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# EXTREME INTRAPLANT VARIATION IN NECTAR SUGAR COMPOSITION IN AN INSECT-POLLINATED PERENNIAL HERB[1]

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Variation in nectar chemistry among plants, flowers, or individual nectaries of a given species has been only rarely explored, yet it is an essential aspect to our understanding of how pollinator-mediated selection might act on nectar traits. This paper describes variation in nectar sugar composition in a population of the perennial herb *Helleborus foetidus* (Ranunculaceae) and dissects it into components due to variation among plants, flowers of the same plant, and nectaries of the same flower. The proportions of sucrose, glucose, and fructose in single-nectary nectar samples collected at two times in the flowering season were determined using high performance liquid chromatography (HPLC). Sugar composition varied extensively among nectaries, and nearly all combinations of individual sugars were recorded. Population-wide variance was mainly accounted for by variation among flowers of the same plant (56% of total), nectaries of the same flower (30%), and only minimally by differences among plants (14%). In absolute terms, intraplant variation was similar to or greater than that ordinarily reported in interspecific comparisons. Results suggest that the prevailing notion of intraspecific constancy in nectar sugar composition may be unwarranted for some species and that more elaborate nectar sampling designs are required to detect and appropriately account for extensive within-plant variance. Within-plant variation in nectar sugar composition will limit the ability of pollinators to exert selection on nectar chemistry in *H. foetidus* and may be advantageous to plants by reducing the number of flowers visited per foraging bout by variance-sensitive, risk-averse pollinators.

**Key words:** *Helleborus foetidus*; hexoses; individual variation; nectar sugar composition; sucrose; variance components; within-plant variation.

Nectar is the most common form of floral reward furnished by animal-pollinated plants to their mutualistic partners (Simpson and Neff, 1983), and considerable correlative evidence links the broad variation in energetic and nutritional contents and chemical composition of angiosperm nectars to differences in the identity of pollinators and their energetic and nutritional needs (Baker and Baker, 1975, 1982, 1983a, 1986). This applies particularly to sugars, which are the dominant chemical constituents of most nectars and whose variation has been thoroughly investigated in relation to differences in pollinator composition (e.g., Baker and Baker 1983b; Baker et al., 1998; Galetto and Bernardello, 2003; Dupont et al., 2004; Jürgens, 2004). The most common sugars in nectar are the disaccharide sucrose and the hexose monosaccharides glucose and fructose. The relative proportion of disaccharides and monosaccharides, i.e., the sucrose to hexose ratio, has been considered a distinctive compositional signature of nectars that tends to be predictably related to pollinator composition. Nectars have been traditionally classified as either sucrose- or hexose-rich (e.g., Freeman et al., 1991; Van Wyk, 1993; Van Wyk et al., 1993; Barnes et al., 1995), and a number of studies have documented a relationship between sucrose : hexose ratio and pollinator type (Baker and Baker, 1983b; Perret et al., 2001; Dupont et al., 2004; but see also, e.g., Galetto and Bernardello, 2003, 2004; Jürgens, 2004).

With very few exceptions, investigations on nectar sugar composition have focused on comparisons at the species level or above. Typically, these investigations have proceeded by characterizing each species by the percentage of glucose, fructose, and sucrose. This single set of figures per species is obtained either from a single analytical determination conducted on a pooled sample combining nectar from a number of flowers and individual plants or by averaging the results of separate analytical determinations conducted on a few (usually $<10$) of such combined samples (e.g., Van Wyk, 1993; Van Wyk et al., 1993; Barnes et al., 1995; Perret et al., 2001). Combining nectar from different flowers and plants for analysis was unavoidable in the early years of nectar investigations because the rather rudimentary analytical procedures available at the time imposed stringent constraints on the minimum amount of nectar that could be reliably assayed (e.g., Wykes, 1952; Percival, 1961). More recently, the practice of averaging a few samples into a single nectar composition figure per species must instead be related to the prevailing, although generally unexpressed notion that nectar chemistry tends to be a relatively invariant species-specific feature and that intraspecific variance is comparatively minor and thus not worthy of particular consideration. This notion of intraspecific constancy in nectar composition, which may be traced back to Wykes (1952) and Percival (1961, 1965), has been rigourously tested on few occasions since then. These studies, however, have revealed that nectar chemistry, including sugar proportions, may differ among individuals, populations, cultivars, or subspecies of the same species (Baker and Baker, 1983b; Severson and Erickson, 1984; Freeman et al., 1985; Reid et al.,

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1985; Freeman and Wilken, 1987; Lanza et al., 1995; Roldán-Serrano and Guerra-Sanz, 2004). One of these investigations also suggested extensive intraplant variation in nectar sugar composition, at least judging from the large coefficients of variation around individual plant means (Tables 2 and 3 in Freeman and Wilken, 1987), but this level of intraspecific variation has been investigated even less often than variation among individuals or populations.

