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POLLINATOR FORAGING MODIFIES NECTAR SUGAR COMPOSITION IN *HELLEBORUS FOETIDUS* (RANUNCULACEAE): AN EXPERIMENTAL TEST[1]

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We experimentally tested the hypothesis that the extensive within-plant variation of nectar sugar composition in *Helleborus foetidus* (Ranunculaceae) and other species results from differences between flowers and nectaries in pollinator visitation history. Experiments were conducted to mimic single-nectary visits by wild-caught individuals of the main bee pollinators of *H. foetidus*, which were assayed for their capacity to modify the sugar composition of natural and artificial nectar. Experimental nectar probing with bee mouthparts induced extensive changes in proportional sugar composition 48 h after treatment, and bee taxa differed widely in their effects. Nectar probing by *Andrena*, medium-sized Anthophoridae, *Apis mellifera,* and *Lasioglossum* had no subsequent effects on nectar sugar composition, while probing by *Bombus terrestris* and *B. pratorum* induced an extensive reduction in percentage sucrose, a marked increase in percentage fructose, and a slight increase in percentage glucose. Results support the hypothesis that stochastic variations among flowers or nectaries in the taxonomic identity of recent visitors and their relative visitation frequencies may eventually generate very small-scale mosaics in nectar sugar composition. Changes in nectar sugar composition following bumblebee probing may be the consequence of nectar contamination with pollinator-borne nectarivorous yeasts.

Key words: bumblebees; *Helleborus foetidus*; nectar sugar composition; nectarivorous yeasts; pollinator visitation; Ranunculaceae; Spain.

Floral nectar is an essential link in the interaction between most insect-pollinated plants and their pollinators, and studies in nectar biology have traditionally considered variations in nectar chemical composition in relation to differences in the identity of pollinators (Nicolson and Thornburg, 2007). In particular, variation in sugars, the dominant chemical constituents of most nectars, has been thoroughly investigated in relation to differences in pollinator composition (e.g., Baker and Baker, 1982, 1983; Galetto and Bernardello, 2003; Petanidou, 2005). With few exceptions, these studies have generally focused on variation at the species level or above. A handful of studies, however, have revealed that nectar chemistry, including sugar proportions, often differs among individuals, populations, cultivars, or subspecies of the same species (Severson and Erickson, 1984; Freeman et al., 1985; Reid et al., 1985; Freeman and Wilken, 1987; Lanza et al., 1995; Roldán-Serrano and Guerra-Sanz, 2004). In addition, two recent investigations have shown that nectar sugar composition may also vary extensively among flowers and nectaries of the same plant. For the bumblebee-pollinated *Helleborus foetidus*, Herrera et al. (2006) showed that the proportions of sucrose, glucose and fructose in individual nectaries of wild plants varied widely among flowers of the same plant and nectaries of the same flower. Similar findings have been reported by Canto et al. (2007) for two species of bumblebee-pollinated *Aquilegia*. In this case, nectar composition varied greatly within wild plants, but not within plants grown in a glasshouse in the absence of flower visitors.

While variation between conspecific plants in average nectar composition may reflect intrinsic differences or responses to environmental variation (Pacini et al., 2003; Mitchell, 2004; Petanidou, 2005; Petanidou et al., 2006), extensive variation among flowers of the same plant or nectaries of the same flower is more difficult to explain. Floral nectar often harbor diverse yeast communities for which pollinators may represent major vehicles for dispersal among flowers (Kevan et al., 1988; Brysch-Herzberg, 2004; and references therein). Yeasts have the capacity to modify the original sugar profile by hydrolyzing the disaccharide sucrose into the monosaccharides glucose and fructose and/or by differential consumption of monosaccharides (Barnett, 1997). On the basis of these considerations, Canto et al. (2007) advanced the hypothesis that extensive within-plant and within-flower variation in nectar sugar composition observed in the field may be the outcome of differences between individual flowers and nectaries in prior pollinator visitation history, which would lead to small-scale variation in frequency of yeast contamination, composition of yeast communities, and/or metabolic characteristics of component yeast species.

