C. ladanifer* (Chaves et al. 2001, Herranz et al. 2006), which would limit or prevent the transfer of *M. reukaufii* from other plants to *C. hypocistis* flowers. Alternatively, intrinsic characteristics of *C. hypocistis* races could be mediating the interactions between nectar and yeasts. Parasitic plants obtain secondary compounds from their hosts that can be transferred to all tissues of the parasites, including nectar, and this uptake depends on the identity of the host (Schneider and Stermitz 1990, Marko and Stermitz 1997, Adler and Wink 2001). If secondary compounds of *C. ladanifer* are transferred to *C. hypocistis* that affect its nectar chemical attributes, then some effects on the patterns of diversity and abundance of nectar-dwelling yeasts could not be ruled out, and similarly a potential detrimental effect on *M. reukaufii*.

Yeasts modify patterns of intra- and interpopulation variation in nectar traits. Such modification can involve an increase of heterogeneity, generating variations in nectar chemical composition among plants, flowers and even nectaries as emphasized by earliest studies (Canto et al. 2007, 2008, Herrera et al. 2008), but also a reduction, as shown here for the first time, homogenizing differences between populations in some nectar attributes. The three races of *C. hypocistis* grow closely in the field under homogeneous climatic conditions and were pollinated by the same pollinators. When analyzing nectar characteristics of *C. hypocistis* prior to pollinator visitation we found differences in nectar sugar concentration among the three races that would reflect underlying race-specific characteristics, ranging from diluted nectar in the race Cl to highly concentrated nectar in the race Hh. However, when yeast-exposed flowers are considered, differences among races disappear, and all races show diluted nectar with a similar concentration. Our results confirm that caution should be exercised when attempting to characterize intrinsic nectar properties of a species from flowers exposed to pollinators. Moreover, our findings raise a number of considerations that deserve further studies in relation to the evolutionary consequences of yeasts at the individual-plant level and community level that may depend on how yeast alters interactions functioning, and the strength of selection.

The ability to transport microbes that dramatically change nectar properties confers to ants yet another potentially significant role for ant–flowers systems besides pollination, nectar thievery, or the discouragement of floral visits by other pollinators (reviewed by Rico-Gray and Oliveira 2007). Ants are ubiquitous visitors of nectar sources in most terrestrial worldwide ecosystems, interacting with a wide diversity of plants, and hence the findings shown here are most probably widespread. While visiting flowers, ants may be conditioning subsequently floral visits due to changes in sugar concentration originated by their own activity. Modification of nectar concentration may deter ant visitation or change patterns of foraging behavior in some way, and ants may prefer 'clean nectar' with higher nectar concentration. Additionally the effects probably go beyond the ant–flower interaction and further affect other pollinators visiting the same flowers as ants do, since many pollinators including insects and birds have shown to prefer nectar rewards with higher sugar concentration than dilute solutions (Butler 1945, Roubik and Buchmann 1984, Roberts 1996, Cnaani et al. 2006).

Besides their possible implications in plant–pollinator mutualisms, nectar-dwelling yeasts provide a novel model system to address ecological questions related to community structure and dynamics (Peay et al. 2012, Belisle et al. 2012). Nectar strongly filters microbial inocula transported by pollinators, leading to phylogenetically clustered microbial communities (Herrera et al. 2010). Nectar filtering was clear for filamentous fungi in our study system. Filamentous fungi were regularly present in ant body surfaces, and have been reported to appear frequently in other pollinators (Batra et al. 1973, Inglis et al. 1993, Belisle et al. 2012). However, they were never recovered from the nectar of either *C. hypocistis* or other plants (Inglis et al. 1993, Pozo et al. 2011, Belisle et al. 2012). Nectar can contain secondary compounds, proteins such as nectarins and pathogenesis-related (PR) enzymes that confer nectar antibiotic properties (Adler 2000, Carter and Thornburg 2004, González-Teuber et al. 2009, 2010, Sasu et al. 2010, Heil 2011). High sugar concentration also protect from microbial infestation. Yeasts may provide additional benefits to plants by inhibiting the growth of other potential pathogenic microorganisms by enriching nectar with alcohol produced by fermentation (Spencer et al. 1997) or through direct competition (Spadaro et al. 2002). All this evidence suggest that only some specialist microbes possessing certain physiological abilities like osmotolerance and/or resistance to certain antibiotic compounds will successfully exploit floral nectar.

