Intraplant variation in nectar traits in *Helleborus foetidus* (Ranunculaceae) as related to floral phase, environmental conditions and pollinator exposure

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ABSTRACT

Factors that contribute to variation in nectar sugar composition, nectar concentration and volume have been a central concern in studies of pollinator assemblages in angiosperms. In an effort to better understand the mechanisms underlying variation in nectar traits, we designed a series of experiments with flowering *Helleborus foetidus* individuals under natural and glasshouse conditions, to identify intraplant variation in nectar traits which depend on both intrinsic (sexual phases of individual flowers) and external (pollinator visits and plant growth conditions) factors. The results showed that nectar volume, sugar composition and concentration in *Helleborus foetidus* varied between floral sexual phases, environmental growing conditions, and levels of flower exposure to pollinator visits. Processes of mate-limitation in male reproductive success or pollen-limitation in female success, as well as flower protogyny and holocrine secretion of nectaries may be involved in nectar variability between floral phases. By comparing different environments we observed that nectar volume and concentration at the nectary and flower level were plastic traits sensitive to external conditions, emphasizing responsiveness to environmental changes and a consequent plasticity in nectar traits such as sugar concentration and volume. Nectar sugar composition did not respond to different growing conditions, suggesting that this is an intrinsic characteristic of this species, but pollinator exposure produced significant changes in the nectar of single nectaries, particularly in the sucrose–fructose balance. Future research on nectar ecology and nectar chemistry will need to consider that nectar traits exhibit different kinds of variation at the intraplant level and under different environmental conditions.

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Introduction

Nectar is one of the most important floral rewards plants offer to pollinators in exchange for pollination (Simpson and Neff, 1983). This floral reward functions as the pivot around which the evolution of plant–pollinator interaction occurred. Pollinator visitation frequency to nectariferous flowers and its duration may depend on two nectar traits, viz. production rate (Biernaskie et al., 2002; Nicolson and Nepi, 2005; Shafir et al., 2003) and chemical composition, this latter including type and relative amounts of sugars, amino acids and lipids (Baker and Baker, 1983a, 1986; Bernardello et al., 1999). Factors that contribute to variation in nectar sugar composition, nectar concentration and volume have been a central concern in studies of pollinator assemblages in angiosperms (e.g. Baker and Baker, 1983b; Baker et al., 1998; Dupont et al., 2004;

Galetto and Bernardello, 2003; Jürgens, 2004). However, most studies have focused on nectar analyses at the species level or above (for a recent review see Herrera et al., 2006), and therefore may not have detected variation at other levels. For example, several studies have revealed that nectar sugar composition, concentration and volume may differ among individuals, populations, cultivars or subspecies of the same species (Baker and Baker, 1983a; Freeman and Wilken, 1987; Freeman et al., 1985; Gottsberger et al., 1989; Lanza et al., 1995; Reid et al., 1985; Roldán-Serrano and Guerra-Sanz, 2004; Schlumpberger et al., 2009; Severson and Erickson, 1984; Stiles and Freeman, 1993; Witt et al., 1999). Intraplant variation in nectar traits can also be extensive and responds to environmental stimuli (e.g. light, water, fertilization, and temperature regimens) (Carroll et al., 2001; Cawoy et al., 2008; Gardener and Gillman, 2001; Mitchell, 2004; Smith et al., 1990). Variation among flowers on the same plant in field populations can be greater than variation among plants under glasshouse conditions (Canto et al., 2007; Freeman and Wilken, 1987). Significant variation has also been reported among different parts of the same flower (Davis et al.,

1998), among nectaries (Herrera et al., 2006) and even between the sexual phases of a single flower (Langenberger and Davis, 2002). This intraplant variation and the effect of different environments on nectar traits has not received as much research attention as interspecies variation.