The experiments reported in this paper were designed to test if differences among flowers and nectaries of the same plant in recent pollinator visitation history may actually account for the

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extensive small-scale variations in nectar sugar composition documented by Herrera et al. (2006) and Canto et al. (2007) for *Helleborus foetidus* and *Aquilegia* spp. Our general goal was to determine whether single pollinator visits can induce short-term modifications in the sugar composition profile of floral nectar. To this end, experiments were performed that mimicked single-nectary visits by wild-caught individuals of different species of *Helleborus foetidus* pollinators, which were assayed for their capacity to modify the sugar composition of both natural and artificial nectar. The following questions are specifically addressed: (1) Does pollinator visitation modify the sugar composition of *H. foetidus* nectar? (2) Do pollinator species differ in their capacity to promote short-term changes in nectar sugar composition of visited flowers?

MATERIALS AND METHODS

Study plant—*Helleborus foetidus* is a perennial herb widely distributed in western and southwestern Europe (Werner and Ebel, 1994). Flowering mainly takes place from January through March, and, anthophorid bees, honeybees and two species of bumblebees (*Bombus pratorum* and *B. terrestris*) are the main pollinators (Herrera et al., 2001). Plants produce one or a few inflorescences, each bearing 25–100 flowers, that open gradually over 1.5–2.5 months. Individual flowers last for 1–3 weeks. The perianth consists of five large, overlapping green sepals. As in other *Helleborus* species, the petals of *H. foetidus* have become modified into nectaries (Tamura, 1993). Each flower generally contains five individual nectaries shaped like flattened horns and hidden deeply inside the perianth. These form a distinct ring between the stamens and the sepals and produce copious nectar (Herrera and Soriguer, 1983; Vesprini et al., 1999). The nectar of unvisited flowers contains mainly sucrose (approximately 90%), with small quantities of glucose (5%) and fructose (5%) (see next section and "early sample" in Herrera et al., 2006).

Study site and methods—This study was conducted in March and April 2006 on a *H. foetidus* population located at 1250–1350 m a.s.l. on wooded slopes near the Roblehondo field station, in the Sierras de Cazorla-Segura-Las Villas Natural Park, Jaén Province, southeastern Spain. Plants were growing in the understory of a mixed pine (*Pinus nigra*) and holm oak (*Quercus ilex*) forest.

During the study period, bees were hand-netted while they were visiting the flowers of five small groups (2–6 plants each) of *H. foetidus* plants growing near the field station. A total of 94 individual bees from nine different species were collected and subsequently used as experimental subjects. For the purpose of the analyses, they will be grouped into the following six classes on the basis of taxonomic affiliation, size, and behavioral similarity: *Andrena* sp. (including *A. bicolor* and *A.* sp.; $N = 6$ individuals), medium-sized Anthophoridae (including *Anthophora plumipes*, *Eucera* near *caspica*, and *Melecta* near *italica*; $N = 8$), *Apis mellifera* ($N = 32$), *Bombus pratorum* ($N = 14$), *Bombus terrestris* ($N = 16$), and *Lasioglossum* sp. ($N = 18$). Differences among species in sample sizes closely reflect their differential abundance at *H. foetidus* flowers. Immediately upon collection, bees were placed individually in sterile, labeled containers and anaesthetized by placing them inside a refrigerator until used in the experiments, generally within a few hours after collection. All bees were alive, albeit torpid, at the time of experiments. In these experiments, trapped bees were used to probe both natural nectar of *H. foetidus* and artificial nectar whose sugar profile was roughly similar to that of natural one in the study area, as detailed next. Bees were killed after the experiments and kept as vouchers at the entomological collection of CMH and deposited at the Estación Biológica de Doñana.

