Yeasts in nectar of an early-blooming herb: sought by bumble bees, detrimental to plant fecundity

Carlos M. Herrera,[1] María I. Pozo, and Mónica Medrano

Estación Biológica de Doñana, CSIC (Consejo Superior de Investigaciones Científicas), Américo Vespucio s/n, E-41092 Sevilla, Spain

Abstract. Through their effects on physicochemical features of floral nectar, nectar-dwelling yeasts can alter pollinator behavior, but the effect of such changes on pollination success and plant reproduction is unknown. We present results of experiments testing the effects of nectar yeasts on foraging patterns of captive and free-ranging bumble bees, and also on pollination success and fecundity of the early-blooming, bumble bee-pollinated *Helleborus foetidus* (Ranunculaceae). Under controlled experimental conditions, inexperienced *Bombus terrestris* workers responded positively to the presence of yeasts in artificial sugar solutions mimicking floral nectar by visiting proportionally more yeast-containing artificial flowers. Free-ranging bumble bees also preferred yeast-containing nectar in the field. Experiments conducted in two different years consistently showed that natural and artificial nectars containing yeasts were more thoroughly removed than nectars without yeasts. Experimental yeast inoculation of the nectar of *H. foetidus* flowers was significantly associated with reductions in number of pollen tubes in the style, fruit set, seed set, and mass of individual seeds produced. These results provide the first direct evidence to date that nectar yeasts can modify pollinator foraging patterns, pollination success, and the quantity and quality of seeds produced by insect-pollinated plants.

Key words: Bombus terrestris; *bumble bee; floral nectar;* Helleborus foetidus; Metschnikowia reukaufii; *nectar yeasts; pollination; pollinator behavior; seed production; Sierra de Cazorla, southeastern Spain.*

Introduction

The majority of angiosperms are pollinated by animals, which are enticed to flowers by some reward provided by the plant. Nectar is the commonest type of floral reward offered to pollinators (Simpson and Neff 1983), and a huge body of literature has built up focusing on patterns of nectar secretion, availability, and composition. Variation in these factors may condition the identity and foraging behavior of pollinators and, ultimately, influence the pollination success and fecundity of plants (Nicolson et al. 2007). Natural variation in ecologically consequential nectar traits (e.g., sugar and amino acid concentration) not only reflects intrinsic features of plant species and individuals, but also depends on the action of a variety of extrinsic abiotic and biotic factors unrelated to the plants themselves (Corbet 1978, Nicolson et al. 2007, Baude et al. 2011). One infrequently acknowledged biotic factor potentially altering nectar features is nectar-dwelling yeasts, which recent studies have shown to abundantly populate animal-pollinated flowers worldwide (Brysch-Herzberg 2004, Herrera et al. 2009). Among other effects, nectar yeasts can alter the composition and concentration of sugars and amino

acids in nectar, contribute to the emission of floral volatiles, and warm the nectar in relation to the surrounding air (Raguso 2004, Herrera et al. 2008, Wiens et al. 2008, Herrera and Pozo 2010, Canto and Herrera 2012, Peay et al. 2012). Microbial alterations of the intrafloral environment might influence the behavior of pollinators, which could in turn impinge on pollination success and fecundity of plants. Investigating how nectar-dwelling yeasts alter pollinator behavior and plant fitness is essential for knowing whether these widespread fungal microbes play some ecological role in plant–pollinator mutualisms, but this aspect remains unexplored. We present here experimental evidence showing that the presence of nectar yeasts alters pollinator behavior and influences pollination success and maternal fecundity in an early-blooming plant.

Methods

Study system

This study focuses on the tripartite system formed by the early-blooming perennial herb *Helleborus foetidus* (Ranunculaceae), the yeasts inhabiting its floral nectar, and the bumble bees that pollinate the plant and disseminate the yeasts. *Helleborus foetidus* inflorescences are produced in early winter, each bearing 20–75 flowers that open gradually over the following 1.5–2.5 months. Individual flowers are hermaphroditic, last for 1–3 weeks, and are pollinated by bumble bees, mostly

Bombus terrestris and *B. pratorum*. Flowers are apocarpous, with 1–3 independent carpels, and contain five nectaries shaped like flattened horns and forming a distinct ring between stamens and perianth (see Herrera et al. 2008: Plate 1a). Each nectary may accumulate up to 5 µL of sucrose-dominated nectar, with a sugar concentration ranging between 25% and 55% (throughout this paper, concentrations are given on a mass-to-mass basis). Further details on *H. foetidus* floral biology and pollination can be found in Herrera and Soriguer (1983), Vesprini et al. (1999), Herrera et al. (2001), and Vesprini and Pacini (2010). The nectar commonly harbors dense populations of specialized nectarivorous yeasts, principally *Metschnikowia reukaufii* (Metschnikowiaceae, Saccharomycetales), the colonizing inocula of which are brought to flowers by foraging bumble bees (Brysch-Herzberg 2004).