Several studies have addressed sources of intraspecific variation in nectar chemistry. For example, Nepi et al. (2003), Herrera et al. (2006), and Vezza et al. (2006) reported differences in sugar content and volume of nectar along the flowering season of *Linaria vulgaris*, *Helleborus foetidus*, and *Hedera helix*. At the intraplant level, in a study of variation in the relative amounts of three main nectar sugars in a *H. foetidus* (Ranunculaceae) field population, Herrera et al. (2006) dissected variation into three components: among plants; among flowers on the same plant; and among nectaries in the same flower. Population-wide variance was mainly accounted for by among flowers variation on the same plant (56% of total) and among nectaries variation on the same flower (30%) with only minimal interplant variation (14%). In another study, Canto et al. (2007) analyzed nectar sugar composition variation in two groups of *Aquilegia pyrenaica* subsp. *cazorlensis* plants, one in the field and the other under glasshouse conditions. Field plants exhibited the most variation, accounted for mainly at the among-flower level (82% of total) with minimal variation originating at the among-nectary (10%) or among-plant levels (9.8%). In contrast, variation in nectar sugar composition was negligible in glasshouse plants. Both studies suggested that nectar sugar composition variation occurs largely at the flower level. Neither study, however, considered important nectar traits such as concentration and volume, or specific target-flower factors such as flower sexual phase and the pollinator-visit regime. Detailed studies incorporating these factors are useful in understanding how environment-mediated selection and plant–pollinator interactions may impact nectar trait variation.

In an effort to better understand the mechanisms underlying variation in nectar traits, we designed a series of experiments to identify variation sources in *H. foetidus* nectar traits which incorporated both intrinsic (sexual phases of individual flowers) and external (pollinator visits and plant growth conditions) factors. If intrinsic plant features are shaping natural nectar trait variation patterns, then similar nectar variation patterns should occur in plants under field and glasshouse conditions; for example, nectar trait variation in different flower phases should be comparable between field and glasshouse plants. If external factors (e.g. pollinator visits) are involved in creating the natural variation in nectar traits, then comparison of plants under field conditions but with different exposure to pollinator visits (exposed and unexposed) should effectively reveal this phenomenon. To explore these ideas, we evaluated intraplant variation in sugar composition, nectar concentration and volume in *H. foetidus* following a hierarchical sampling method (Herrera et al., 2006) and comparing hierarchical patterns in nectar variation (1) between flower phases, (2) between plants grown under field or glasshouse conditions, and (3) between field-grown plants with different pollinator exposures.

Materials and methods

Study species

Helleborus foetidus L. is a perennial herb distributed widely in western and southwestern Europe (Weber and Ebel, 1994). It flowers mainly from January through March. Normally one and sometimes more inflorescences are produced, each bearing 25–100 greenish flowers that open gradually over 1.5–2.5 months. Individual flowers are protogynous, extremely long-lived (up to 20 d), hermaphroditic and apocarpous. During anthesis, the flower initially exposes the female phase (receptive stigma) for approxi-

mately 6–15 d, followed by the male phase for the remaining flower lifespan. Flowers are pollinated mainly by medium- and large-sized bees such as bumble bees and anthophorid bees (Herrera et al., 2001; Vesprini and Pacini, 2010). 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 deep 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 contains mainly sucrose (approximately 90%) with small quantities of glucose and fructose (Herrera et al., 2006).

Study site and methods

We evaluated the influence of intraplant variation on nectar traits using flowering *H. foetidus* individuals under natural and glasshouse conditions between November 2005 and March 2006. In early November, 12 reproductive individuals bearing a growing inflorescence meristem were transplanted to a glasshouse from a field population located at ca. 1220 m a.s.l. in a hilly wooded area known as Las Navillas, approximately 10 km from the Roblehondo Field Station in the Sierras de Cazorla-Segura-Las Villas Natural Park, Jaén Province, south-eastern Spain. Plants in the field were growing in the understory of pine (*Pinus pinaster* and *P. nigra*) and evergreen oak (*Quercus ilex*) mixed woodlands with limestone-derived lithosols and clays soil. Climate of the area is cool Mediterranean type with 790 mm of mean annual rainfall, 13 °C of mean annual temperature, and 50–80% air humidity. In the glasshouse, plants were cultivated in polyethylene flowerpots of 3.8 l capacity all containing the same mixture of growing substrate (75% peat moss and 25% vermiculite to improve soil aeration while retaining the moisture and nutrients necessary to feed roots for faster growth). They all were subject also to the same growing conditions: watering – three times per week to 100% of flowerpot field capacity, light – natural daylight, temperature – approximately 27 °C, air humidity – approximately 46%. Plants were allowed to free growing and flowering without pruning. To control for the effect of biotic factors on nectar traits, neither pollinators nor herbivores were allowed access to glasshouse plants during the experiment.