In summary, the present study provides compelling evidence that ants play a decisive role as dispersal agents for yeasts, some of which may exert strong effects on nectar traits by altering the energetic value of this food source.

The ant–plant–yeast interactions may be a widespread phenomenon. Through their influence on plant functional traits yeasts have the potential to alter nectar consumption, pollinator foraging behavior and ultimately plant reproduction. The outcome of the tripartite ant–plant–yeast interaction can be mediated by biological and ecological characteristics of plant populations, and has shown to be highly dependent on the identity of the participants, even to the extent of influencing the species composition of the ternary system. The study on the net effect of nectar microbial communities on ecosystem functioning is still on its infancy, and much work remains to be done.

Acknowledgements – We thank P. Bazaga, E. López, I. M. García and M. García for technical assistance in the laboratory. Special thanks to Dr. R. G. Albaladejo for helpful comments on the manuscript. This work was supported by funds from Consejería de Innovación, Ciencia y Empresa, Junta de Andalucía (Proyecto de Excelencia P09-RNM- 4517 to CMH), and Ministerio de Ciencia e Innovación (grant CGL2010-15964 to CMH and Juan de la Cierva Program to CdV).

References

Adler, L. S. 2000. The ecological significance of toxic nectar. – Oikos 91: 409–420.

Adler, L. S. and Wink, M. 2001. Transfer of alkaloids from hosts to hemiparasites in two *Castilleja–Lupinus* associations: analysis of floral and vegetative tissues. – Biochem. Syst. Ecol. 29: 551–561.

Alm, J. et al. 1990. Preference of cabbage white butterflies and honey bees for nectar that contains amino acids. – Oecologia 84: 53–57.

Armbruster, W. S. 1997. Exaptations link evolution of plant–herbivore and plant–pollinator interactions: a phylogenetic inquiry. – Ecology 78: 1661–1672.

Ba, A. S. and Phillips Jr, S. A. 1996. Yeast biota of the red imported fire ant. – Mycol. Res. 100: 740–746.

Baker, H. G. and Baker, I. 1983. Floral nectar sugar constituents in relation to pollinator type. – In: Jones, C. E. and Little, R. J. (eds), Handbook of experimental pollination biology. Van Nostrand Reinhold, pp. 117–141.

Batra, L. R. et al. 1973. The mycoflora of domesticated and wild bees (Apoidae). – Mycopathol. Mycol. Appl. 49: 13–44.

Belisle, M. et al. 2012. Flowers as islands: spatial distribution of nectar-inhabiting microfungi among plants of *Mimulus aurantiacus*, a hummingbird-pollinated shrub. – Microb. Ecol. 63: 711–718.

Blüthgen, N. and Fiedler, K. 2004a. Preferences for sugars and amino acids and their conditionality in a diverse nectar feeding ant community. – J. Anim. Ecol. 73: 155–166.

Blüthgen, N. and Fiedler, K. 2004b. Competition for composition: lessons from nectar-feeding ant communities. – Ecology 85: 1479–1485.

Botha, A. 2006. Yeasts in soil. – In: Rosa, C. and Peter, G. (eds), Biodiversity and ecophysiology of yeasts. Springer, pp. 221–240.

Brody, A. K. 1997. Effects of pollinators, herbivores, and seed predators on flowering phenology. – Ecology 78: 1624–1631.

Bronstein, J. L. 2001. The exploitation of mutualisms. – Ecol. Lett. 4: 277–287.

Brysch-Herzberg, M. 2004. Ecology of yeasts in plant–bumblebee mutualism in central Europe. – FEMS Microbiol. Ecol. 50: 87–100.

Butler, C. G. 1945. The influence of various physical and biological factors of the environment on honeybee activity – an examination of the relationship between activity and nectar concentration and abundance. – J. Exp. Biol. 21: 5–12.

