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INVISIBLE FLORAL LARCENIES: MICROBIAL COMMUNITIES DEGRADE FLORAL NECTAR OF BUMBLE BEE-POLLINATED PLANTS

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Abstract. The ecology of nectarivorous microbial communities remains virtually unknown, which precludes elucidating whether these organisms play some role in plant–pollinator mutualisms beyond minor commensalism. We simultaneously assessed microbial abundance and nectar composition at the individual nectary level in flowers of three southern Spanish bumble bee-pollinated plants (*Helleborus foetidus*, *Aquilegia vulgaris*, and *Aquilegia pyrenaica cazorlensis*). Yeasts were frequent and abundant in nectar of all species, and variation in yeast density was correlated with drastic changes in nectar sugar concentration and composition. Yeast communities built up in nectar from early to late floral stages, at which time all nectaries contained yeasts, often at densities between 10^4 and 10^5 cells/mm^3. Total sugar concentration and percentage sucrose declined, and percentage fructose increased, with increasing density of yeast cells in nectar. Among-nectary variation in microbial density accounted for 65% (*H. foetidus* and *A. vulgaris*) and 35% (*A. p. cazorlensis*) of intraspecific variance in nectar sugar composition, and 60% (*H. foetidus*) and 38% (*A. vulgaris*) of variance in nectar concentration. Our results provide compelling evidence that nectar microbial communities can have detrimental effects on plants and/or pollinators via extensive nectar degradation and also call for a more careful interpretation of nectar traits in the future, if uncontrolled for yeasts.

Key words: Aquilegia pyrenaica cazorlensis; Aquilegia vulgaris; *bumble bee pollination; floral microbiology;* Helleborus foetidus; *mutualism exploitation; nectar concentration; nectar sugar composition; yeast communities.*

Introduction

Placing mutualisms into a community context will advance our understanding of the ecology and evolution of species interactions (Stanton 2003, Strauss and Irwin 2004). This improved approach should not only consider the mutualistic communities themselves, but also those organisms that exploit the mutualistic interactions (Bronstein 2001, Bronstein et al. 2003). In plant–animal mutualisms mediated by food reward provisioning (e.g., pollination, seed dispersal), exploitation often implicates the consumption or spoilage of food rewards by non-mutualists that do not return any benefit to plants. Because such exploitation can have some direct or indirect detrimental effects on plant fitness, certain plant traits involved in mutualistic interactions can be partly explained as the outcome of selection to reduce the impact of exploiters on plant fitness (Herrera 1982, Irwin et al. 2004). This applies, for example, to the toxic substances often found in fleshy fruit pulps and floral nectars, which may function as defenses against frugivorous and nectarivorous microbes, respectively (Herrera 1982, Cipollini and Levey 1997*a*, *b*, Adler 2000). A key assumption underlying this interpretation is that microbial degradation of fruits or nectar can be sufficiently frequent and severe to select for antimicrobial compounds, despite potentially detrimental side effects on attractiveness to mutualists. Considerable empirical information supports this assumption for fleshy fruits (Herrera 1982, Cipollini and Stiles 1992), yet a similar confirmation is so far lacking for floral nectar. The presence of microbes in the nectar of wild plants is known to microbiologists (Sandhu and Waraich 1985, Brysch-Herzberg 2004) and plant ecologists (Kevan et al. 1988, Eisikowitch et al. 1990, Ehlers and Olesen 1997). Nevertheless, the ecology of nectarivorous microbial assemblages, including basic aspects like abundance, distribution patterns, and effects on