The importance of the flower sexual phase on nectar traits was investigated by selecting three buds per glasshouse plant, and during anthesis closely monitoring them to identify when they were in the female and male phases. When single buds opened and the female phase began (i.e., the pistil was elongated beside the sepal aperture and some of the external-whorl anthers were elongated but still closed), three nectaries were selected per flower and the nectar contained was extracted using 1 mm × 20 mm paper-wicks (Whatman 3MM). After 24 h, the nectar accumulated in the selected nectaries was individually extracted with calibrated capillary tubes and its volume measured using the capillary column length. Each nectar sample was placed in a plastic vial and stored at −80 °C until chemical analysis by HPLC (see below). When the buds began the male phase (i.e., anthers elongated beside the sepal aperture and pollen grains released), this same procedure was repeated.

Variation in nectar traits under different environmental conditions was evaluated using 12 glasshouse plants and ten field plants in the same wild population from which the glasshouse plants had been collected. Three separate flowers in the early male phase (i.e., when anthers from the most external whorl release pollen) were selected on each plant. Three nectaries were selected within each flower and nectar extracted from them with paper-wicks. After 24 h, this same procedure was used to extract, measure and store the accumulated nectar for later HPLC analysis.

Table 1
Summary statistics of relative amounts of individual sugars, total sugar concentration and volume (24 h accumulation) of nectar in individual nectaries of *Helleborus foetidus* flowers by sexual phase, growing conditions and flower exposure to pollinator visits. Flower phase nectar data are only from glasshouse plants.

Nectar trait	Flower phase		Growing conditions	Pollinator Exposure	
	Female	Male	Glasshouse	Field/Unexposed	Field/Exposed
Glucose (%)					
Mean ± s.d.	0.4 ± 1.1	3.5 ± 4.6	0.5 ± 0.7	0.2 ± 0.2	0.4 ± 0.6
Interquartile range	0 – 0.2	0.5 – 6.1	0.1 – 0.5	0 – 0.2	0.1 – 0.4
Fructose (%)					
Mean ± s.d.	0.7 ± 0.9	2.9 ± 3.2	1.1 ± 0.8	1.3 ± 0.6	4.2 ± 11.6
Interquartile range	0.2 – 0.7	0.7 – 4.2	0.6 – 1.2	1.0 – 1.8	0.6 – 2.3
Sucrose (%)					
Mean ± s.d.	99.0 ± 1.9	93.7 ± 7.8	98.5 ± 1.5	98.5 ± 0.8	95.5 ± 12.1
Interquartile range	99.1 – 99.6	90.6 – 98.8	98.4 – 99.3	98.0 – 99.0	97.3 – 99.3
Total sugar concentration (nM)					
Mean ± s.d.	$2 \times 10^6 \pm 5.3 \times 10^5$	$1.8 \times 10^6 \pm 4.8 \times 10^5$	$2.2 \times 10^6 \pm 9.3 \times 10^5$	$1.1 \times 10^6 \pm 4.6 \times 10^5$	$8.8 \times 10^5 \pm 3.7 \times 10^5$
Interquartile range	$1.6 \times 10^6 - 2.3 \times 10^6$	$1.4 \times 10^6 - 2.1 \times 10^6$	$1.3 \times 10^6 - 3.0 \times 10^6$	$7.7 \times 10^5 - 1.5 \times 10^6$	$6.3 \times 10^5 \pm 1.1 \times 10^6$
Nectar volume (µL/24h)					
Mean ± s.d.	1.6 ± 0.7	2.3 ± 1.2	1.1 ± 0.6	2.0 ± 1.0	2.1 ± 0.9
Interquartile range	1.1 – 2.0	1.6 – 3.1	0.7 – 1.4	1.2 – 2.6	1.5 – 2.7

Flower phase survey: 109 nectar samples from single-nectaries during female phase; 107 samples from the same single-nectaries during male phase. A total of 36 flowers sampled from 12 plants. Growing conditions/pollinator exposure surveys: Glasshouse, 144 samples from single-nectaries in 47 flowers on 12 plants; field (pollinator unexposed), 119 samples from 40 flowers on 10 plants; and field (pollinator exposed) 120 samples from 40 flowers on 10 plants.