Cahill, J. F. et al. 2008. Disruption of a belowground mutualism alters interactions between plants and their floral visitors. – Ecology 89: 1791–1801.

Canto, A. et al. 2007. Intraplant variation in nectar sugar composition in two *Aquilegia* species (Ranunculaceae): contrasting patterns under field and greenhouse conditions. – Ann. Bot. 99: 653–660.

Canto, A. et al. 2008. Pollinator foraging modifies nectar sugar composition in *Helleborus foetidus* L. (Ranunculaceae): an experimental test. – Am. J. Bot. 95: 315–320.

Carter, C. and Thornburg, R. W. 2004. Is the nectar redox cycle a floral defense against microbial attack? – Trends. Plant Sci. 9: 320–324.

Chao, A. 1987. Estimating the population size for capture–recapture data with unequal catchability. – Biometrics 43: 783–791.

Chaves, N. et al. 2001. Identification and effects of interaction phytotoxic compounds from exudate of *Cistus ladanifer* leaves. – J. Chem. Ecol. 27: 611–621.

Cnaani, J. et al. 2006. Flower choice and learning in foraging bumblebees: effects of variation in nectar volume and concentration. – Ethology 112: 278–285.

Colwell, R. K. and Coddington, J. A. 1994. Estimating terrestrial biodiversity through extrapolation. – Phil. Trans. R. Soc. B 345: 101–118.

de Vega, C. 2007. Reproductive biology of *Cytinus hypocistis* L.: host–parasite interactions. – PhD thesis, Univ. of Seville, Spain.

de Vega, C. et al. 2007. The endophytic system of Mediterranean *Cytinus hypocistis* (Cytinaceae) developing on five host Cistaceae species. – Ann. Bot. 100: 1209–1217.

de Vega, C. et al. 2008. Genetic races associated with the genera and sections of host species in the holoparasitic plant *Cytinus hypocistis* (Cytinaceae) in the western Mediterranean basin. – New. Phytol. 178: 875–887.

de Vega, C. et al. 2009a. Yeasts in floral nectar of some South African plants: quantification and associations with pollinator type and sugar concentration. – S. Afr. J. Bot. 75: 798–806.

de Vega, C. et al. 2009b. The ant–pollination system of *Cytinus hypocistis* (Cytinaceae) a Mediterranean root holoparasite. – Ann. Bot. 103: 1065–1075.

de Vega, C. et al. 2011. Endozoochory by beetles: a novel seed dispersal mechanism. – Ann. Bot. 107: 629–637.

Eisikowitch, D. et al. 1990. The effect of the natural assemblage of microorganisms and selected strains of the yeast *Metschnikowia reukaufii* in controlling the germination of pollen of the common milkweed *Asclepias syriaca*. – Can. J. Bot. 68: 1163–1165.

Ehlers, B. K. and Olesen, J. M. 1997. The fruit–wasp route to toxic nectar in *Epipactis* orchids? – Flora 192: 223–229.

Fierer, N. et al. 2007. Metagenomic and small-subunit rRNA analyses reveal the genetic diversity of bacteria, archaea, fungi and viruses in soil. – Appl. Environ. Microbiol. 73: 7059–7066.

Galen, C. 1983. The effects of nectar thieving ants on seedset in floral scent morphs of *Polemonium viscosum*. – Oikos 41: 245–249.

Gange, A. C. and Smith, A. K. 2005. Arbuscular mycorrhizal fungi influence visitation rates of pollinating insects. – Ecol. Entomol. 30: 600–606.

García, M. B. et al. 1995. Ant pollination of the paleoendemic dioecious *Borderea pyrenaica*. Dioscoreaceae. – Plant. Syst. Evol. 198: 17–27.

Gómez, J. M. et al. 1996. Experimental study of pollination by ants in Mediterranean high mountain and arid habitats. – Oecologia 105: 236–242.

González-Teuber, M. et al. 2009. Pathogenesis-related proteins protect extrafloral nectar from microbial infestation. – Plant. J. 58: 464–473.