Captive bumble bee responses to yeasts in artificial nectar

This laboratory experiment was designed to elucidate whether inexperienced bumble bees responded to yeast presence in nectar. We used workers of commercial *Bombus terrestris* colonies kept in an unheated greenhouse and connected to a 60 × 60 × 60 cm flight cage. The flight cage held a 42 × 42 cm green plexiglas board where a grid of 1 mm diameter and 3 mm deep wells had been drilled. Artificial "flowers" were created by sticking yellow-painted, 2 cm diameter round plexiglas pieces around the entrance of 12 wells. Flowers were arranged into four experimental groups, each containing three flowers, located near the corners of the board. Pollen was continuously available to bees inside the hive, and a sterile 33% sucrose–16% glucose–16% fructose solution was supplied in a dripping bag outside the nest. Prior to the experiments, bees lacked experience with either natural flowers or the experimental arena, and were subjected to a training period before engaging them in the choice trials. Individuals were allowed to access the flight cage singly, and were left there for 30 minutes with access to artificial flowers. The latter had been cleaned with ethanol and filled with 6 µL of 30% sucrose sterile solution. Bees probing the sucrose solution of some flower during the training phase were marked individually with a numbered tag on the back and subsequently were used in choice trials.

In each trial, a bee was exposed to artificial flowers filled with artificial nectar with (treatment; two three-flower groups) or without (control; two three-flower groups) yeasts. Each flower in the treatment group was filled with 3 µL of a suspension of living yeast cells in artificial nectar consisting of 12% sucrose, 0.3% glucose, 0.3% peptone, and 0.3% DIFCO yeast extract (Becton, Dickinson and Company, 38800 Le Pont de Claix, France). Control flowers received 3 µL of the same, albeit yeast-free, medium. The use of low-sugar artificial nectar in choice trials was motivated by our expectation that reducing the energy reward per nectar volume unit could promote between-flower movements by bees and improve the likelihood of detecting responses to yeasts. Sugar concentrations can alter bumble bee responses to other nectar components (Gegear et al. 2007), but the correspondence between our laboratory and field results (see *Results*) suggests that differences in sugar concentration do not alter bee response to yeasts in this system. Artificial nectar of treatment flowers contained 8×10^3 yeast cells/mm^3, which is near the lower limit of the natural range of yeast densities in *H. foetidus* nectar (range 10^3–10^6 cells/mm^3; Herrera et al. 2008, 2010). Yeast cells in the artificial nectar of treatment flowers belonged to one of three species, namely *Metschnikowia reukaufii* (21 trials involving 12 different bees), *M. gruessii* (21 trials, 13 bees), and *Candida bombi* (5 trials, 3 bees). The three species occur naturally in nectar of *H. foetidus*, although *M. reukaufii* is by far the most abundant (Brysch-Herzberg 2004, Herrera et al. 2010). Whenever possible, each bee was tested twice on the same day, the spatial location of treatment and control flower groups being reversed between trials. Between two trials, all flowers were emptied and cleaned with ethanol and individual bees were kept in a flight cage similar to the one used for trials. After completing the two trials, bees were not returned to the colony until all the trials of the day were completed to prevent short-term communication with other bees to be tested later on the same day. Maximum trial duration, counted from the time the bee probed the first flower, was set to 10 minutes.

Bumble bee behavior during trials was video-recorded using a digital camera. Video-recorded sessions allowed us to distinguish effective flower visits, where nectar was actually probed and consumed, from those where bees hovered in front of flowers without extending the proboscis and actually probing nectar. For each trial, we recorded the number of effective visits to control (without yeasts) and treatment (with yeasts) flowers, as well as the total time spent consuming nectar from each flower type. Because both measures were closely correlated across trials, we will report only the proportion of flowers visited. Trials where experimental bees did not effectively probe at least one flower of each type were discarded from analyses.