The effect of pollinator visits to flowers and nectaries was analyzed in the field using ten pairs of plants, each pair being nearest neighbors. In each pair, one plant was left exposed to pollinator visits while the other was covered with mosquito net to prevent visits. In each plant, three flowers and three nectaries within single flowers were selected, and the nectar-sampling method described above applied to single nectaries.

Nectar analysis

Sucrose, glucose and fructose proportions were measured for 599 nectar samples ($N = 360$ from glasshouse plants, $N = 239$ from field plants) using high performance liquid chromatography (HPLC). The nectar-containing vials were thawed and different volumes of HPLC-grade water added to each to complete to 1 mL of solution. For each sample, 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, CA, USA). The HPLC system was equipped with an effluent degas module, a GP 40 gradient pump, a CarboPac PA10 (4 mm × 50 mm) guard column and a CarboPac PA10 (4 mm × 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 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 and separately for D-glucose, D-fructose and sucrose (Sigma–Aldrich, Madrid, Spain) by injecting 1 to 10 µL of a combination of several calibration solutions each containing one of these sugars and one of these concentrations: 0.1–0.025 mM L^{-1}.

The proportions of the three sugars (glucose, fructose, and sucrose) in each analyzed sample were estimated by integrating the area under the chromatogram peaks. Two independent HPLC measurements were done on each sample, and replicate results averaged for the analyses. Only sucrose, glucose, and fructose appeared in all samples. Overall sugar concentration for a single nectar sample was estimated by fitting linear regression models to the data of standard sugar concentration solutions and comparing it to the integrated area of each sugar contained in the individual analyzed samples. The resulting partial concentrations of glucose, fructose and sucrose were then summed per individual sample and corrected according to each sample's volume. Finally, overall sugar concentrations were converted to nanomoles (nM) as suggested by Petanidou (2005).

Statistical analysis

All statistical analyses were done with the SAS statistical package (SAS Institute, Cary, NC, USA). Analysis of intraplant variation in the three studied nectar traits (nectar sugar composition, nectar sugar concentration, and nectar volume) was done by applying a hierarchical partition to divide total variance into three levels of variation: among plants, among flowers in the same plant, and among nectaries in the same flower. Variance components were calculated using the COVTEST statement in the MIXED procedure, and statistical significance estimated with the RANDOM statement of the GLM procedure, which produces an unbiased F-test for each hierarchical level (Herrera et al., 2006). Hierarchical partition analyses were run separately for flower phases, growing conditions and pollinator exposures data sets. Differences in nectar sugar composition were evaluated between flower phases, growing conditions and pollinator exposure. Glucose, fructose and sucrose percentages

in the nectar of single nectaries were arc-sine transformed and analyzed in a multivariable context using a MANOVA procedure. Differences in nectar concentration (untransformed nanomole values) and nectar volume (untransformed microliter values) between flower phases, growing conditions and pollinator exposures were tested using mixed models fitted to the data with the MIXED procedure and applying restricted maximum likelihood estimation (REML; Littell et al., 1996).

Results

Nectar sugar composition

Nectar sugar composition between flower phases was studied only in glasshouse plants. In both female and male phases, single nectaries contained sucrose-dominated nectar with minimal glucose and fructose proportions. Application of a MANOVA analysis showed significant differences (Hotelling's $T = 0.48$, $F_{3, 202} = 32.59$, $P < 0.0001$) in sugar composition between phases. Glucose and fructose contents were lower in the female phase than in the subsequent male phase, while sucrose content was higher during the female phase than in the male phase (Table 1). Hierarchical variance partition of the relative proportions of individual nectar sugars revealed a significant variance component only during the male phase and at two levels: among flowers on the same plant and among nectaries in the same flower (Fig. 1a and b, Table 2).