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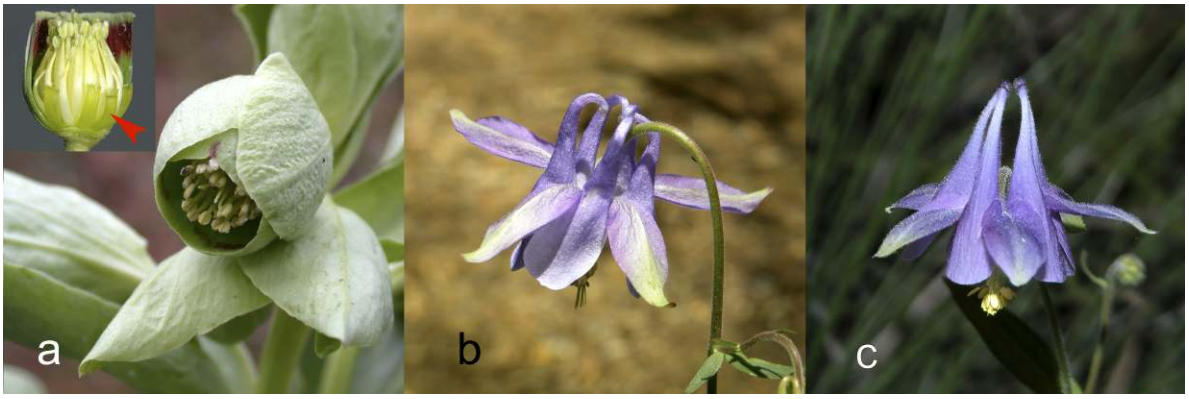

PLATE 1. (a) Flowers of *Helleborus foetidus*, (b) *Aquilegia vulgaris*, and (c) *Aquilegia pyrenaica cazorlensis*. The three species are similar in having five separate, independent nectaries per flower. In *H. foetidus* the nectaries (red arrow in a) are hidden inside the corolla, forming a ring between the stamens and the sepals. In *Aquilegia* the nectaries are located at the tip of the elongated spurs. Nectar samples for this study were collected from individual nectaries. Photo credits: C. M. Herrera.

floral nectar remain unexplored, as revealed by the conspicuous absence of these topics in recent reviews (Ngugi and Scherm 2006, Rosa and Péter 2006, Nicolson et al. 2007). This dearth of empirical information has so far precluded solving the dilemma of whether these organisms are innocuous commensals or exploiters having some impact on plant–pollinator mutualisms (Antonovics 2005). By combining very small-scale, nectary-level nectar sampling with a split-sample analytical approach, we show in this paper that the floral nectar of three southern Spanish bumble bee-pollinated plants often harbors very dense yeast communities, and that variation in yeast density among nectaries of the same species runs parallel to drastic changes in important nectar features such as sugar composition and total sugar concentration. Our results provide novel evidence suggesting that nectarivorous microbes can become influential exploiters of plant–pollinator mutualisms, and also call for a more careful interpretation of nectar traits in the future.

MATERIALS AND METHODS

Floral nectar samples of the perennial herbs *Helleborus foetidus*, *Aquilegia vulgaris*, and *Aquilegia pyrenaica cazorlensis* (Ranunculaceae) (see Plate 1) were collected during March–June 2007 at three separate localities in the Sierra de Cazorla, Jaén province, southeastern Spain. The two nearest sampling sites were 7.5 km apart, and the two most distant ones were at 17 km. The three species differ widely in flowering time (February–April, May–June, and June–July, respectively) and habitat type (pine forest understory, damp meadows, and bare patches of sandy soil under limestone cliffs, respectively). They have the same bumble bee species (*Bombus terrestris*, *B. pratorum*) as main pollinators, and per-flower pollinator visitation rates range from extremely low (*H. foetidus*, 0.005–0.030 visits·flower^{-1}·min^{-1}; Herrera et al. 2001) to low (*Aquilegia*, 0.025–0.075 visits·flower^{-1}·min^{-1}; C. M. Herrera, *unpublished data*). The three species are similar in having five separate, independent nectaries per flower. In *H. foetidus* the nectaries are shaped like flattened horns and are deeply hidden inside the corolla, forming a ring between the stamens and the sepals. In *Aquilegia* the nectaries are located at the tip of elongated spurs. Flowers of *H. foetidus* are protogynous, while those of *A. vulgaris* and *A. p. cazorlensis* are protandrous. The three species generally produce between 20 and 75 flowers per inflorescence. Further details on the autoecology, floral biology, and pollination ecology of the study plants in the Sierra de Cazorla region can be found in Herrera et al. (2001, 2006), Medrano et al. (2006), and Canto et al. (2007).