Field experiment: yeast effect on artificial nectar removal

This experiment aimed to test whether free-ranging, wild bumble bees discriminated between flowers with yeast-containing and yeast-free artificial nectar. Experiments were conducted at a *H. foetidus* population in the Sierra de Cazorla, southeastern Spain ("Las Navillas," 1220 m elevation). Plants grew there in the understory of a mature *Pinus nigra* woodland. *Bombus terrestris* and *B. pratorum* were the main floral visitors and pollinators of *H. foetidus* at the site, and *M. reukaufii* was the dominant nectar yeast. The effect of yeasts on removal of artificial nectar by wild bumble bees was tested on 20, 22, and 24 March 2012, when most plants were at peak blooming. On each date, 10–13 widely spaced *H.*

foetidus plants were randomly chosen, and 4–6 flowers were marked on a single inflorescence of each plant and assigned to either control or treatment groups. Because flowers had been exposed to natural bumble bee visitation during the preceding days, nectaries contained no or very little (<1 μL) nectar. Early in the morning, all nectaries of control flowers were filled with 3.5 μL of artificial nectar consisting of 13% sucrose, 13% glucose, 13% fructose, 0.5% peptone, 0.2% $MgSO_4$, and 0.3% KH_2PO_4. Nectaries of treatment flowers received the same volume of a fresh suspension of living *M. reukaufii* cells in the same medium. Suspensions with 1×10^4, 2×10^4, and 10×10^4 cells/mm^3 were used on different dates. Although *H. foetidus* produces sucrose-dominated nectar, nectar of flowers exposed to natural bumble bee visitation in the field consists of variable mixtures of glucose, fructose, and sucrose as a consequence of the metabolic activity of nectar-dwelling yeasts (Canto et al. 2008, 2011, Herrera et al. 2008). Because free-ranging bumble bees foraging on *H. foetidus* flowers at Las Navillas site were probably most familiar with mixed-sugar nectars, the artificial nectar used in this experiment consisted of a sugar mixture rather than sucrose alone. After nectary filling, flowers remained exposed to natural visitation by bumble bees throughout the daytime and were collected before sunset (exposure period ~8 h). Mean volume of nectar remaining in the nectaries of treatment (artificial nectar + yeasts) and control (artificial nectar) flowers was determined using calibrated micropipettes.

Field experiment: yeast effects on pollination and fecundity

The main objective of this experiment was to determine whether, under field conditions, flowers of *H. foetidus* with yeast-contaminated and yeast-free natural nectar differed in pollination success and fecundity. The design prioritized resemblance to a natural situation in which the nectar of some flowers was colonized by yeasts while that of other flowers was not. Secondarily, the experiment also allowed us to test whether free-ranging bumble bees discriminated between yeast-free and yeast-containing flowers when yeasts occurred in natural nectar (in contrast to other experiments, where artificial nectar was used). The experiment was conducted during February–June 2011 at the same Las Navillas population where yeast effect on artificial nectar removal was tested in 2012. Inflorescences from widely spaced plants were selected at the beginning of the flowering season, and were bagged after removing any open flower, which ensured that only flowers with yeast-free nectar would be present thereafter ($N = 14$ inflorescences bagged). Two weeks later, newly open flowers within bagged inflorescences were randomly assigned to one of two groups, namely with yeasts excluded or with yeasts added ($N = 33$ flowers in total in each group). All flowers had nectaries full of nectar to the rim. Every nectary of all flowers in

the yeasts-added group received a small starting inoculum (0.8 μL, ~15% of nectary content) of a fresh *M. reukaufii* cell suspension (1.9×10^4 cells/mm^3), after a similar volume of nectar was removed (flowers with "yeasts added," hereafter). The vehicle for the inoculum was a 1:2 blend of sterile, natural *H. foetidus* nectar from local plants and artificial nectar as previously described for the 2012 experiment. Flowers in the yeasts-excluded group were handled identically to those with yeasts added, except that nectaries received neither yeasts nor the vehicle used in inoculations. To allow for the natural buildup of yeast populations in inoculated flowers, which would simulate population growth after initial colonization, all flowers were kept bagged for two days after inoculation, then were unbagged and exposed for two days to natural pollinator visitation, and were bagged again to preclude further visits. Nectar composition of the two experimental groups at the time of exposure to pollinators was evaluated by replicating treatments in the laboratory and analyzing the nectar two days past inoculation, allowing yeasts to metabolize nectar components as they would in the field manipulation. At that time, the combined sucrose, fructose, and glucose concentration of yeast-added *H. foetidus* nectar had been reduced by 17.5% relative to yeast-free nectar, and the small amount of peptone introduced with the inoculum was not detectable in yeast-treated nectar. Had non-inoculated flowers also received sterile vehicle for the sake of design, persistence of artificial peptone would have led to a spurious difference between the nectars of the two treatments at the time of exposure to pollinators.