Sucrose dominated nectar composition in single nectaries under both glasshouse and field growing conditions, although differences in nectar sugar proportions (mainly for glucose and fructose) were detected between growing conditions by the MANOVA analysis (Hotelling's $T = 0.42$, $F_{2, 260} = 36.56$, $P < 0.0001$). Overall, variability in sugar composition was minimal between growing conditions (Fig. 1c and d). The variance partition analysis detected significant variance in nectar sugar composition mainly at the among-plant level under glasshouse conditions, whereas under field conditions variance was present at the among-plant and among-flower levels (Table 2).

Nectar sugar composition remained sucrose-dominated under different pollinator exposures, although significant differences in sugar composition were identified at the among-plant level between exposed and unexposed plants (Hotelling's $T = 0.95$, $F_{3, 236} = 4.01$, $P = 0.008$). These differences were the result of reductions in the sucrose percentage and increases in the fructose percentage (Table 1). The hierarchical analysis of variance showed that most of the significant variance in nectar composition originated among flowers on the same plant (Fig. 1d and e, Table 2).

Nectar sugar concentration

Nectar sugar concentration was high in single nectaries at both flower phases (glasshouse plants), but was slightly higher during the female phase than during the male phase ($t = 3.54$, df = 164, $P = 0.0005$, Table 1). The hierarchical partition of variance identified significant variance in sugar concentration at all three levels only during the female phase (Table 3).

Nectar sugar concentration was twice as high in the single nectaries of glasshouse plants than in those of field plants ($t = 11.21$, df = 268, $P < 0.0001$, Table 1). Most of the nectar concentration variance in glasshouse plants originated at the among-plant and among-flower levels, while in field plants it originated only at the among-plant level (Table 3).

Pollinator exposure affected sugar concentrations such that concentrations in unexposed flowers were higher than in exposed flowers ($t = 3.69$, df = 268, $P = 0.0003$, Table 1). In unexposed plants,

variance in nectar sugar concentration occurred only at the among-plant level, but in exposed plants none of the analyzed intraplant hierarchical levels accounted for the significant variance in concentration (Table 3).

Nectar volume

At the flower phase level, 24-h nectar accumulation in single nectaries was lower during the female phase than in the male ($t = 6.85$, df = 174, $P < 0.0001$, Table 1). During the female phase, both the among-plant and among-flower levels accounted for nectar volume variance, but during the male phase only the among-flower level accounted for most of the variance (Table 3).

Nectar volume in single nectaries was lower in glasshouse plants than in field plants ($t = 8.99$, df = 268, $P < 0.0001$, Table 1); under both conditions most of the nectar volume variance originated at the among-plant and among-flower levels (Table 3). Nectar volume in single nectaries was slightly lower in pollinator unexposed plants versus pollinator exposed plants ($t = 3.02$, df = 268, $P = 0.0028$, Table 1). In unexposed plants, most of the variance occurred at the among-plant and among-flower levels, while in exposed plants it occurred at the among-flower level (Table 3).

Discussion

Helleborus foetidus nectar sugar composition, concentration and volume differed between flower sexual phases, growing conditions and pollinator exposures. Nectar response under the different situations studied here indicates that mechanisms both intrinsic and extrinsic to the plant control nectar variability. Two questions are prompted by these results: 'What mechanisms are involved in observed nectar variation?' and 'Does the observed hierarchical intraplant variation ultimately reflect the type of mechanism involved in the different nectar variation scenarios?'

Gender-biased nectar traits

Gender-bias in nectar is an intrinsic characteristic reported for plants families with dichogamous or heterostylous flowers, including the Ranunculaceae family (Carlson and Harms, 2006; Cawoy et al., 2008; Nepi et al., 2001; Ornelas et al., 2004; Symes and Nicolson, 2008). Gender-bias may be prompted and maintained by sexual selection or inbreeding avoidance (Carlson, 2008; Carlson and Harms, 2006), implying that mechanisms of mate-limitation in male reproductive success or pollen-limitation in female success may enhance nectar trait performance in one of a flower's sexual functions. In the present results, *H. foetidus* nectar sugar concentration was female-biased but nectar production (volume) was male-biased. The higher sugar concentrations observed during the female phase, and the nectar volume increases observed during the male phase, plausibly constitute a complementary time-lag between flower sexual functions that would simultaneously promote both male-biased nectar volume through sexual selection and female-biased sugar concentration in nectar through intraplant inbreeding avoidance.