Investigating intraspecific variation in nectar chemistry is important for at least two reasons. First, evaluating the relative magnitude of inter- and intraspecific variation is necessary to interpret interspecific patterns of variation in average nectar sugar composition in a proper evolutionary context. Second, a detailed knowledge of the proportions of total intraspecific variance in nectar composition due to variation among individual plants and to smaller-scale variation occurring within individual plants (i.e., among flowers and among separate nectaries within a flower) is crucial to our understanding of how pollinator-mediated selection might act on nectar traits. To our knowledge, no previous investigation has dissected intraspecific variation in nectar chemistry from this latter perspective. In this paper, we adopt this approach to analyze variation in nectar sugar composition in the insect-pollinated perennial herb *Helleborus foetidus* L. (Ranunculaceae). We will quantify variation at the among- and within-plant levels for a single southeastern Spanish population of this species using individual nectaries as the sampling units for nectar composition analyses. Each flower of *H. foetidus* bears five independent, separate nectaries. By obtaining separate nectar samples from different nectaries of the same flower, we will be able to extend the partition of within-plant variance in nectar composition down to the within-flower level. Despite the limited spatial and temporal scope of this study, results demonstrate extreme intraspecific variation in nectar sugar composition, with most of this variance occurring within individual plants (among flowers and among the nectaries of the same flower).

## MATERIALS AND METHODS

***Study plant***—*Helleborus foetidus* is a perennial herb widely distributed in western and southwestern Europe (Werner and Ebel, 1994). In the Iberian Peninsula, it typically occurs in clearings, forest edges, and the understory of montane forests, mainly on limestone substrates. Flowering mainly takes place from January through March. Bumble bees and anthophorid bees are the main pollinators (Herrera et al., 2001). Plants consist of one or a few ramets that develop a terminal inflorescence after several seasons of vegetative growth. Each inflorescence, made of 3–7 cymes ("laterals" hereafter), produces 25–100 flowers that open gradually over 1.5–2.5 mo. The green, pendant, bell-shaped flowers are 14–19 mm long and 12–17 mm wide. The outer floral whorl consists of five large, overlapping sepals, which are green at anthesis. As in other species of the genus *Helleborus*, the petals of *H. foetidus* have become modified into nectaries (Tamura, 1993). There are five nectaries per flower, which are shaped like flattened horns and are deeply hidden inside the corolla. They form a distinct ring between the stamens and the sepals and produce copious nectar (Herrera and Soriguer, 1983; Vesprini et al., 1999). The structure of *H. foetidus* flowers, including the nectaries, is depicted in Corbet et al. (1979), Vesprini et al. (1999), and Herrera et al. (2002).

***Study site and field methods***—This study was conducted on March–May 2005 at a large, more or less continuous population of *H. foetidus* located at 950–1100 m elevation in wooded slopes around the small village of Vadillo-Castril, in the Parque Natural Sierras de Cazorla-Segura-Las Villas, Jaén Province, southeastern Spain. Plants were growing in the understory of pine (*Pinus pinaster* and *P. nigra*) and holm oak (*Quercus ilex*) mixed woodlands. Nectar samples were collected on two separate dates, which roughly corresponded to the beginning of the flowering period (9 March) and slightly past the flowering peak for the study population (4 May). In 2005, the flowering season of *H. foetidus* was considerably delayed with respect to the usual January–March flowering period (Herrera et al., 2001). On the first collecting date ("early sample" hereafter), few flowers were still open in the population, the weather was cool and rainy, and bumblebees were only rarely seen visiting flowers. As a consequence, nectar was abundant in the nectaries, and plants did not need protecting from floral visitors in order for us to obtain nectar samples. Five inflorescences, each from a different plant, were cut, taken to the laboratory in sealed plastic bags, and the nectar was sampled within a few hours of collection. On the second collecting date ("late sample" hereafter), in contrast, many more flowers were open at the population, bumblebees were visiting them very frequently, and the amount of nectar in individual nectaries was generally too small for individual sampling. To exclude consumers and allow nectar to build up in the nectaries, six plants each bearing a single inflorescence were enclosed with 1-mm-mesh black tulle. After 48 h, inflorescences were cut, taken to the laboratory in sealed plastic bags, and the nectar sampled.