The volume of nectar remaining in every nectary of flowers with yeasts added and excluded ($N = 177$ nectaries in each group) at the end of the two-day exposure to pollinator visitation was measured with calibrated micropipettes, and an average value was obtained per flower. Withered styles of all experimental flowers were collected 10 days after completing exposure to pollinator visitation, and the number of fully developed pollen tubes in each of them was counted using epifluorescence microscopy (Herrera 2002). Fecundity of experimental flowers was assessed in mid-June, shortly before fruits would have dehisced naturally. We determined the proportion of carpels in each flower that eventually produced mature follicles with at least one sound seed ("fruit set," hereafter). For seed-containing follicles, we estimated the proportion of initial ovules in the carpel that eventually produced seeds ("seed set," hereafter). All seeds produced by experimental flowers ($N = 952$) were collected, dried at room temperature, and weighed individually.

Data analysis

Results from trials with captive bumble bees were analyzed by fitting an intercept-only mixed model to the proportion of total visits paid to flowers with yeasts, treating individual bees as a random factor. Computa-
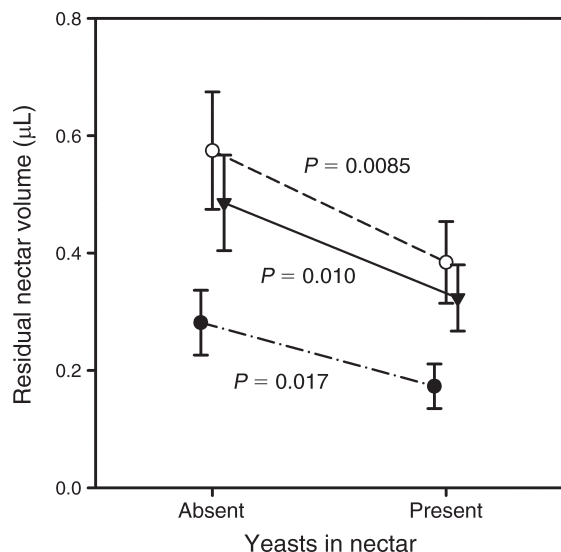
FIG. 1. Interaction graph depicting the effects of experimental date and presence of yeasts in artificial nectar (present, absent) on mean residual volume per nectary in *Helleborus foetidus* flowers after ~8 h posttreatment exposure to natural visitation by free-ranging bumble bees in the 2012 field experiments. Symbols are model-adjusted, least-squares means ± SE. *P* values are from tests of significance for the simple main effects involved. Each line corresponds to a different experimental date.

tions were performed using SAS procedure MIXED and restricted maximum likelihood estimation. Nonrandomness of flower selection was tested by comparing the intercept estimated by the model with 0.5, the expected value under random flower selection, using a standard two-tailed Student's *t* test.

In the 2011 and 2012 field experiments, significance of the effect of yeast presence on nectar consumption (estimated inversely by residual nectar volume) and pollination- and fecundity-related parameters were tested by fitting generalized linear mixed models to the data with SAS procedure GLIMMIX (SAS Institute 2006). In the 2011 experiments, yeast treatment was the only fixed effect included. Binomial error distributions were used for proportions (fruit set, seed set). In the 2012 experiments, yeast treatment, experimental date, and their interaction were the fixed effects considered. Plants were always included as a random effect in models. In *H. foetidus*, within-flower variance of reproductive parameters associated with repeated structures within flowers (styles, carpels) characteristically exceeds variance due to differences between flowers in the same plant (e.g., Herrera [2002] for pollen tubes per style). This suggests considerable functional autonomy of homologous structures in the same flower and provides biological justification for not including flowers as another random effect in models analyzing response variables that refer to such repeated structures (pollen tubes per styles, seed set per carpel). The incorporation of flowers as an additional random effect in models,

however, only slightly impaired the significance levels of some tests without altering the main conclusions of the experiments (results not shown). Analyses of residual nectar volume (2011 and 2012 experiments) were based on mean per flower values. Negative binomial error distribution provided the best fit to residual nectar data and this error distribution was used in these analyses. Model-adjusted, least-squares cell means of response variables for different factor level combinations and their standard errors were obtained with the LSMEANS statement and the ILINK option. In the 2012 experiments, significance of "simple main effects" (i.e., the effects of a given factor at different levels of the other factor; Pedhazur 1982) was tested with the SLICE option.