Nectar sugar composition was also gender-biased. During the male phase, variance in hexose proportions increased in comparison to the female phase. We are aware of few studies addressing gender-bias in nectar composition: Witt et al. (1999) on two dioecious *Silene* species, and Langenberger and Davis (2002) on the dichogamous *Carum carvi*. The proportion of hexoses increased during the male phase in these studies, as found here for *H. foetidus*. Given that pollinators can respond to minimal variations in food sources, the greater sugar composition variation in the nectar during the male phase may indicate a complementary time lag between female and male phase that could enhance reproductive
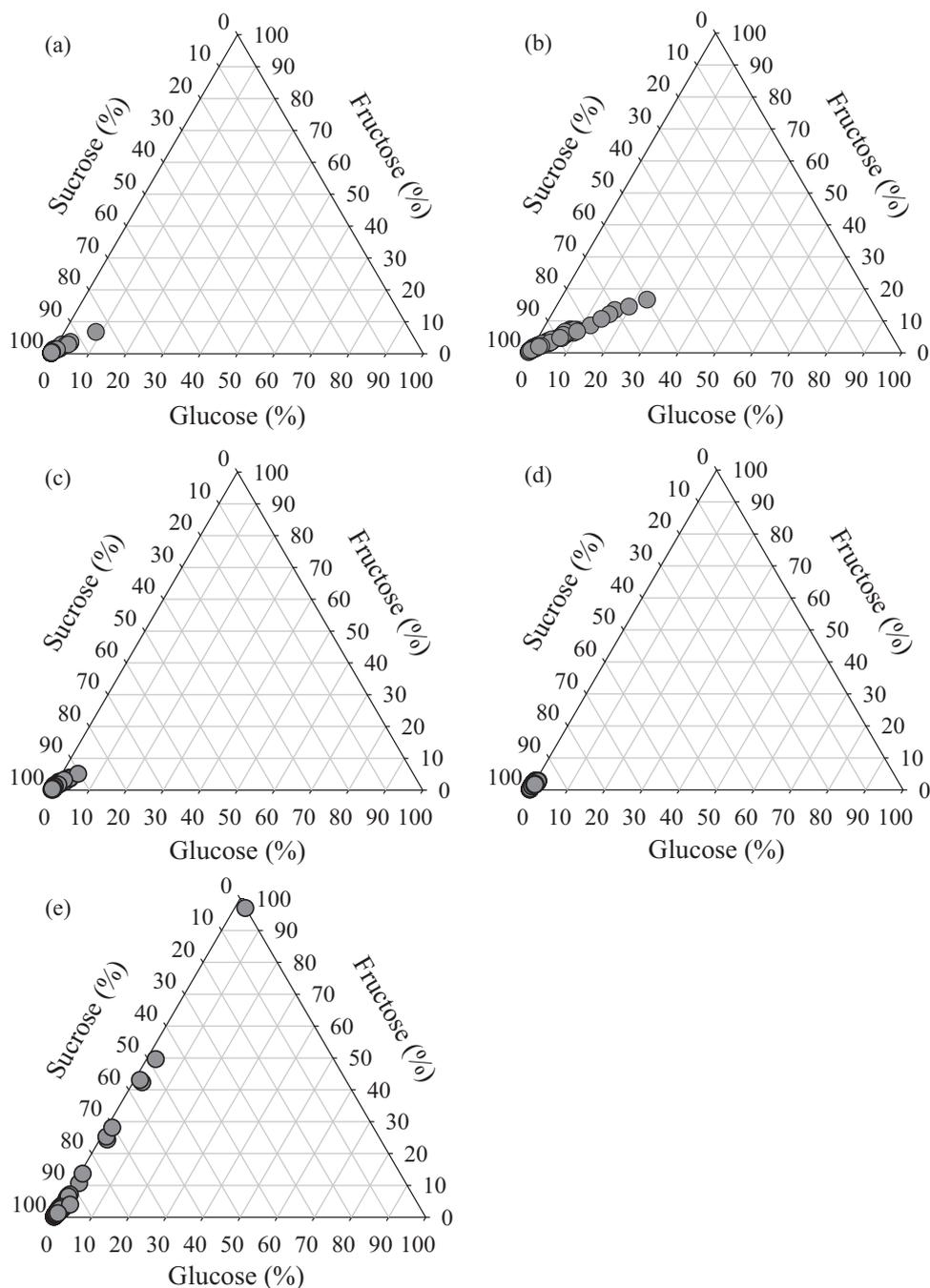
Fig. 1. Ternary diagrams showing variation in the relative amounts (%) of sugars in nectar from single nectaries of *Helleborus foetidus* at the (a) female and (b) male flower phases; under (c) glasshouse and (d) field (pollinator unexposed) growing conditions; and in field plants (d) unexposed and (e) exposed to pollinator visits. Each point represents the proportional nectar sugar composition in each sampled nectary. The distance of a point from a side of the triangle is proportional to the relative importance of that sugar in the sample.