To ensure availability of "clean" natural nectar from unvisited flowers for the experiments, 10 plants of *H. foetidus* bearing inflorescences were bagged at the beginning of the study, before any flower had opened. Flower buds were monitored daily until sepals began to spread open. A clean cotton swab was then firmly plugged into the flower entrance to further protect nectaries from possible visitation by small insects accidentally entering the exclosures and nectar contamination by airborne microorganisms. When plugged flowers became functional (as denoted by elongation of anther filaments), they were cut shortly before use in the bee visitation experiments and kept refrigerated inside small containers with cotton plugs in place until used, usually within a few hours of collection. We cut whole flowers, rather than excising individual nectaries, to minimize the risks of nectary contamination during manipulation and transport. We prepared artificial nectar whose sugar composition and concentration mimicked that of natural *H. foetidus* fresh nectar. The average sugar molarity (M) of nectar from three populations close to our study site was calculated as suggested by Bolten et al. (1979), using data in Herrera and Soriguer (1983). Then a proportional M was computed for each component sugar based on the mean percentage of glucose, fructose, and sucrose reported for *H. foetidus* nectar by Herrera et al. (2006). The proportional sugar molarities thus obtained were then used to determine the mass of each sugar required to produce an artificial nectar 1.66 M and containing approximately 5% glucose, 5% fructose and 90% sucrose. Fresh artificial nectar was prepared daily over the course of the experiments by dissolving in sterile distilled water preweighted amounts of the artificial mix of sugars made from reagent grade glucose, fructose, and sucrose.

Four nectar samples (two natural, two artificial) were assigned to each collected bee and randomly allocated to each of four treatment combinations arising from a two-way factorial design with visitation (probed and unprobed) and nectar type (natural and artificial) as two-level main effects. The glossa of each individual bee was carefully extended using fine forceps beyond the tip of the maxillary galeae and introduced into an excised floral nectary containing natural nectar (probed-natural nectar treatment combination) and into a droplet of artificial nectar placed close to the rim of a microcentrifuge tube (probed-artificial nectar treatment combination). To minimize possible biases, we alternated the order of probing of natural and the artificial nectar with bee tongues in successive assays. The forceps used to handle bee tongues were carefully cleaned between probes using ethanol. After each single experiment, the four nectar samples (i.e., probed-natural nectar, probed-artificial nectar, unprobed-natural nectar, unprobed-artificial nectar) were incubated at room temperature (range 16–26°C, mean 21°C) for 48 h. After that, the two samples of natural nectar were drawn from the nectaries using sterile microcapillaries and placed individually in sterile microcentrifuge tubes. All samples were then hermetically sealed and kept deep-frozen at −80°C until chemical analysis.

Nectar analysis—The proportions of sucrose, glucose, and fructose in each of the $N = 376$ nectar samples obtained (= 94 bees × 4 samples/bee) were determined using high performance liquid chromatography (HPLC). The nectar-containing tubes were thawed and different volumes of HPLC-grade water added to each to complete 1 mL of solution. Two independent, replicate measurements were done for each sample. For each replicate, 5 μL of solution was filtered through a 0.4 μm polyvinylidenedifluoride (PVDF) filter (Análisis Vínicos SL, Tomelloso, Spain) and injected into a Dionex DX 500 HPLC system (Dionex, Sunnyvale, California, USA). The HPLC system was equipped with an effluent degas module, a GP 40 gradient pump, a CarboPac PA10 (4 x 50 mm) guard column and a CarboPac PA10 (4 × 250 mm) analytical column. It also had an ED 40 electrochemical detector for pulsed amperometric detection in integrated amperometric mode, with the normal preloaded wave form for sugar detection (Dionex Corp, 1994); detector output range was set to 100 nC. The column was eluted (flow rate 1 $mL \cdot min^{-1}$) isocratically with 40 mM NaOH (50% solution; J. T. Baker, Deventer, The Netherlands) and kept at 24°C during analysis. Retention times were calibrated daily for D-glucose, D-fructose and sucrose (Sigma-Aldrich, Madrid, Spain) by injecting 10 μL of a calibration mixture containing 5.5 ppm, 13.75 ppm, and 13.75 ppm of these sugars, respectively. The proportions of the three different sugars (glucose, fructose, sucrose) in each analyzed sample were estimated by integrating the area under the chromatogram peaks. Only sucrose, glucose, and fructose appeared in all samples.