Nectar extraction, storage, and analytical procedures were the same for the early and late collections. All nectar samples were obtained by the same person. Individual flowers in similar stages (midway in the male phase, with about half the anthers open) were dissected by removing the sepals, and 1–3 individual nectaries were excised using fine forceps. A separate nectar sample was obtained from each nectary. The nectar was forced to emerge as a droplet in the nectary entrance by gently squeezing the nectary base and was immediately blotted onto a 10 × 2 mm Whatman 3MM paper wick. To avoid sample contamination, particular care was taken to avoid tissue damage (which could cause other plant fluids to leak into the nectar) and to ensure exclusive contact between the nectar and the wick. Immediately after nectar absorption, wicks were individually placed into clean, small paper envelopes. Until analysis, these were stored in a sealed plastic box full of silica gel at ambient temperature. On the first sampling date, only 1–3 flowers could be sampled per inflorescence, and it was not possible to stratify samples among different positions in the inflorescence. This was possible on the second sampling date, when there were sufficient flowers at appropriate stages for sampling on the same inflorescence. Six flowers were sampled per inflorescence. Flowers were taken from three laterals located at basal, middle, and distal positions in the inflorescence. In each lateral, two flowers were sampled, located in basal and distal positions. This sampling allowed us to test for position-dependent intraplant variation in nectar composition. In total, 21 early and 104 late, single-nectary nectar samples were analyzed.

***Analysis of nectar sugars with high performance liquid chromatography (HPLC)***—Nectar-containing wicks were individually placed into 2-mL Eppendorf tubes, and 500 μL of HPLC grade water was added to each one. Each sample was measured independently three times. For each measurement, 10 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 eluent degas module, a GP 40 gradient pump, a guard column CarboPac PA10 (4 × 50 mm), and an analytical column CarboPac PA10 (4 × 250 mm), as well as an ED 40 electrochemical detector for pulsed amperometric detection (PAD) in integrated amperometric mode, with the normal preloaded wave form for sugar detection (Dionex, 1994). The output range of the detector was set to 100 nC. The column was eluted (flow rate 1 mL/min) isocratically with 40 mM NaOH (50% solution obtained from J. T. Baker, Deventer, The Netherlands) as eluent 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. Trace amounts of a fourth, unidentified sugar appeared in a few samples, and is not considered here.

***Statistical analyses***—Statistical analyses were conducted using the SAS statistical program (SAS Institute, Cary, North Carolina, USA). Significance of the variation between the early and late nectar samples in average sugar composition was tested in a multivariate context with a MANOVA. Variance partitions and tests of statistical significance of variance components were carried out separately for the early and late samples, using a fully nested,

TABLE 1. Summary statistics for the relative amount of individual sugars in single-nectary nectar samples of *Helleborus foetidus* at a southeastern Spanish population.

| Collection date[a] | Statistic | Glucose (%) | Fructose (%) | Sucrose (%) |
|---|---|---|---|---|
| Early sample | Mean ± SD | 21.9 ± 13.5 | 24.8 ± 20.8 | 53.3 ± 31.8 |
| | Range | 3.9–52.5 | 1.6–69.9 | 1.1–94.4 |
| | Interquartile range | 11.4–24.2 | 10.5–39.8 | 22.6–77.0 |
| Late sample | Mean ± SD | 9.1 ± 8.5 | 64.4 ± 30.2 | 26.5 ± 31.1 |
| | Range | 0–55.6 | 0–100 | 0–97.9 |
| | Interquartile range | 4.5–10.2 | 45.3–91.8 | 0–42.9 |

[a] Early sample: 9 March 2005, $N = 21$ nectaries from 12 flowers and 5 plants. Late sample: 4 May 2005, $N = 104$ nectaries from 36 flowers and 6 plants.

random effects hierarchical ANOVA. Variance components of the proportions of the three main sugars at the various hierarchical levels considered (plant, flower within plant, nectary within flower) were estimated using restricted maximum likelihood (REML; e.g., Searle et al., 1992), as implemented in SAS procedure MIXED (Littell et al., 1996). The three replicate measurements of sugar proportions obtained for each single-nectary sample allowed us to estimate measurement error and thus to assess the variance component and statistical significance of the within-flower, among-nectary component of variation in nectar composition. The MIXED procedure provides approximate standard errors of variance component estimates and two-tailed tests of significance (i.e., departure from zero) based on standard normal deviates, but these tests may be unreliable (Littell et al., 1996). Instead, we used the $F$ tests produced by the RANDOM statement in the SAS procedure GLM to test the statistical significance of variation among plants, among flowers within plants, and among nectaries within flowers.