success in this species. This greater variation may produce a mosaic of food availability to which pollinators respond. Plant pollinators, bumble bees in the case of *H. foetidus*, are expected to detect and respond to the food availability variation mosaic during foraging forays (Waddington, 2001). In response to greater variability in this mosaic, pollinators may forage more widely and increase mating among plants via the male function and outcrossing-pollination via the female function (Carlson, 2008; Carlson and Harms, 2006). Therefore, both sexual selection and inbreeding avoidance are plausible explanations for the variation in sugar composition, nectar concentration and volume observed between male and female flower phases in *H. foetidus*.

Growing conditions and pollinator exposure

Because we did not directly measure differences between glasshouse and field conditions for environmental factors such as light, water, soil nutrients, temperature and moisture the results cannot be discussed in terms of the effect of specific environmental variables on nectar traits. The results can, however, be generally interpreted in relation to nectar trait response to the different plant environments. In addition, the glasshouse plants had been transplanted from the same geographical location as the field plants, meaning no genetic or local effects on nectar traits were assumed and only environmental effects were expected. Nec-

Table 2
Hierarchical partition of variance in the relative sugar proportions (%) of nectar in individual nectaries of *Helleborus foetidus* flowers by sexual phase, growing conditions and flower exposure to pollinator visits. Relative contribution of variance, *F*-test, and statistical significance results are shown.

Nectar trait	Flower phase						Growing conditions						Pollinator Exposure		
	Female			Male			Glasshouse			Field/Unexposed			Field/Exposed		
	Variance %	F	P	Variance %	F	P	Variance %	F	P	Variance %	F	P	Variance %	F	P
Glucose (%)															
Plant	20.1	1.1	0.42	12	1.4	0.23	63.6	2.4	0.029	0	0.6	0.76	0	0.9	0.58
Flower in same plant	0	0.8	0.69	68.2	3.7	<0.0001	33.7	1.2	0.29	100	1.3	0.22	99.8	4.5	<0.0001
Nectary in same flower	79.9	1.3	0.27	20	5.2	0.0003	2.7	0.7	0.76	0	0.1	0.99	0.2	0.9	0.53
Fructose (%)															
Plant	26.7	1.2	0.37	11	1.4	0.27	96.5	3.8	0.001	62.1	6.6	<0.0001	0	0.7	0.68
Flower in same plant	67.9	1	0.51	70.3	3.5	<0.0001	3.6	1	0.55	37.9	2.8	0.0005	97.2	1.9	0.017
Nectary in same flower	5.4	0.7	0.65	18.7	4.4	0.001	0	0.7	0.79	0	0.3	0.99	2.6	1	0.48
Sucrose (%)															
Plant	0	0.9	0.55	0	0.6	0.80	82.6	3.5	0.003	32.3	3.4	0.007	6.9	1.1	0.42
Flower in same plant	0	0.7	0.86	37	3	0.0006	12.4	1	0.50	67.8	2.9	0.0002	92.3	3.9	<0.0001
Nectary in same flower	100	0.7	0.63	63	3.1	0.011	5	0.7	0.75	0	0.4	0.95	0.9	1	0.45