Statistical analyses—All statistical analyses were done with the SAS statistical package (SAS Institute, Cary, North Carolina, USA). Separate analyses were conducted for each combination of the three component sugars (sucrose, fructose, and glucose) with each of the six bee taxa (*Andrena* sp., Anthophoridae, *A. mellifera*, *B. pratorum*, *B. terrestris* and *Lasioglossum* sp.) and all the taxa combined into a single sample. In each analysis, a mixed model was fitted to the data using SAS procedure MIXED with restricted maximum likelihood estimation (REML; Littell et al., 1996). Percentage sugar (log-transformed) was used as the dependent variable, and visitation treatment (probed and unprobed), nectar type (natural and artificial), and their interaction, were included in the model as fixed effects. Replicate measurements for each sample were entered as a random effect. No detectable variance in the percentages of the three nectar sugars was accounted by this measurement error component. The nectar type main effect was included in the analyses to statistically account for possible differences between artificial and natural nectar in average sugar composition. The visitation × nectar type interaction effect provides a test of

whether the effect of bee visitation on nectar sugar composition differed for natural and artificial nectar.

RESULTS

Not unexpectedly, natural and artificial nectar used in the experiments differed slightly in mean sugar composition. When only the unprobed samples are considered, artificial nectar contained significantly more glucose (mean ± SE = 5.3 ± 0.1% vs. 0.4 ± 0.1%; $F_{1,93} = 2509$, $P < 0.0001$), more fructose (4.9 ± 0.6% vs. 2.3 ± 0.6%; $F_{1,93} = 8.9$, $P = 0.004$), and less sucrose (89.8 ± 0.7% vs. 97.3 ± 0.7%; $F_{1,93} = 67.0$, $P < 0.0001$), on average than natural nectar. Results of the experiments, however, will be unaffected by these differences, since nectar type was included as a main effect in all the models tested and differences in mean contents of individual sugars were thus accounted for.

Experimental nectar probing with bee mouthparts induced extensive changes in sugar composition. For all bee taxa combined, there was a significant effect of visitation on the (log-transformed) percentages of glucose, fructose, and sucrose. Nectar samples that had been experimentally probed with bee tongues had slightly greater percentages of glucose (5.1 ± 0.4% vs. 2.8 ± 0.4%; $F_{1,185} = 46.6$, $P < 0.0001$), considerably higher percentages of fructose (24.3 ± 2.6% vs. 3.8 ± 2.6%; $F_{1,185} = 46.5$, $P < 0.0001$), and much lower percentages of sucrose (70.0 ± 2.7% vs. 93.8 ± 2.7%; $F_{1,185} = 32.1$, $P < 0.0001$) than unprobed samples.

Bee taxa differed widely in their effects on nectar sugar composition, as revealed by the contrasting results shown in Table 1. Experimentally probing nectar with the mouthparts of *Andrena* sp., Anthophoridae, *A. mellifera*, and *Lasioglossum* sp. had no significant effect on subsequent nectar sugar composition, as revealed by nonsignificant visitation main effects on percentage sucrose, fructose, and glucose. Natural and artificial nectars were similarly unaffected by bee visitation, as revealed by nonsignificance of the visitation × nectar type interaction effects (Table 1). In marked contrast, probing nectar with the mouthparts of *Bombus pratorum* and *B. terrestris* had highly significant effects on the percentage content of all the three sugars. In addition, significant visitation × nectar type interaction effects were found for the percentage glucose (*B. pratorum* and *B. terrestris*) and fructose (*B. pratorum* only) content, which suggests differential responses of natural and artificial nectar to visitation by bumblebees.

The two bumblebee species had nearly identical effects on nectar sugar composition. Nectar probing resulted in an extensive reduction in percentage sucrose, a marked increase in fructose content, and a weak increase in percentage glucose (Fig. 1). The nearly pure-sucrose initial nectar thus shifted to a fructose-dominated one 48 h after being probed with bumblebee mouthparts. Consequently, the mean sucrose to hexoses ratios were much lower in probed (2.4 ± 0.7 and 6.1 ± 2.4 for *B. pratorum* and *B. terrestris*, respectively) than unprobed nectars (67.2 ± 13.4 and 50.7 ± 10.9 for *B. pratorum* and *B. terrestris*, respectively). The mean glucose to fructose ratios of probed nectars (0.15 ± 0.03 and 0.30 ± 0.07 for *B. pratorum* and *B. terrestris*, respectively) was also substantially lower than those of unprobed nectars (0.73 ± 0.10 and 0.68 ± 0.10 for *B. pratorum* and *B. terrestris*, respectively), thus denoting that nectar probing induced a strong imbalance in the relative proportions of the two hexoses.