For each species, 8–12 flowering individuals growing within a 75–150 m^2 area were bagged with fine mesh early in the morning to exclude pollinators and allow for nectar accumulation in the nectaries. Twenty-four hours later, a random sample of $N = 20$ flowers was collected from different plants (1–3 flowers/plant), and kept refrigerated until dissected in the laboratory a few hours later. All collected flowers were already open, and thus had been exposed to pollinator visits, by the time of bagging. Each sampled flower was assigned to one of three consecutive floral stages: female, transitional (both female and male verticils functional), and male in protogynous *H. foetidus*; male, transitional, and female in protandrous *Aquilegia*. Average time elapsing between early and late floral stages are 8, 3, and 4 d for *H. foetidus*, *A. vulgaris,* and *A. p. cazorlensis*, respectively. Two noncontiguous nectaries were excised from each flower and visually inspected for nectar. Empty nectaries or those with minute amounts of nectar were discarded, and replaced with others from the same flower whenever possible. Final samples consisted of $N = 40$, 40, and 35 nectaries for *H. foetidus*, *A. vulgaris*, and *A. p. cazorlensis*, respectively.

The nectar from each nectary was split into two subsamples, which were used for characterizing the size

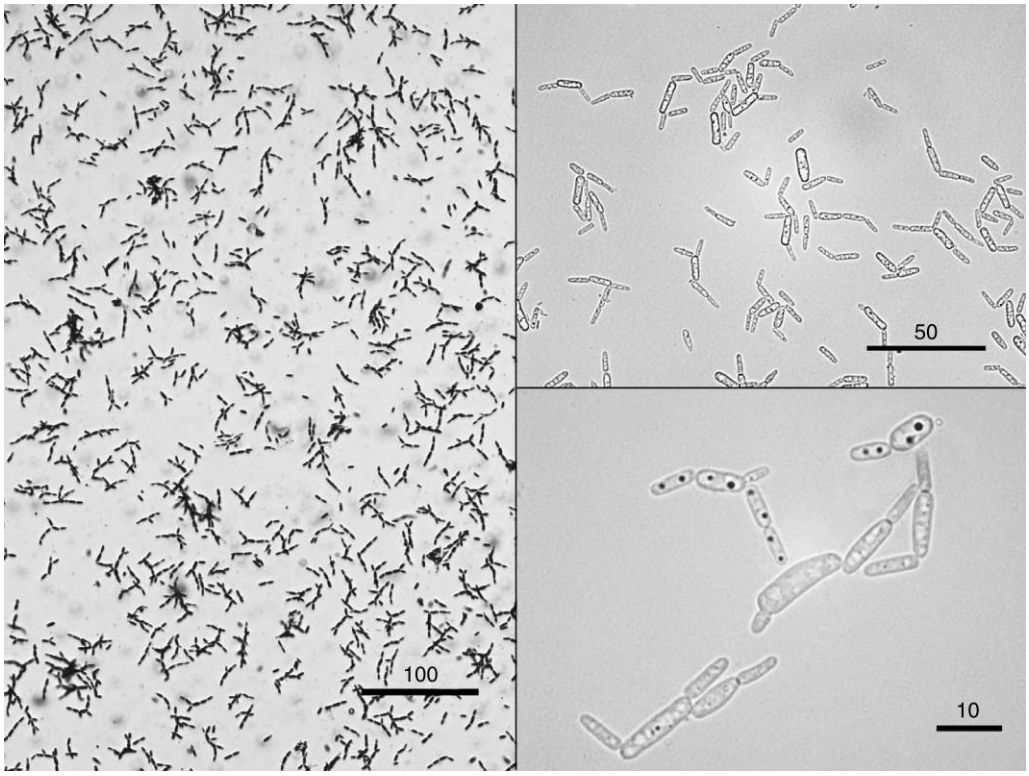

FIG. 1. Yeast cells in field-collected nectar of *Helleborus foetidus*, most likely belonging to species of *Metschnikowia* and *Candida*. Cells in the left photomicrograph were stained with cotton blue with lactophenol and illustrate the microscopical aspect of a densely populated nectar. Unstained cells in the two photomicrographs on the right show diagnostic cell features (e.g., large vacuoles with highly refractive corpuscles, visible as dark spots). Scale bars are in μm.