González-Teuber, M. et al. 2010. Glucanases and chitinases as causal agents in the protection of *Acacia* extrafloral nectar from infestation by phytopathogens. – Plant. Physiol. 152: 1705–1715.

Gotelli, N. J. and Colwell, R. K. 2001. Quantifying biodiversity: procedures and pitfalls in the measurement and comparison of species richness. – Ecol. Lett. 4: 379–391.
Haber, W. A. et al. 1981. Ants like flower nectar. – Biotropica 13: 211–214.
Heil, M. 2011. Nectar: generation, regulation, and ecological functions. – Trends. Plant. Sci. 16: 191–200.
Herranz, J. M. et al. 2006. Effect of allelopathic compounds produced by *Cistus ladanifer* on germination of 20 Mediterranean taxa. – Plant Ecol. 184: 259–272.
Herrera, C. M. and Pozo, M. I. 2010. Nectar yeasts warm the flowers of a winter-blooming plant. – Proc. R. Soc. B 277: 1827–1834.
Herrera, C. M. et al. 1984. Nectar thievery by ants from southern Spanish insect-pollinated flowers. – Insect. Soc. 31: 142–154.
Herrera, C. M. et al. 2002. Interaction of pollinators and herbivores on plant fitness suggests a pathway for correlated evolution of mutualism- and antagonism-related traits. – Proc. Natl Acad. Sci. USA 99: 16823–16828.
Herrera, C. M. et al. 2008. Invisible floral larcenies: microbial communities degrade floral nectar of bumble bee-pollinated plants. – Ecology 89: 2369–2376.
Herrera, C. M. et al. 2009. Yeasts in floral nectar: a quantitative survey. – Ann. Bot. 103: 1415–1423.
Herrera, C. M. et al. 2010. Inhospitable sweetness: nectar filtering of pollinator-borne inocula leads to impoverished, phylogenetically clustered yeast communities. – Proc. R. Soc. B 277: 747–754.
Hughes, J. B. et al. 2001 Counting the uncountable: statistical approaches to estimating microbial diversity. – Appl. Environ. Microbiol. 67: 4399–4406.
Hurlbert, S. H. 1984. Pseudoreplication and the design of ecological field experiments. – Ecol. Monogr. 54: 187–211.
Inglis, G. D. et al. 1993. Aerobic microorganisms associated with alfalfa leafcutter bees. *Megachile rotundata*. – Microbiol. Ecol. 6: 125–143.
Irwin, R. E. and Brody, A. K. 1998. Nectar robbing in *Ipomopsis aggregata*: effects on pollinator behaviour and plant fitness. – Oecologia 116: 519–527.
Josens, R. B. et al. 1998. Nectar feeding by the ant *Camponotus mus*: intake rate and crop filling as a function of sucrose concentration. – J. Insect Physiol. 44: 579–585.
Kevan, P. G. et al. 1988. Yeast-contaminated nectar and its effects on bee foraging. – J. Apicult. Res. 27: 26–29.
Klinkhamer, P. G. L. and de Jong, T. J. 1990. Effects of plant size, plant-density and sex differential nectar reward on pollinator visitation in the Protandrous *Echium vulgare* (Boraginaceae). – Oikos 57: 399–405.
Kurtzman, C. P. and Fell, J. W. 1998. The yeasts, a taxonomic study, 4th edn. – Elsevier.
Kurtzman, C. P. and Robnett, C. J. 1998. Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit 26S ribosomal DNA partial sequences. – Anton. Leeuw. Int. J. G. 73: 331–371.
Lachance, M. A. 2006. Yeast biodiversity: how many and how much? – In: Rosa, C. A. and Peter, G. (eds), Biodiversity and ecophysiology of yeasts. Springer, pp. 1–9.
Lee, S.-M. and Chao, A. 1994. Estimating population size via sample coverage for closed capture–recapture models. – Biometrics 50: 88–97.
Lin, Y. and Tanaka, S. 2006. Ethanol fermentation from biomass resources: current state and prospects. – Appl. Microb. Biotech. 69: 627–642.