The three statistically significant visitation × nectar type interaction effects shown in Table 1 (on fructose and glucose content for *B. pratorum*; on glucose content for *B. terrestris*) are discernible in the corresponding graphs of Fig. 1. In quantitative terms, however, these interaction effects are negligible in comparison with the magnitude of main effects, and it seems reasonable to interpret our results as denoting essentially similar effects of bumblebee probing on the sugar profiles of natural and artificial nectars.

DISCUSSION

The wild bees used in our experiments were caught while they were foraging in *H. foetidus* flowers. With the only possible exception of *Lasioglossum* sp., all were regular and effective pollinators of *H. foetidus*; the two bumblebees were the most important in most sites and years (Herrera et al., 2001). Individual *H. foetidus* flowers are very long-lived, lasting for as much as 1–3 wk, and their nectaries produce nectar during most of this time (Vesprini et al., 1999). The 48-h interval between our experimental treatments and the assessment of nectar composition was thus much shorter than the natural length of time nectaries are exposed to pollinators. Ambient temperature of nectar samples during incubation was roughly similar to average daytime temperatures experienced by *H. foetidus* plants during the flowering season (Sánchez-Lafuente et al., 2005). Furthermore, because of *H. foetidus*' early blooming time, flower visits by pollinators are infrequent (Herrera et al., 2001), and abundant nectar often accumulates in the nectaries (Herrera and Soriguer, 1983). This will generally allow for insertion of bumblebee mouthparts into the nectar as mimicked in our experiments. For the preceding reasons, the experiments

TABLE 1. Summary of mixed model statistical analyses testing the effects of experimental bee visitation (probed vs. unprobed nectar), and the visitation × nectar type (natural nectar *vs.* artificial nectar) interaction, on the (log-transformed) percentage of sucrose, fructose and glucose in nectar 48 h past the experimental treatment. Statistical tests were conducted separately for each of the six bee taxa used for the experiments. Significance levels were Bonferroni-corrected to account for the multiplicity of simultaneous tests and are coded as follows: **, $P < 0.01$; ***, $P < 0.001$; ns, not significant. Significant effects are shown in boldface type.

	% Sucrose		% Fructose		% Glucose	
Bee taxa	Visitation	Visitation × nectar type	Visitation	Visitation × nectar type	Visitation	Visitation × nectar type
Andrena sp.	$F_{1,10} = 4.3^{ns}$	$F_{1,10} = 2.3^{ns}$	$F_{1,10} = 0.9^{ns}$	$F_{1,10} = 0.1^{ns}$	$F_{1,10} = 8.4^{ns}$	$F_{1,10} = 0.03^{ns}$
Anthophoridae	$F_{1,14} = 0.05^{ns}$	$F_{1,14} = 11.4^{ns}$	$F_{1,14} = 0.2^{ns}$	$F_{1,14} = 1.3^{ns}$	$F_{1,14} = 1.9^{ns}$	$F_{1,14} = 7.5^{ns}$
Apis mellifera	$F_{1,61} = 3.6^{ns}$	$F_{1,61} = 1.4^{ns}$	$F_{1,61} = 6.8^{ns}$	$F_{1,61} = 7.3^{ns}$	$F_{1,61} = 6.9^{ns}$	$F_{1,61} = 6.2^{ns}$
Bombus pratorum	**$F_{1,26} = 29.2^{***}$**	$F_{1,26} = 5.3^{ns}$	**$F_{1,26} = 123.3^{***}$**	**$F_{1,26} = 27.9^{***}$**	**$F_{1,26} = 43.3^{***}$**	**$F_{1,26} = 140.5^{***}$**
Bombus terrestris	**$F_{1,30} = 42.7^{***}$**	$F_{1,30} = 0.7^{ns}$	**$F_{1,30} = 36.4^{***}$**	$F_{1,30} = 1.1^{ns}$	**$F_{1,30} = 44.7^{***}$**	**$F_{1,30} = 17.2^{**}$**
Lasioglossum sp.	$F_{1,34} = 11.4^{ns}$	$F_{1,34} = 0.3^{ns}$	$F_{1,34} = 10.6^{ns}$	$F_{1,34} = 1.9^{ns}$	$F_{1,34} = 1.5^{ns}$	$F_{1,34} = 4.2^{ns}$