of microbial communities and nectar composition. A 1-μL subsample (except for *A. p. cazorlensis*) was taken using a calibrated microcapillary, placed inside a microcentrifuge tube, and kept frozen until used for chemical analyses, as described in the next paragraph. The other subsample consisted of the rest of nectar in the nectary. After measuring its volume using a calibrated micropipette (usually 0.5–1 μL, except for *A. p. cazorlensis*), it was diluted up to 5 μL by the addition of 0.1% Safranin water solution (Sigma-Aldrich, Madrid, Spain), which facilitated microscopical examination. Microbial cell density (cells/mm^3 of nectar volume) was estimated directly for each of these subsamples under a microscope at 400× using a Neubauer chamber (Auxilab, Beriain, Navarra, Spain) and standard cell counting methods. Most nectaries of *A. p. cazorlensis* contained insufficient nectar for consistently applying the preceding protocol. For this reason, sample size is slightly smaller and total sugar concentration data are missing for this species.

Nectar sugar composition of all samples ($N = 115$) was determined using ion-exchange high performance liquid chromatography (HPLC), following the analytical procedures and equipment described in detail by Herrera et al. (2006) and Canto et al. (2007). Two independent HPLC measurements were done on each sample, and results of replicates were averaged for the analyses. Only sucrose, glucose, and fructose appeared in the analyses. For each sample, the proportions of individual sugars were obtained by integrating the area under chromatogram peaks. HPLC results also allowed us to compute separate estimates of glucose, fructose, and sugar concentration in each nectar sample on a mass of solute to mass of solution basis (except for *A. p. cazorlensis*). Total sugar concentration of nectar was then computed by summing up these partial figures.

RESULTS

Microbial communities in nectar

Microscopical examinations revealed microbial communities in most nectar samples of all species. Although a rigorous identification of the organisms involved would have required culturing and isolation (e.g., Brysch-Herzberg 2004), morphological features unequivocally characterized them as yeasts in all instances (Fig. 1). This coarse level of taxonomic resolution was sufficient for the purposes of this study.

The proportion of nectar samples containing yeasts was very high: 90.0%, 60.0%, and 62.9% for *H. foetidus*, *A. vulgaris*, and *A. p. cazorlensis*, respectively. Yeast incidence increased over successive floral stages in all species, and nectar from flowers in their latest stages

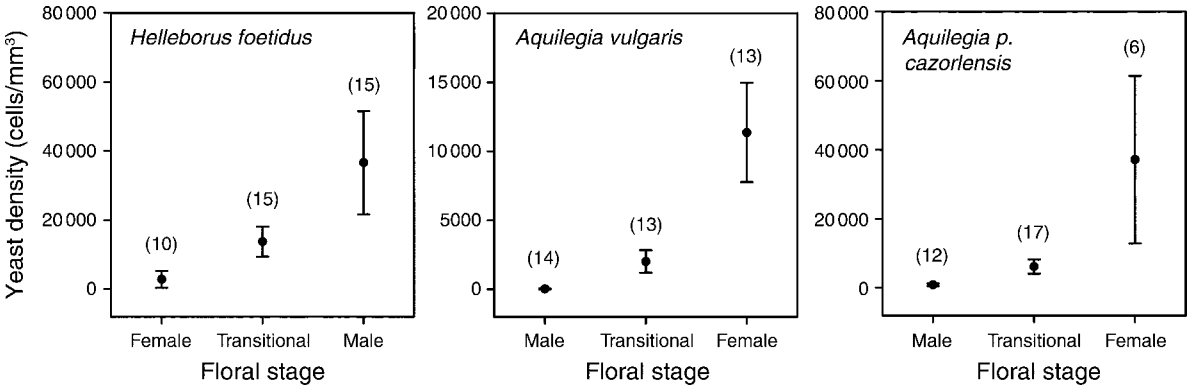

FIG. 2. Mean yeast cell density increases steadily across successive floral stages in the three species studied. *Helleborus foetidus* flowers are protogynous, while those of *Aquilegia* spp. are protandrous; thus flower age increases from left to right in each plot. Average time elapsing between early and late floral stages' midpoints are 8, 3, and 4 d for *H. foetidus*, *A. vulgaris*, and *A. p. cazorlensis*, respectively. Dots represent mean values, and vertical segments extend over ±SE. Numbers in parentheses are sample sizes. Variation among floral stages in microbial density was statistically significantly in all species ($\chi^2 = 13.9$, 27.5, and 11.1 for *H. foetidus*, *A. vulgaris*, and *A. p. cazorlensis*, respectively, df = 1, $P \leq 0.004$; Kruskal-Wallis nonparametric analyses of variance).

always contained yeasts. In the protogynous *H. foetidus*, yeast-containing nectar samples were less frequent in the female (60%) than in the transitional (100%) and male (100%) stages ($P = 0.002$, Fisher exact probability test). In the protandrous *Aquilegia*, yeast incidence increased from male through transitional to female stages (*A. vulgaris*, 7.1%, 76.9%, and 100%, respectively, $P < 0.001$; *A. p. cazorlensis*, 33.3%, 70.6%, and 100%, respectively, $P = 0.01$).