Because late samples were stratified according to inflorescence lateral and position within the lateral, it was possible to investigate whether within-plant variation had some predictable spatial component. This was done by fitting a mixed model to the data, with plant treated as a random factor and position of the inflorescence lateral (basal, middle, distal) and relative position of the flower within the lateral (basal, distal) as fixed effects.

## RESULTS

Differences between the results of the three replicate HPLC measurements for each single-nectary sample were negligible, thus corroborating the high repeatability of the analytical procedure. Variation between replicate measurements of the same sample were responsible, on average, for only 0.004–0.13% and 0.04–0.28% of the total sample-wide variance in the proportions of individual sugars in the early and late samples, respectively.

Summary statistics for the variation among individual nectaries in nectar sugar proportions were based on a single set of values for sugar proportions for each nectary, obtained by averaging the results from replicate measurements (Table 1). On average, the nectar was dominated by sucrose (53%) in the early sample, with glucose (22%) and fructose (25%) sharing second place. The average composition changed markedly in the late sample; fructose (64%) was the dominant sugar, sucrose (26%) was second, and glucose (9%) fell to very low levels. Differences between sampling dates for average proportions of sugar composition are statistically significant, as shown by results of MANOVA (Wilk's $\lambda = 0.637$, $F_{2,122} = 34.76$, $P < 0.0001$).

Average values describe the central tendency for the "population" of nectaries on each sampling occasion, but there was extreme variation around these mean values on each sampling date. The broad ranges shown in Table 1 denote that, when the nectar of individual nectaries is analyzed separately,

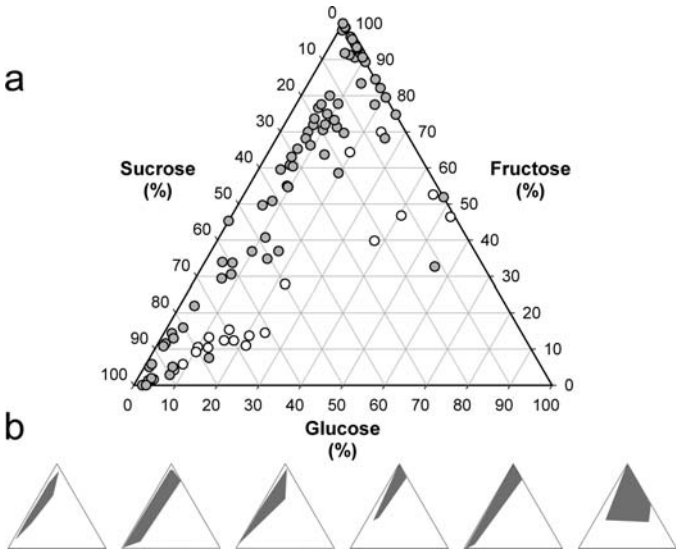


Fig. 1. Variation in nectar sugar composition in *Helleborus foetidus*. (a) Ternary diagram of the distribution of analyzed samples ($N = 125$) over the plane defined by axes corresponding to the percentage of glucose, fructose, and sucrose. Each point depicts the proportional sugar composition of the nectar from a single nectary. The distance of a point from a side of the triangle is proportional to the relative importance of that sugar in the sample. Open circles, early samples (March); shaded circles, late samples (May). (b) Schematic representation of the distribution of data points for each of the six plants sampled in May (shaded polygons).

almost any possible combination of sugars occurred in the sample. These include glucose-, fructose- and sucrose-dominated nectars, as well as nectars containing virtually only sucrose or fructose. Glucose was the only major sugar that was never found by itself in our nectar samples. The extreme variation in sugar composition among individual nectaries, and the occurrence of broadly contrasting sugar combinations in the nectaries sampled, is clearly illustrated by the ternary diagram in Fig. 1a.