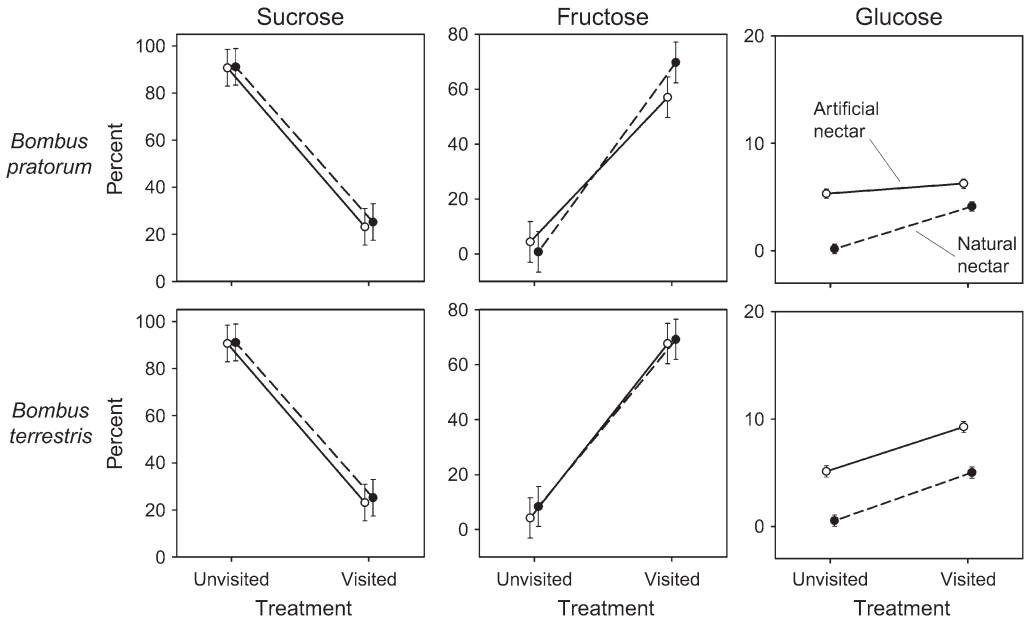

Fig. 1. Effects of experimentally probing natural and artificial nectar of *Helleborus foetidus* with the mouthparts of wild-caught *Bombus pratorum* (upper graphs) and *B. terrestris* (lower graphs) on percentage sucrose, fructose, and glucose content of nectar 48 h past the treatment. Unvisited and visited treatments correspond to unprobed and probed nectar, respectively. Continuous lines with open circles refer to artificial nectar, and dashed lines with filled circles refer to natural nectar. Circles denote model-adjusted cell means, and vertical bars extend over ±1 SE of estimates. Note the different vertical scales used for the three sugars. See Table 1 for a summary of statistical tests.

conducted in this study can be considered as reasonably realistic from a biological viewpoint, and changes in nectar sugar composition similar to those observed in this study subsequent to experimental nectar probing with bumblebee mouthparts are also expected to occur in the nectaries of field-grown plants exposed to bumblebee visitation. Given that different floral visitors have been shown to differ in their short-term effects on the nectar sugar profile, our results clearly support the hypothesis of Canto et al. (2007) that stochastic variations among individual flowers or single nectaries in the taxonomic identity of recent visitors and their relative visitation frequencies may ultimately generate very small-scale mosaics in nectar sugar composition that are independent of the plants and largely out of their control. Interestingly, the three mosaics of this sort so far documented refer to plants (*H. foetidus*, *Aquilegia vulgaris*, *A. pyrenaica*) whose main pollinators are bumblebees, the only bees shown in this study to possess the ability to induce modifications in nectar sugar profiles (Herrera et al., 2006; Canto et al., 2007).