Mean yeast cell density also increased steadily from early through transitional to late stages in the three species (Fig. 2). At the latest floral stages, the nectar of *H. foetidus*, *A. vulgaris*, and *A. p. cazorlensis* contained on average (±SE, range in parentheses) 36 612 ± 14 956 (455–219 545) cells/mm^3, 11 362 ± 3603 (1445–43 003) cells/mm^3, and 37 166 ± 24 284 (280–156 800) cells/mm^3, respectively.

Variation in nectar features

Nectar characteristics varied widely among samples of the same species. Variation in sugar composition took place along an axis defined by pure-sucrose nectar on one extreme and pure-fructose nectar on the other. Nectars exemplifying every possible combination of the two sugars occurred in each species, including pure-sucrose and pure-fructose ones (Fig. 3). Percentage glucose was always low, and varied much less among samples than the other sugars. Nectar sugar concentration (percentage mass of sugar per mass of solution) varied also widely in the two species with data available. In *H. foetidus*, sugar concentration ranged between 0.3% and 19.7% (8.0% ± 0.6%, mean ± SE), and in *A. vulgaris* between 11.8% and 47.2% (28.7% ± 1.3%).

Variation in nectar characteristics among nectaries of the same species was correlated with variation in yeast cell density, and the slopes of the regressions linking a

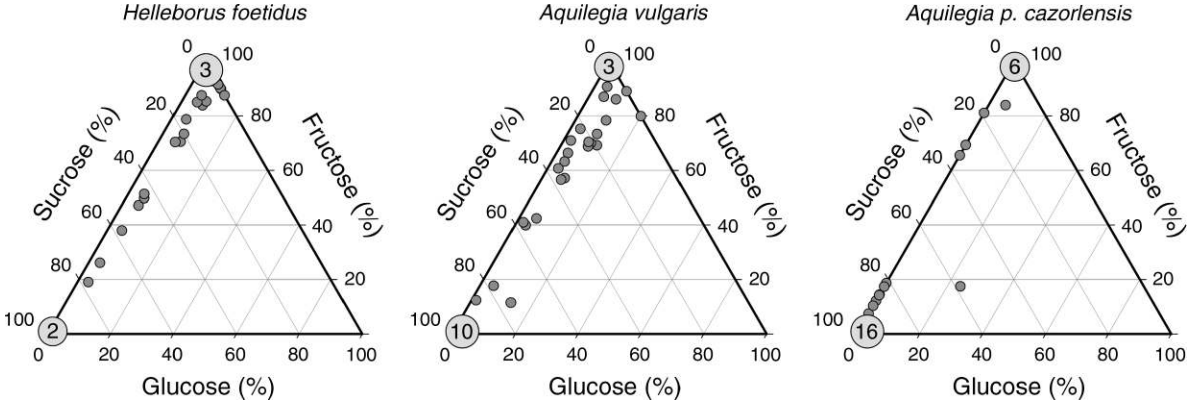

FIG. 3. Ternary diagrams showing the distribution of nectar samples over the plane defined by axes corresponding to the percentage amount of glucose, fructose, and sucrose for the three species studied. Each point depicts the proportional sugar composition of the nectar from a single nectary. Circled numbers denote the number of coincident points at the top (100% fructose) and bottom-left (100% sucrose) vertices.