In the population studied, most variation in the sugar composition of nectar from individual nectaries occurred within individual plants. This can be appreciated visually in Fig. 1b, where separate ternary graphs are shown for the six plants of the late sample. In all cases, the range of nectar sugar compositions in the nectaries of a single plant was nearly as broad as the full range for all sampled flowers (Fig. 1a). Variance component estimates provide a rigorous assessment of the relative importance of the different sources of variation considered (Table 2). Irrespective of sampling date, most population-wide variance in the relative amounts of glucose, fructose, and sucrose in the nectar of individual nectaries was due to variation among flowers of the same plant, followed by variation among nectaries of the same flower. On average (for the two sampling dates and the three sugars), differences among individual plants accounted for only 14% of total variance in nectar sugar composition and were statistically nonsignificant (five instances) or barely significant (one instance, glucose in the late sample). In contrast, differences among flowers of the same plant accounted for 56% of the population-wide variance and were statistically significant in five of six instances. Furthermore, differences among nectaries of the same flower were responsible for an additional 30% of the variance and were statistically significant in three instances

TABLE 2. Statistical significance of, and proportion of total variance due to: variation among plants, flowers within plants, and nectaries within flowers in the relative amounts of glucose, fructose, and sucrose in single-nectary nectar samples of *Helleborus foetidus*.

| | | Glucose (%) | | | Fructose (%) | | | Sucrose (%) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Significance of variation | | | Significance of variation | | | Significance of variation | | |
| Collection date | Source of variation | *F* | *P* | Percentage of variance[a] | *F* | *P* | Percentage of variance | *F* | *P* | Percentage of variance |
| Early | Plant | 2.7 | 0.12 | 26.7 | 1.5 | 0.30 | 11.9 | 1.8 | 0.24 | 17.8 |
| | Flower within plant | 1.0 | 0.50 | 11.3 | 3.5 | 0.04 | 55.3 | 3.0 | 0.05 | 48.4 |
| | Nectary within flower | 1495.7 | <0.0001 | 61.8 | 4496.2 | <0.0001 | 32.8 | 2531.4 | <0.0001 | 33.8 |
| Late | Plant | 5.0 | 0.04 | 18.8 | 1.1 | 0.43 | 3.2 | 1.4 | 0.33 | 7.2 |
| | Flower within plant | 4.2 | 0.005 | 54.5 | 32.7 | <0.0001 | 84.8 | 29.0 | <0.0001 | 81.1 |
| | Nectary within flower | 1.1 | 0.32 | 26.3 | 0.3 | 0.99 | 12.0 | 0.4 | 0.99 | 11.7 |

[a] Figures for percentage of total variance do not always equal 100 because of the small component due to measurement error (i.e., to differences between replicate HPLC measurements on the same nectar sample)

(Table 2). These figures indicate, therefore, that within-plant variation was responsible on average for 86% of all variance in nectar sugar composition occurring at the study population.

The possibility that within-plant variation had some predictable spatial component was investigated for the late samples, which were stratified according to inflorescence lateral and position within the lateral. Separate analyses were run for glucose, fructose, and sucrose. The effect of flower position within the lateral was not significant for any of the sugars ($P > 0.10$ in all cases, results not shown). The effect of position of the lateral along the inflorescence axis was significant for glucose ($F_{2,95} = 3.96$, $P = 0.022$), but not for fructose ($F_{2,95} = 2.13$, $P = 0.12$) or sucrose ($F_{2,95} = 2.82$, $P = 0.065$). The average proportion of glucose tended to increase from the basal to medial to distal laterals of the inflorescence (mean ± SE = 6.6 ± 2.1%, 9.0 ± 2.2% and 11.5 ± 2.2%, respectively).

## DISCUSSION

Despite its limited spatial and temporal scope, this investigation has shown that proportions of different sugars in the nectar of *H. foetidus* have considerable intraspecific variation and that most of this variation takes place within individual plants. Implications of these results are twofold, as we will discuss later. First, the extreme variation observed in nectar sugar composition shows that the assumption of species specificity and intraspecific constancy in nectar chemistry generally implicit in interspecific comparisons is unwarranted for some species. Second, the fact that within-plant variation is by far the most important source of intraspecific variance in nectar sugar composition raises a number of considerations in relation to the interaction between *H. foetidus* plants and their nectar-seeking pollinators.