The design of this study did not allow for rigorously identifying the proximate mechanism responsible for nectar sugar modification following bumblebee probing. Our results, however, unambiguously demonstrated that (1) the causal agent of nectar sugar modification is unrelated to the plants or the nectaries themselves. Otherwise, nectar modification would have occurred in the natural nectar (i.e., produced by, and still contained inside the nectary) but not in the artificially synthesized one; and (2) the causal agent involved is associated with the action of nectar being probed by the bees' mouthparts, which suggests that it is transferred from the mouthparts of bumblebees into the nectar. Sugar-hydrolyzing enzymes from the bumblebees' hypopharingeal glands (e.g., invertases; Von der Ohe, 1994; Pereboom, 2000) and sugar-metabolizing microorganisms brought into the nectar by foraging bees (e.g., Gilliam et al., 1983; Kevan et al., 1988; Eisikowitch et al., 1990; Brysch-Herzberg, 2004) are the two most likely candidate agents of nectar change in our experiments. Considerable circumstantial evidence points to the second possibility. First, if nectar modification were exclusively due to the action of invertases produced by the bees, then the resulting proportions of glucose and fructose should be equal. Our results, however, clearly contradict this expectation because nectar modification following bumbleebee probing not only involved a decline in sucrose content, but also a marked inequality in the proportions of glucose and fructose. This finding can be parsimoniously explained by the fact that many yeasts, in addition to hydrolyzing sucrose into glucose and fructose, often metabolize preferentially one of these monosaccharides, eventually leading to nonstoichiometric proportions departing from the 1 : 1 ratio expected from simple sucrose hydrolysis (Von der Ohe, 1994; Barnett, 1997). Second, microbiological studies by Brysch-Herzberg (2004; see also Herzberg et al., 2002; Hong et al., 2003) have revealed that individuals of *B. terrestris* and *B. pratorum* ordinarily harbor dense and taxonomically diverse yeast populations on their bodies and mouthparts. Although we did not look systematically for yeasts on the bodies or mouthparts of our experimental bumblebees, anecdotal observations conducted after completion of the study revealed that at least some of them carried dense aggregations of yeast cells on the glossa (Fig. 2). And third, a large number of yeast species have been isolated from the nectar of *H. foetidus* in central Europe (Schoellhorn, 1919; Herzberg et al., 2002; Hong et al., 2003; Brysch-Herzberg, 2004). Although systematic work on the size and composition of the yeast communities associated with *H. foetidus* nectar in our study region has been not conducted so far, preliminary microscopic screenings of field-collected nectar samples of this species have revealed that yeast cell densities frequently exceed 10^4 cells/μL (C. M. Herrera, unpublished data). All these

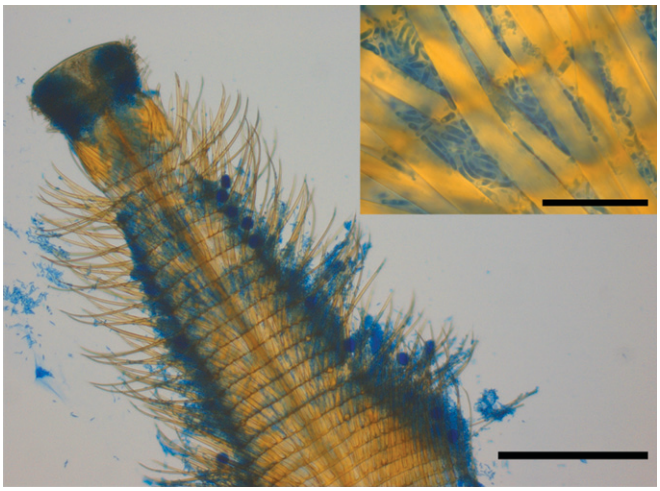

Fig. 2. Photomicrographs of the terminal portion of the glossa of an individual *Bombus pratorum* used in the experiments, stained with lacto cotton blue without phenol. Apart from a few easily discernible pollen grains, the rest of the blue areas in the main image are very dense aggregations of yeast cells on the glossa surface and around the setae lining it (bar = 200 μm). The high density and close association of yeast cells with the setae is best appreciated in the close-up in the inset (bar = 20 μm).

considerations, although admittedly circumstantial in nature, strongly suggest the interpretation that changes in nectar sugar composition following bumblebee probing are the consequence of contamination of nectar with nectarivorous yeasts caused by bumblebee probing. Studies are currently in progress to test directly this possibility.