RESULTS

Captive bumble bees preferred yeast-containing artificial flowers

When exposed to a similar number of flowers containing artificial nectar with and without yeasts, worker bumble bees included in their foraging bouts a significantly greater proportion of yeast-containing flowers than expected if they had foraged at random with regard to yeast presence (proportion of visits to yeast-containing flowers = 0.588 ± 0.027, mean ± SE; t = 3.26, df = 27, P = 0.003). Foraging responses of bumble bees to yeasts depended on yeast species, as revealed by separate analyses of trials involving the two yeasts with larger sample sizes (*M. reukaufii* and *M. gruessii*). Trials involving *M. reukaufii* showed significant yeast effects on the proportion of visits to yeast-containing flowers (0.660 ± 0.055, t = 2.91, df = 11, P = 0.014). In trials involving *M. gruessii*, the proportion of visits to yeast-containing flowers was lower and did not depart significantly from that expected by chance (0.544 ± 0.028, t = 1.57, df = 12, P = 0.14).

Wild bumble bees preferentially consumed yeast-containing artificial nectar

The set of single-day experiments conducted in March 2012 revealed consistent, significant effects of yeasts on the consumption of artificial nectar by bumble bees foraging naturally on *H. foetidus* flowers. After ~8 h of flower exposure to free-ranging bumble bees, there was a strong, highly significant effect of yeasts on mean volume of residual nectar per nectary (F = 19.20, df = 1, 110, P < 0.0001). Although the mean volume of residual nectar varied significantly among experimental dates (F = 5.15, df = 2, 31, P = 0.011), the effect of yeasts remained consistent across dates, as denoted by nonsignificance of the yeasts × date interaction effect (F = 0.06, df = 2, 110, P = 0.94). On every date, flowers with yeasts had their nectaries more thoroughly depleted by the end of the day than did flowers without yeasts (Fig. 1), which denoted preferential consumption of yeast-containing nectar by bumble bees visiting experimental plants. This preference was unaffected by variation

TABLE 1. Summary of generalized linear mixed models testing for the effects of yeasts on response variables related to nectar removal during exposure to pollinators, pollination success, and maternal fecundity of experimental *Helleborus foetidus* flowers, in 2011 experiments.

Dependent variable	Yeasts excluded	Yeasts added	df	F	P
Residual nectar (μL)	2.53 ± 0.34	2.17 ± 0.34	1, 51	6.34	0.015
Number of pollen tubes per style	9.6 ± 1.2	7.8 ± 1.2	1, 126	13.46	0.0004
Fruit set (%)	95.4 ± 3.3	75.7 ± 11.5	1, 50	9.22	0.0038
Seed set (%)	72.0 ± 6.8	56.7 ± 8.4	1, 98	23.18	<0.0001
Individual seed mass (mg)	16.1 ± 0.5	15.7 ± 0.5	1, 937	9.28	0.0024

Notes: Residual nectar refers to the mean volume remaining per nectary in experimental flowers by the end of the two-day exposure to natural pollinator visitation, which provides an inverse estimate of removal by free-ranging bumble bees. Values (mean ± SE) for yeasts refer to model-adjusted, least-squares means and associated standard errors for treatment levels.

among dates in cell density of the yeast suspension used in the experiments.

Yeasts enhanced nectar consumption, impaired pollination, and reduced fecundity

In the experiment conducted in 2011, flower inoculation with yeasts had a significant effect on the volume of nectar remaining in nectaries at the end of the two-day exposure of flowers to bumble bee visitation. As found in 2012 for nectaries filled with artificial nectar, presence of yeasts was associated with a significant reduction in the amount of natural nectar remaining in the nectaries after exposure to pollinators (Table 1), which likewise demonstrates a more thorough removal by bumble bees of yeast-contaminated nectar in comparison to nectar without yeasts. The inoculation of *H. foetidus* flowers with yeasts had statistically significant effects on the number of pollen tubes per style, fruit set, seed set, and individual seed mass (Table 1). In all cases, the effects were detrimental to pollination success and fecundity, as they involved reductions in number of pollen tubes, fruit set, seed set, and individual seed size (Table 1).