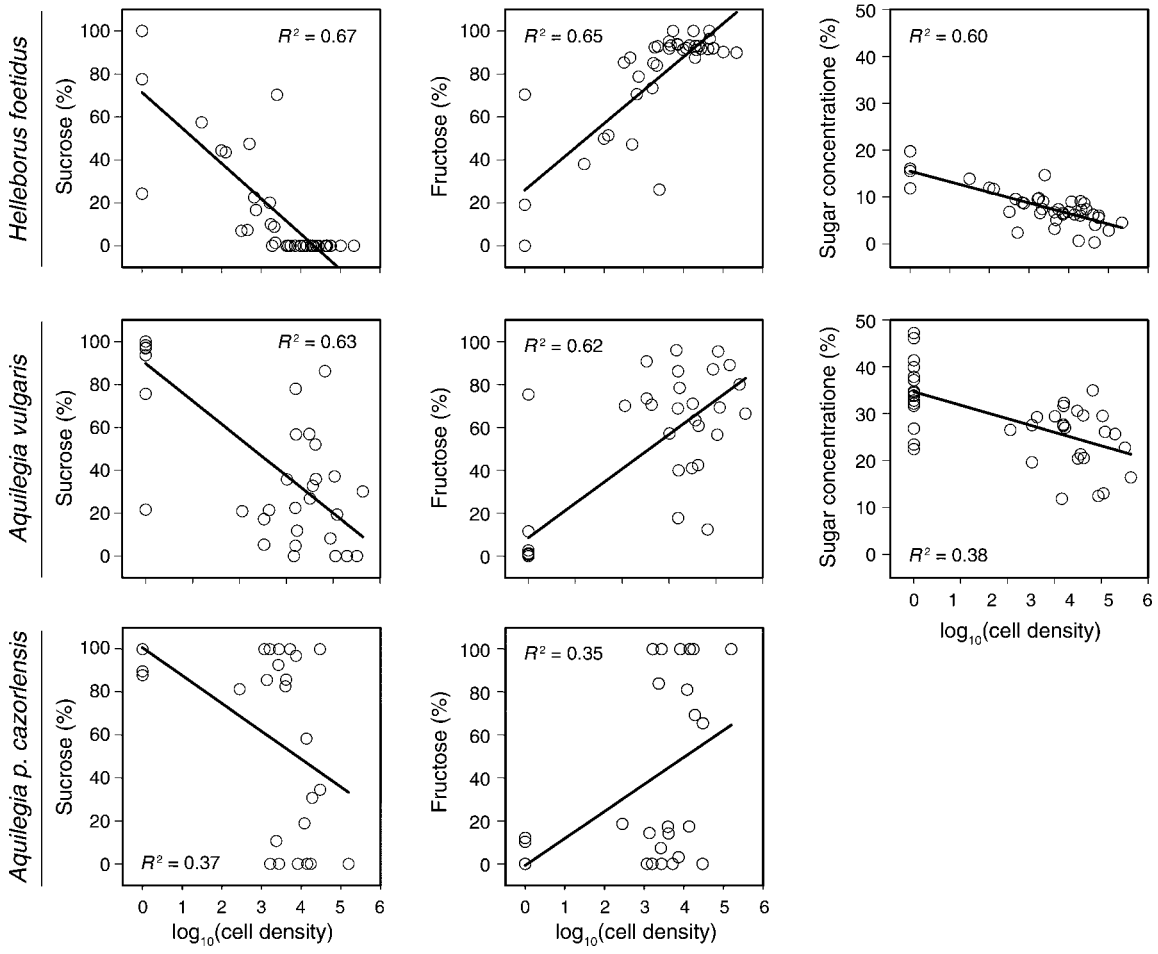

FIG. 4. Relationships between percentage sucrose (left), percentage fructose (center), and total sugar concentration (right), and yeast cell density in single-nectary nectar samples of the three species studied. The proportion of variance accounted for by least-squares fitted linear regressions (lines) is shown for each graph (R^2). All the depicted relationships are statistically significant ($P < 0.0001$ in all cases; significance tested using rank correlations). For *Helleborus foetidus*, $r_s = 0.672$, -0.854, and -0.700, for fructose, sucrose, and sugar concentration, respectively; for *Aquilegia vulgaris*: $r_s = 0.680$, -0.700, and -0.602; for *Aquilegia p. cazorlensis*: $r_s = 0.650$ and -0.655.

given nectar feature with microbial density were remarkably similar in the three species (Fig. 4). Percentage sucrose content declined, and percentage fructose increased, with increasing density of yeast cells. Around 65% (*H. foetidus* and *A. vulgaris*) and 35% (*A. p. cazorlensis*) of intraspecific, among-nectary variance in percentage content of these two sugars was accounted for by differences in microbial density. Nectars without yeasts almost invariably had only sucrose, while nectars with dense microbial communities either had only fructose (*H. foetidus*) or were fructose-dominated (*Aquilegia*). Intraspecific variation in total sugar concentration also ran parallel to differences in yeast density. In the two species with data available, sugar concentration declined significantly with increasing yeast density (Fig. 4). The effect was most pronounced in *H. foetidus*, where some nectars with very dense yeast communities contained <1% sugar.