Results of this study suggest that, in *H. foetidus*, average nectar sugar composition vary between regions and, within a given locality, also between times of the flowering period. Working on British plants, Percival (1961) and Corbet et al. (1979) classed the nectar of *H. foetidus* in the category of those made up entirely of sucrose. Vesprini et al. (1999) likewise reported that sucrose was the main nectar sugar in plants from central Italy, with fructose and glucose contributing less than 5% on average. In our southern Spanish population, in contrast, sucrose (53%) predominated in the early sample, while in the late sample the nectar became dominated by fructose (64%), with sucrose ranking second (26%). Differences between early and late samples should be related to variable patterns in sugar secretion and/or to variation in postsecretory processes taking place in the nectary. The large nectaries of *Helleborus* species are green structures with considerable photosynthetic competence (Vesprini et al., 1999; Pacini et al., 2003; Aschan et al., 2005), and variation between early and late samples in the relative importance of phloem-supplied vs. locally supplied sugars due, e.g., to changes in temperature and insolation, could lead to variation in the relative amounts of the different sugars. Postsecretory effects might also contribute to observed differences between early and late samples, particularly the remarkable shift in relative proportions of sucrose and hexoses. Variation in the concentration or activity of invertases (due, e.g., to changing ambient temperature), the enzymes hydrolizing sucrose into glucose and fructose (Heil et al., 2005), might eventually alter the proportions of sucrose and hexoses in nectar. It is unlikely that the different field procedures used with the early (unbagged) and late (bagged) experimental plants contributed substantially to variations in sugar composition between early and late nectar samples. Tulle exclosures intercepted 15% of photosynthetically active radiation incident on the plants (C. M. Herrera, unpublished data) and presumably limited air movement, which perhaps altered intrafloral microclimate in relation to unenclosed plants on the same dates. These differences, however, should be insignificant in comparison to the large differences in incident radiation and ambient temperature between early and late sampling occasions.

The most significant result of this study is the demonstration that, when chemical analyses are conducted on nectar samples, each corresponding to the production of one elemental secretory structure (i.e., individual nectaries), nectar sugar composition emerges as extraordinarily variable, with most of the variation taking place at the restricted within-plant scale. The magnitude of intraplant variation in nectar sugar composition reported here for *H. foetidus* is similar or even greater than that ordinarily found in interspecific comparisons (e.g., Baker and Baker, 1983b). As noted in the Introduction, the notion of intraspecific constancy prevailing in most recent literature on nectar sugar composition may be traced back to Percival (1961, 1965) and particularly to Wykes (1952). In their pioneering contributions, however, these authors clearly showed that some species may have considerable intraspecific variation in nectar chemistry. Wykes (1952) reported seasonal variation in a few of the species studied, and Percival (1961) noted that at least 61 of the 893 species that she investigated had variable nectars. This figure most likely underestimates the

proportion of species with variable nectars, because it probably reflects only extreme cases where variation was large enough as to be detected by the rather rudimentary analytical methods used by these earlier investigations. In a few subsequent investigations with increasingly sophisticated analytical tools, nectar sugar composition varied between separate populations of the same species or among periods of the flowering season for the same population (Baker and Baker, 1983b; Freeman et al., 1985; Reid et al., 1985; Freeman and Wilken, 1987; Lanza et al., 1995; Roldán-Serrano and Guerra-Sanz, 2004). Most of these studies, however, tended to focus on individual differences as a source of intraspecific variation in nectar sugar composition, without separately analyzing intraplant variation as an additional relevant source. Perhaps for this reason, the magnitude of intraspecific variation reported by these studies appears relatively small in comparison to that documented here for *H. foetidus*. Only Freeman and Wilken's (1987) detailed investigation on *Ipomopsis longiflora* nectar has previously documented intraplant variation in nectar sugar composition. Lanza et al. (1995) failed to detect statistically significant within-plant variation in nectar constituents, but their results should be interpreted with caution in view of the small sample sizes used. Our study has shown that, at the population studied, within-plant variation was the main source of local variation in nectar sugar composition, by far exceeding variation among individual plants. The quantitative importance of variation among flowers of the same plant and among nectaries of the same flower would have remained undetected had we analyzed pooled samples from different nectaries and flowers. As shown by this study, variation in nectar composition among individual plant means may actually be negligible, yet extensive variation may still occur at a very fine-grain spatial scale in local populations due to differences between flowers and nectaries of the same plant. Disclosing this source of variation requires a sampling unit that is commensurate with the spatial scale at which it takes place.