DISCUSSION

Recent observations that nectar yeasts can significantly alter the physicochemical characteristics of floral nectar and the intrafloral environment prompted the hypothesis that their presence at flowers can influence pollinator behavior and translate into measurable pollination and fecundity consequences for plants (Herrera et al. 2008, Herrera and Pozo 2010). One possible scenario under this hypothesis, for example, would be that yeasts impair pollinator service and thus behave as detrimental parasites of the plant–pollinator mutualism, but the reverse situation in which yeasts improve pollinator service and plant reproduction may also be envisaged (Goodrich et al. 2006, Wiens et al. 2008, Herrera and Pozo 2010). Contrasting ecological and evolutionary implications will ensue depending on whether nectar yeasts play consequential or inconsequential roles for plants, pollinators, or both, and on whether the consequences are favorable or unfavorable. Investigating the fitness consequences for plants of nectar-dwelling yeasts and the mechanisms involved is therefore central to a better understanding of the

ecological role of these fungal microbes in plant–pollinator interactions. Using a combination of laboratory and field experiments involving artificial and natural flowers, as well as natural and artificial nectar, we have shown here that, through effects on bumble bee pollinators, nectar yeasts may have important consequences for the fecundity of *Helleborus foetidus*.

The only study known to us examining the possible effect of nectar yeasts on pollinator foraging failed to find evidence of yeasts influencing flower choice by honey bees (Kevan et al. 1988), although yeast density in nectar of contaminated flowers was not known and could have been too low to induce a discriminatory response. The present study has shown that, under controlled experimental conditions, inexperienced *Bombus terrestris* workers detected the presence of yeasts in artificial nectar and responded positively by paying proportionally more visits to yeast-containing flowers. Interestingly, the magnitude of the response depended on the yeast species involved, preference being greatest for artificial nectars containing *Metschnikowia reukaufii*, the dominant nectar-living yeast in flowers of *H. foetidus* and many other plants (Brysch-Herzberg 2004, Pozo et al. 2011). The preference exhibited by captive bumble bees for *M. reukaufii*-containing nectar was corroborated in a real-world scenario by the field experiments. Despite being conducted in different years and with differences in design, length of exposure time to pollinators, and type of nectar involved (natural vs. artificial), the two field experiments consistently demonstrated that flowers with nectaries containing *M. reukaufii* populations had their nectar most thoroughly depleted by bumble bees. Because all nectaries were similarly filled with nectar at the beginning of the exposure period, and nectaries with and without yeasts secrete nectar at similar rates (Canto et al. 2011), the most parsimonious explanation for yeast-related differences in residual amount of nectar is that bumble bee foragers discriminated between flower types and preferred nectar with *M. reukaufii*. Rigorously testing this interpretation would require direct observations of insect visits to treated and control flowers, but unfortunately this possibility was precluded by the extraordinarily low pollinator visitation rates to *H. foetidus* flowers (Herrera et al. 2001).

Reports

Bombus terrestris is known to discriminate between flowers differing in temperature, scent composition, and nectar sugar concentration and composition (Dyer et al. 2006, Whitney et al. 2008, Suchet et al. 2011). Captive and wild bumble bees in our study thus could have relied for yeast detection on some cue correlated with presence of yeasts in nectar, such as increased temperature, volatile emissions, yeast metabolites (e.g., ethanol), taste alterations, or sugar and amino acids profiles (Herrera et al. 2008, Herrera and Pozo 2010, Canto et al. 2011, Peay et al. 2012). At present, we can only speculate about the sensory mechanism(s) involved in yeast detection by bumble bees, and their elucidation will require additional experimentation. Regardless of the proximate cues involved in yeast detection, however, the innate preference of *B. terrestris* for nectars with *M. reukaufii* raises some intriguing questions about the possible adaptive value of this behavior. Considering that energetic constraints are a key driver of bumble bee behavior (Heinrich 1979), and the fact that the metabolism of dense *M. reukaufii* populations reduces the energetic reward of nectar as shown in this study and in Herrera et al. (2008), the preference for nectars harboring this yeast would seem maladaptive. Factors other than energetics may explain the attraction exhibited by bumble bees toward *M. reukaufii*-containing nectars. For example, increased availability of vitamins, amino acids, or metabolites (e.g., ethanol, antibiotics) in nectar with yeasts might compensate for reduced energetic reward, within certain limits. The possibility that bumble bee behavior is maladaptive also should be considered. For example, yeasts could be manipulating bumble bee behavior to their benefit by luring them and making the bees help them to disperse to new flowers (T. Fukami, *personal communication*).