Results of this study have a number of implications in relation to the study of nectar composition and plant–pollinator interactions. Assessing their actual significance will require additional investigations that evaluate the generality of our findings, yet it is worth pointing out some of these implications here. Literature reports indicate that nectars of many animal-pollinated plants are characterized by unequal proportions of glucose and fructose, as found here for bumblebee-probed samples (e.g., Perret et al., 2001; Galetto and Bernardello, 2003; Petanidou, 2005; Chalcoff et al., 2006). When they have been explicitly considered, nonstoichiometric proportions of hexoses have been generally treated as species-specific features of nectar. The imbalance of hexoses has been related, for example, to the action of invertases in nectary tissue cell walls in combination with possible differential reabsorption by nectariferous tissue (Nicolson, 1995). Our results suggest that nectar contamination by pollinator-borne yeasts can be another mechanism leading to hexose imbalance, which could represent a sort of chemical signature of yeast presence and recent metabolic activity, rather than an intrinsic property of the nectar under direct control of the plant.

As discussed by Herrera et al. (2006), variance sensitivity and risk-aversion seem almost universal among animals foraging in patchy environments (Kacelnik and Bateson, 1996). Animal pollinators are not an exception to this rule because they generally respond negatively to variability in nectar volume and concentration (Shafir, 2000; Shafir et al., 2003). In self-compatible hermaphrodite plants, extensive within-plant variance in nectar production and standing crop may reduce the number of sequentially visited flowers and, consequently, the costs derived from geitonogamous pollinations (Pleasants, 1983; Biernaskie et al., 2002; Biernaskie and Cartar, 2004). Extensive within-plant variation in nectar sugar composition may likewise be advantageous to plants by decreasing the number of flowers visited per plant by variance-sensitive, risk-averse pollinator foragers tending to avoid plants with highly variable nectars. The results of this study suggest, on one side, that insofar as within-plant variation in nectar composition seems to be an indirect outcome of pollinator-mediated yeast contamination, it will be difficult to interpret such variation only as an adaptive plant trait selected for by pollinators. And on the other, that the foraging activity of the pollinators themselves would ultimately be responsible for the decline in attractiveness of plants with highly variable nectars.

Nectarivorous yeasts are known to exist in association with a variety of bees, both solitary and social (Sandhu and Waraich, 1985; Lachance et al., 2001; Brysch-Herzberg, 2004; Pimentel et al., 2005). The association seems particularly tight between certain yeast and bee lineages, as suggested by Brysch-Herzberg (2004) for species of *Bombus* and ascomycetous yeasts of the *Metschnikowia* clade. Results of our experiments show that the capacity to induce modifications in *H. foetidus* nectar sugar profiles was pollinator-dependent, and that of the six bee taxa assayed, only *B. pratorum* and *B. terrestris* were effective at modifying nectar sugar composition. Because these two species are the main floral visitors and pollinators of *H. foetidus* over most of its range, our findings may reflect a predictable three-way ecological association involving *H. foetidus*, its bumblebee pollinators, and the yeasts associated with the latter.

Pollinators frequently act as vectors of plant pathogenic fungi (Roy, 1993; Pfunder and Roy, 2000; and references therein). The current study provides compelling evidence that certain pollinators may also play decisive roles as vehicles of microbial agents that drastically change nectar composition. In contrast with the plant–pathogen–pollinator triad, where the nature (e.g., mutualistic, antagonistic) of each binary relationship involved is relatively straightforward, the signs of the binary interactions involved in a putative plant–yeast–pollinator triad are much less evident, and a variety of plausible ecological scenarios may be envisaged. Whether the incorporation of a third microbial interactor in the study of the relationships between *H. foetidus* and bumblebees—thus conceptually scaling up a binary system to a ternary one—will disclose hitherto unknown features of the interaction between this plant and its main pollinators remains to be investigated.

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