Little, A. E. F. and Currie, C. R. 2008. Indirect interaction web reveals how black yeast symbionts compromise the efficiency of antibiotic defenses in fungus-growing ants. – Ecology 89: 1216–1222.
Marko, M. D. and Stermitz, F. R. 1997. Transfer of alkaloids from *Delphinium* to *Castilleja* via root parasitism. Norditerpenoid alkaloid analysis by electrospray mass spectrometry. – Biochem. Syst. Ecol. 25: 279–285.
Norgate, M. et al. 2010. Ambient temperature influences Australian native stingless bee (*Trigona carbonaria*) preference for warm nectar. – PLoS One 5(8): e12000. doi:10.1371/journal.pone.0012000.
Peay, K. G. et al. 2012. Phylogenetic relatedness predicts priority effects in nectar yeast communities. – Proc. R. Soc. B 279: 749–758.
Pozo, M. I. et al. 2011. Species richness of yeast communities in floral nectar of southern Spanish plants. – Microb. Ecol. 61: 82–91.
Puterbaugh, M. N. 1998. The roles of ants as flower visitors: experimental analysis in three alpine plant species. – Oikos 83: 36–46.
Retana, J. et al. 1987. Foraging ecology of the nectarivorous ant *Camponotus foreli* (Hymenoptera, Formicidae) in a savanna-like grassland. – Misc. Zool. 11: 187–193.
Rico-Gray, V. and Oliveira, P. S. 2007. The ecology and evolution of ant–plant interactions. – Univ. of Chicago Press.
Rico-Gray, V. et al. 1998. Geographical and seasonal variation in the richness of ant–plant interactions in Mexico. – Biotropica 30: 190–200.
Roberts, W. M. 1996. Hummingbird's nectar concentration preferences at low volume: the importance of time scale. – Ann. Behav. 52: 361–370.
Romeis, J. and Wäckers, F. L. 2000 Feeding responses by female *Pieris brassicae* butterflies to carbohydrates and amino acids. – Physiol. Entomol. 25: 247–253.
Roubik, D. W. and Buchmann, S. L. 1984. Nectar selection by *Melipona* and *Apis mellifera* (Hymenoptera: Apidae) and the ecology of nectar intake by bee colonies in a tropical forest. – Oecologia 61: 1–10.
Sasu, M. A. et al. 2010. Antimicrobial nectar inhibits a florally transmitted pathogen of a wild *Cucurbita pepo* (Cucurbitaceae). – Am. J. Bot. 97: 1025–1030.
Schloss, P. D. et al. 2009. Introducing MOTHUR: open-source, platform-independent, community supported software for describing and comparing microbial communities. – Appl. Environ. Microbiol. 75: 7537–7541.
Schneider, M. J. and Stermitz, F. R. 1990. Uptake of host plant alkaloids by root parasitic *Pedicularis* ssp. – Phytochemistry 29: 1811–1814.
Spadaro, D. et al. 2002. Mechanisms of action and efficacy of four isolates of the yeast *Metschnikowia pulcherrima* active against postharvest pathogens on apples. – Postharvest. Biol. Technol. 24: 123–134.
Spencer, J. F. T. et al. 1997. Yeasts as living objects: yeast nutrition. – In: Spencer, J. F. T. and Spencer, D. M. (eds), Yeasts in natural and artificial habitats. Springer, pp. 68–79.
Strauss, S. Y. et al. 1999. Ecological costs of plant resistance to herbivores in the currency of pollination. – Evolution 53: 1105–1113.
Wiens, F. et al. 2008. Chronic intake of fermented floral nectar by wild treeshrews. – Proc. Natl Acad. Sci. USA 105: 10426–10431.
Yarrow, D. 1998. Methods for the isolation, maintenance and identification of yeasts. – In: Kurtzman, C. P. and Fell, J. W. (eds), The yeast, a taxonomic study. Elsevier, pp. 77–100.
Zalar, P. et al. 2008. Redefinition of *Aureobasidium pullulans* and its varieties. – Stud. Mycol. 61: 21–38.

Supplementary material (available online as Appendix O20295 at <www.oikosoffice.lu.se/appendix>). Appendix A1.