Occurrence of yeasts in flowers significantly reduced all pollination and fecundity parameters considered. Number of pollen tubes per style, probability of carpels producing a seed-bearing follicle, probability of ovules producing a seed, and individual seed mass, all were significantly smaller for flowers with yeasts. These results clearly denote a distinct fecundity disadvantage to plants of harboring nectar-dwelling yeasts in their flowers. We tentatively interpret these findings as the combined consequence of the following: (1) during our 2011 study period, *H. foetidus* maternal fecundity was most likely limited by pollen quality rather than pollen quantity; and (2) the preference of pollinators for yeast-containing flowers may have led to longer individual visits to these flowers, resulting in impaired pollination quality. Although *H. foetidus* flowers are self-compatible, selfed flowers produce fewer and smaller seeds than outcrossed ones (Vesprini and Pacini 2000). Longer visits by pollinators to yeast-containing flowers would enhance the probability of "facilitated autogamy" within flowers (Owen et al. 2007) and, hence, the proportion of self pollen in stigmatic pollen loads,

which would explain the reduction in the number and size of seeds produced.

We have considered in this study only the maternal component of reproduction, but in hermaphrodite plants the effects of nectar yeasts on pollinator behavior may also influence the paternal component through effects on pollen export and seed siring success (Stanton et al. 1986), a possibility that deserves investigation. Despite this acknowledged limitation of our study, results have clearly shown that the yeast *M. reukaufii* is more than a neutral, ecologically inconsequential element in the system formed by early-blooming *H. foetidus* and its bumble bee pollinators, because its presence has measurable effects on both the pollinators and the plants. We hypothesize that the eventual effects of nectar-dwelling yeasts on plant maternal fitness will be strongly context dependent, depending on a delicate interplay of several ecological factors. These factors will most likely include the species of yeast involved, its degree of attractiveness to the plants' main pollinators, and the extent (limited vs. non-limited) and nature (quality vs. quantity limitation) of pollen limitation experienced by the plants. Because these factors will most likely vary in time and space, concomitant variations are expected in the role played by nectar yeasts in plant reproduction. For example, in situations in which seed production is limited by pollen quantity rather than quality, a greater attractiveness of yeast-containing flowers to pollinators could result in greater seed production. The dissection of the tripartite relationship linking plants, nectar yeasts, and pollinators offers new angles for deepening our understanding of the ecology of plant reproduction.

Concluding Remarks

One important implication of this study is that some of the parameters customarily examined in studies of pollinator behavior, pollination success, and plant maternal fecundity may sometimes reflect the cryptic influence of nectar-dwelling yeasts rather than, or in addition to, intrinsic pollinator or plant characteristics. In *H. foetidus*, for example, reported variation between flowers, individuals, and populations in number of pollen tubes in the style (Herrera 2002) might be partly accounted for by the patchy distribution of *M. reukaufii* at different spatial scales. Nectar yeast abundance and variability levels comparable to or greater than those generated experimentally in this study seem to be the rule in natural habitats worldwide (Brysch-Herzberg 2004, Herrera et al. 2008, Belisle et al. 2012, Canto and Herrera 2012). This implies that the functional links between nectar yeasts, pollinator behavior, and plant reproductive success found here may also hold for other animal-pollinated plants elsewhere. Explicit consideration of this possibility in future pollination ecology studies perhaps may disclose an unrecognized layer of microbially mediated complexity associated with some plant–pollinator interfaces.

LITERATURE CITED

Baude, M., J. Leloup, S. Suchail, B. Allard, D. Benest, J. Mériguet, N. Nunan, I. Dajoz, and X. Raynaud. 2011. Litter inputs and plant interactions affect nectar sugar content. Journal of Ecology 99:828–837.

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

Brysch-Herzberg, M. 2004. Ecology of yeasts in plant–bumblebee mutualism in Central Europe. FEMS [Federation of European Microbiological Societies] Microbiology Ecology 50:87–100.

Canto, A., and C. M. Herrera. 2012. Micro-organisms behind the pollination scenes: microbial imprint on floral nectar sugar variation in a tropical plant community. Annals of Botany 110:1173–1183.

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

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

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

Dyer, A. G., H. M. Whitney, S. E. J. Arnold, B. J. Glover, and L. Chittka. 2006. Bees associate warmth with floral colour. Nature 442:525.

Gegear, R. J., J. S. Manson, and J. D. Thomson. 2007. Ecological context influences pollinator deterrence by alkaloids in floral nectar. Ecology Letters 10:375–382.

Goodrich, K. R., M. L. Zjhra, C. A. Ley, and R. A. Raguso. 2006. When flowers smell fermented: the chemistry and ontogeny of yeasty floral scent in pawpaw (*Asimina triloba*: Annonaceae). International Journal of Plant Sciences 167:33–46.