DISCUSSION

By simultaneously assessing microbial abundance and nectar features at the scale of individual nectaries, we have been able to show that dense microbial communities frequently occur in the nectar of all species studied, and that among-nectary patchiness in microbial density is correlated with drastic changes in several nectar characteristics. Results were remarkably similar for the three species despite the contrasting habitat types, flowering time, and distance separating sampling sites. Dense yeast communities built up in nectar from early to late floral stages, at which time all the nectaries contained yeasts at densities often falling in the range 10^4–10^5 cells/mm^3. The increased incidence of yeasts with flower aging is most likely the combined consequence of a protracted cell multiplication period and increased cumulative probability of immigration due to prolonged exposure to bumble bee

visitation. In the study region, foragers of *Bombus terrestris* and *B. pratorum* often carry dense aggregations of viable yeasts in their mouthparts, and their probing of nectar causes microbial contamination and subsequent alteration of nectar characteristics, as shown by Canto et al. (2008) for *H. foetidus* (see also Brysch-Herzberg 2004).

Nectar characteristics varied extensively among nectaries of the same species (see also Herrera et al. 2006, Canto et al. 2007). The proportions of sucrose, glucose, and fructose in single-nectary nectar samples varied dramatically, falling all along the continuum running from pure sucrose to pure fructose. Present results verify the suggestion of Canto et al. (2007; see also Canto et al. 2008) that small-scale, extensive intraspecific variation in nectar sugar composition in these species in the field is the outcome of patchiness in microbial communities. The more dense the yeast community in a nectary, the greater the departure of nectar composition relative to the composition of nectar in "clean" nectaries, which were high in total sugar and consistently had sucrose as the dominant or only sugar. This closely agrees with the known sugar composition of clean nectars from plants of the three study species grown under controlled glasshouse conditions and not exposed to pollinator visitation (Vesprini et al. 1999, Canto et al. 2007). Alterations of this initial composition implied drastic declines in total sugar concentration and percentage sucrose content, and a concomitant increase in percentage fructose content, with increasing yeast cell density. In the most densely populated nectars, yeasts nearly completely depleted all sugar, as exemplified by some *H. foetidus* nectar samples that contained only residual amounts ($<1\%$) of fructose. Changes in nectar sugar concentration and composition with increasing yeast cell density are most parsimoniously explained as a consequence of the microbial hydrolysis of the disaccharide sucrose into the monosaccharides glucose and fructose, followed by metabolism of the resulting hexoses (Phaff et al. 1978). The highly nonstoichiometric proportions of fructose and glucose that characterize the most thoroughly transformed nectars (see also Canto et al. 2007, 2008), with proportions departing widely from the 1:1 ratio expected from simple sucrose hydrolysis, can be explained by preferential metabolism of glucose over fructose (Berthels et al. 2004).