Our results have several methodological and conceptual implications. From a methodological viewpoint, extensive intraplant variability in nectar sugar composition calls for more elaborate sampling designs to capture that source of variance. Elementary sampling theory warns us that misleading results and statistical artifacts may arise if the potentially most important source of variance in a trait of interest remains insufficiently sampled (e.g., Steel and Torrie, 1980). We contend that nectar composition studies should be concerned with the same sampling problems long known to researchers on other reiterated plant structures (e.g., leaves, fruits) that likewise have considerable amounts of within-plant variability (e.g., Wood, 1972; de Silva and Ball, 1997; de Silva et al., 2000; Temesgen 2003). The warning issued long ago by Baten (1936) to students of floral morphology that "one should be very careful when taking a random sample of flowers, for flowers at different positions on certain plants are different, and distributions pertaining to them should not be mixed," should be also taken seriously by students of nectar composition. Technical limitations are not a constraint on nectar sampling designs any longer. As illustrated by this study, accurate hierarchical dissections of nectar composition variance into components down to the elementary secretory unit, the individual nectary, are feasible with the chemical analytical tools currently available.

The extreme intraplant variation in nectar sugar composition in this study for *H. foetidus* and the small spatial scale at which it occurs also prompt for some considerations in relation to insect pollinators. After arriving at an *H. foetidus* inflorescence, individual bumblebees visit most or all open flowers and, in each of these, sequentially probe most or all individual nectaries. From the perspective of individual foragers, therefore, the elemental reward unit is the individual nectary rather than the flower. The hierarchical organization of nectar variability in sugar composition depicted by this study is thus congruent with that presumably perceived by a foraging bumblebee. Variation among nectaries within flowers, and among flowers in the same plant, might be perceived by bumblebee foragers as resembling random noise because differences among *H. foetidus* plants are slight at most, and the spatial patterning of variation within inflorescences was very weak. In one and the same inflorescence, a bumblebee will typically find in successively probed nectaries a highly heterogenous, unpredictable series of pure-sucrose, pure-fructose, sucrose-dominated, and fructose-dominated nectars. Even if bumblebees preferred some particular sugar combinations over others, as traditionally implied (Baker and Baker, 1983b), the predominant within-plant components of variance will render unlikely any behavioral adjustments aimed at maximizing encounters with the preferred nectar types. On the other hand, even if pollinators were able to detect in advance some informative cue on nectar sugar composition (e.g., smell) and exert some prior discrimination, this selection would take place mostly at the within-plant rather than the between-plant level; the between-plant level is in principle the only sort of selection apt to have some evolutionary significance in relation to the evolution of nectar characteristics. This means, therefore, that extensive within-plant variation in nectar sugar composition of the sort documented here for *H. foetidus* will generally limit the selective potential of pollinators on that floral trait.

Our study joins a handful of investigations showing that within-plant variation is a major source of population-wide variance in structural and functional floral traits known to influence pollinator behavior, like size of perianth parts (Campbell, 1992; Williams and Conner, 2001), nectar secretion rate (Feinsinger, 1983; Boose, 1997; Real and Rathcke, 1988), and nectar standing crop (Herrera and Soriguer, 1983; Shmida and Kadmon, 1991). Variance sensitivity and risk-aversion seem almost universal among animals foraging in patchy environments (Kacelnik and Bateson 1996, 1997), and 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; and references therein). It has been suggested that, in hermaphroditic plants, extensive within-plant variance in nectar production and standing crop is an adaptive feature reducing the number of sequentially visited flowers and, consequently, the costs derived from geitonogamous pollinations (Pleasant, 1983; Biernaskie et al., 2002; Biernaskie and Cartar, 2004). It is tempting therefore to speculate that extensive within-plant variation in nectar sugar composition, acting alone or in concert with variation in nectar volume, may likewise be advantageous to plants by decreasing the number of flowers visited per plant by variance-sensitive, risk-averse pollinator foragers, thus representing one further adaptive mechanism reducing geitonogamy. In this case, it may be predicted that within-plant variability in nectar sugar composition should increase with the number of flowers simultaneously open on a plant, as found by Biernaskie and Cartar (2004) for within-plant variability in nectar production. Additional studies on *H. foetidus* and their pollinators are needed to substantiate these suggestions and on

intraspecific nectar sugar variation in other species to assess the generality of the patterns described in this paper.

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