Heinrich, B. 1979. Bumblebee economics. Harvard University Press, Cambridge, Massachusetts, USA.

Herrera, C. M. 2002. Censusing natural microgametophyte populations: variable spatial mosaics and extreme fine-graininess in winter-flowering *Helleborus foetidus* (Ranunculaceae). American Journal of Botany 89:1570–1578.

Herrera, C. M., A. Canto, M. I. Pozo, and P. Bazaga. 2010. Inhospitable sweetness: nectar filtering of pollinator-borne inocula leads to impoverished, phylogenetically clustered yeast communities. Proceedings of the Royal Society B 277:747–754.

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

Herrera, C. M., I. M. García, and R. Pérez. 2008. Invisible floral larcenies: microbial communities degrade floral nectar of bumble bee-pollinated plants. Ecology 89:2369–2376.

Herrera, C. M., and M. I. Pozo. 2010. Nectar yeasts warm the flowers of a winter-blooming plant. Proceedings of the Royal Society B 277:1827–1834.

Herrera, C. M., A. M. Sánchez-Lafuente, M. Medrano, J. Guitián, X. Cerdá, and P. Rey. 2001. Geographical variation in autonomous self-pollination levels unrelated to pollinator service in *Helleborus foetidus* (Ranunculaceae). American Journal of Botany 88:1025–1032.

Herrera, C. M., and R. C. Soriguer. 1983. Inter- and intra-floral heterogeneity of nectar production in *Helleborus foetidus* L. (Ranunculaceae). Botanical Journal of the Linnean Society 86:253–260.

Kevan, P. G., D. Eisikowitch, S. Fowle, and K. Thomas. 1988. Yeast-contaminated nectar and its effects on bee foraging. Journal of Apicultural Research 27:26–29.

Nicolson, S. W., M. Nepi, and E. Pacini, editors. 2007. Nectaries and nectar. Springer, Dordrecht, The Netherlands.

Owen, K., G. Vaughton, and M. Ramsey. 2007. Facilitated autogamy and costs of selfing in the perennial herb *Bulbine bulbosa* (Asphodelaceae). International Journal of Plant Science 168:579–585.

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

Pedhazur, E. J. 1982. Multiple regression in behavioral research. Second edition. Holt, Rinehart and Winston, Fort Worth, Texas, USA.

Pozo, M. I., C. M. Herrera, and P. Bazaga. 2011. Species richness of yeast communities in floral nectar of southern Spanish plants. Microbial Ecology 61:82–91.

Raguso, R. A. 2004. Why are some floral nectars scented? Ecology 85:1486–1494.

SAS Institute. 2006. The GLIMMIX procedure, June 2006. SAS Institute, Cary, North Carolina, USA.

Simpson, B. B., and J. L. Neff. 1983. Evolution and diversity of floral rewards. Pages 142–159 *in* C. E. Jones and R. J. Little, editors. Handbook of experimental pollination biology. Van Nostrand Reinhold, New York, New York, USA.

Stanton, M. L., A. A. Snow, and S. N. Handel. 1986. Floral evolution: attractiveness to pollinators increases male fitness. Science 232:1625–1627.

Suchet, C., L. Dormont, B. Schatz, M. Giurfa, V. Simon, C. Raynaud, and J. Chave. 2011. Floral scent variation in two *Antirrhinum majus* subspecies influences the choice of naïve bumblebees. Behavioral Ecology and Sociobiology 65:1015–1027.

Vesprini, J. L., M. Nepi, and E. Pacini. 1999. Nectary structure, nectar secretion patterns and nectar composition in two *Helleborus* species. Plant Biology 1:560–568.

Vesprini, J. L., and E. Pacini. 2000. Breeding systems in two species of the genus *Helleborus* (Ranunculaceae). Plant Biosystems 134:193–197.

Vesprini, J. L., and E. Pacini. 2010. Pollination ecology in sympatric winter flowering *Helleborus* (Ranunculaceae). Flora 205:627–632.

Whitney, H. M., A. Dyer, L. Chittka, S. A. Rands, and B. J. Glover. 2008. The interaction of temperature and sucrose concentration on foraging preferences in bumblebees. Naturwissenschaften 95:845–850.

Wiens, F., A. Zitzmann, M. A. Lachance, M. Yegles, F. Pragst, F. M. Wurst, D. von Holst, S. L. Guan, and R. Spanagel. 2008. Chronic intake of fermented floral nectar by wild tree shrews. Proceedings of the National Academy of Sciences USA 105:10426–10431.

Reports