The reduction in sugar concentration and alteration of the sugar profile associated with yeasts imply a deterioration of the nectar's food value and, possibly, also its attractiveness from the viewpoint of pollinators. Bumble bees are more sensitive to reductions in nectar sugar concentration than to reductions in nectar volume, quickly learning to disregard flowers with dilute nectar when others with more concentrated nectar are available (Cnaani et al. 2006). In addition, bumble bees and honey bees are responsive to variation in nectar sugar composition, preferring pure-sucrose nectars over pure-glucose or pure-fructose ones, or sugar mixtures where sucrose predominates over hexoses (Wykes 1952, Waller 1972, Loper et al. 1976, Roldán-Serrano and Guerra-Sanz 2005), which is in close accordance with the differential electrophysiological responses to different sugars of their mouthpart chemoreceptors (Whitehead and Larsen 1976). There are thus reasons to consider that the reduction in total sugar concentration, which at times amounts to a nearly complete obliteration of its food value, and the shift from sucrose to fructose dominance imply an exploitative degradation by yeasts of the food reward that mediates the plant–pollinator mutualism. It is therefore reasonable to expect that nectar alterations caused by yeasts will have a detrimental effect on plant fitness via reductions in the pollinator service received by individual flowers and whole plants, e.g., as a consequence of within-plant heterogeneity in nectar quality (Herrera et al. 2006). Factors other than nectar sugar characteristics, however, can come into play and complicate the plant–pollinator interaction when yeasts are present. For example, yeasty scents emanating from nectar could influence pollinator attraction to flowers (Raguso 2004, Goodrich et al. 2006). The only study known to us examining the possible effect of nectar yeast contamination on pollinator foraging provided no significant evidence that yeasts in nectar influence pollinator choice (Kevan et al. 1988), but it is not known whether contaminated and uncontaminated experimental flowers actually differed in nectar characteristics. Further investigations are obviously needed to confirm, on a species-by-species basis, the hypothesis that nectar degradation caused by yeasts has some effects on pollinator behavior and plant reproductive success.

The possible biological significance of nectar-inhabiting microorganisms was once played down on the argument that antimicrobial substances in nectar would suppress their growth (Gilliam et al. 1983, Kevan et al. 1988; but see Eisikowitch et al. 1990). This reasoning, however, is nearly as implausible as interpreting the occurrence of allelochemicals in leaves as an indication of the biological insignificance of folivory. Instead, the occurrence of potentially defensive substances in nectar can precisely attest its susceptibility to the deleterious action of non-mutualistic consumers, as implicated by the antimicrobial hypothesis for the presence of toxic substances in nectar (Adler 2000). A key assumption of this hypothesis is that microorganisms can actually have some deleterious effects on plants via extensive nectar degradation. Our results provide supporting evidence by showing that nectar yeast communities can become sufficiently dense to (1) drastically alter nectar sugar composition, and perhaps more importantly, (2) compete for sugar with mutualistic consumers, reducing the nectar's food value down to nearly zero levels. In addition, results for *H. foetidus* also illustrate that toxic substances in nectar do not confer a perfect protection against microbial consumers (see also Manson et al. 2007), in the same way as allelochemicals in other plant

parts do not guarantee protection against specialized herbivores. *Helleborus foetidus* nectar contains protoanemonin (R. Pérez, I. M. García, and C. M. Herrera, *unpublished data*), an unsaturated lactone that inhibits the growth of many generalist, widespread yeasts, including some nectarivorous ones (Mares 1987). The observation that some of the yeasts associated with *H. foetidus* nectar and its bumble bee pollinators are specialists (Brysch-Herzberg 2004) could explain their tolerance to protoanemonin, just like specialist herbivores are immune to allelochemicals of their host plants (Bowers and Puttick 1988). No information is available on the possible presence of toxic substances in the nectar of *Aquilegia*.

It is not possible at present to evaluate the generality of our results. There are, however, some suggestions that microbial communities in nectar are probably more frequent and considerably more consequential for plant–pollinator mutualisms, than hitherto acknowledged. First, the few microbiological surveys that have quantified the incidence of yeasts in nectar samples from wild plants have invariably reported frequencies of occurrence as high as those found here (reviewed in Brysch-Herzberg 2004). Second, reports of nectar changes with flower age resembling those shown here to be associated with increasing yeast densities are not rare. These include steady decline in percentage sucrose content, reduction of the sucrose/hexoses ratio, increasing non-stoichiometry of glucose and fructose, decline of total sugar concentration, or some combination of these (Loper et al. 1976, Petanidou et al. 1996, Nepi et al. 2003, Roldán-Serrano and Guerra-Sanz 2004). And third, the nectar of many species is characterized by very unequal proportions of glucose and fructose (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 characteristic of the plants themselves (Canto et al. 2008; see *Results*). Taken together, these observations suggest that phenomena similar to those reported here, whereby dense yeast communities can alter substantially the sugar composition of floral nectar and depress its food value, probably are more frequent in nature than currently acknowledged. Were this expectation verified by future studies, nectar yeast communities could eventually emerge as an invisible, yet influential "dark matter" in plant–pollinator mutualisms.

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