tar traits responded differently to glasshouse or field conditions, respectively. In plants not exposed to pollinators, nectar sugar composition changed little between the glasshouse and field conditions, which suggests that it is a fixed, intrinsic trait in *H. foetidus*. Sugar concentration and volume, in contrast, did respond to environmental conditions, suggesting the presence of an important plasticity mechanism in their expression. As expected, nectar concentration was higher and less variable in the glasshouse plants, which can be attributed to the optimum growth conditions in this environment. However, nectar volume was unexpectedly high in the field environment, where conditions were assumed to limit

growth. It is likely that variability in field abiotic conditions *per se* played a role in the nectar volume variability which produced the differences between growing conditions observed here (e.g. as the nectar parenchyma of *H. foetidus* has chloroplasts, its photosynthetic activity could be affected by the shade/light mosaic prevailing under field conditions; Vesprini et al., 1999). However, we did not measure abiotic variables and therefore our conclusions must remain limited.

Under field conditions, nectar sugar composition exhibited a decrease in sucrose and an increase in fructose, which contrasts with the lack of differences in sugar composition observed with

Table 3
Hierarchical partition of variance in total sugar concentration and volume (24 h accumulation) of nectar in individual nectaries of *Helleborus foetidus* flowers by sexual phase, growing conditions and flower exposure to pollinator visits. Relative contribution of variance, *F*-test, and statistical significance results are shown.

Nectar trait	Flower phase						Growing conditions						Pollinator Exposure		
	Female			Male			Glasshouse			Field/Unexposed			Field/Exposed		
	Variance %	F	P	Variance %	F	P	Variance %	F	P	Variance %	F	P	Variance %	F	P
Total sugar concentration (nM)															
Plant	46.5	3.1	0.012	56.4	1.9	0.10	44.3	2.3	0.036	42	7.6	<0.0001	29.7	2.1	0.07
Flower in same plant	34.5	2.2	0.009	43.6	1.7	0.06	55.7	2.3	0.001	58	1	0.48	70.3	1.6	0.07
Nectary in same flower	19	3.6	0.004	0	1.3	0.26	0	1	0.52	0	12	0.68	0	0.7	0.68
Nectar volume (µL/24h)															
Plant	38.4	2.8	0.022	31.8	2.0	0.07	41.5	5.2	0.0002	70.5	7.6	<0.0001	29.4	1.9	0.09
Flower in same plant	59.2	7.7	<0.0001	65.7	3.1	0.0002	53.7	3.7	<0.0001	29.5	4.6	<0.0001	70.6	4.1	<0.0001
Nectary in same flower	2.4	2.1	0.06	2.5	1.3	0.27	4.8	1.6	0.09	0	0.9	0.54	0	1.7	0.08

glasshouse plants and field plants not exposed to pollinators. Similar results were reported for *Ipomopsis longiflora* (Freeman and Wilken, 1987), suggesting pollinators may trigger changes in nectar composition by visiting flowers. Herrera et al. (2006) reported extreme variability in nectar sugar composition in *H. foetidus* single-nectaries under a changing scenario of pollinator-visits: scarce at the beginning of the flowering season and abundant at the end of the season. They found that nectar sugar composition varied from almost pure sucrose in the early flowering season to a fructose-dominated solution at its end, when pollinators visits were very frequent. Canto et al. (2008) also reported that nectar sugar composition in single nectaries changed dramatically from sucrose to fructose dominance when the glossa and labial palpus of field-captured bumble bees were introduced into single nectaries containing virgin nectar. Herrera et al. (2008) reported large increases in fructose and decreases in sucrose correlating to increases in yeast density in the nectar of *H. foetidus* plants exposed to pollinators. They concluded that the changes in nectar sugar composition were the result of nectar contamination with nectarivorous yeasts harbored in the bee proboscides. Changes in nectar sugar composition occurred when the bees' proboscides were inserted into the nectary and nectar thus became contaminated with yeasts. The effect of yeasts on nectar sugar composition is an external mechanism in which yeasts hydrolyze sucrose into its two component monosaccharides. The yeasts then selectively consume the glucose in the nectar, leaving fructose to dominate in the extracellular environment (Trumbly, 1992). This agrees with the unbalanced nectar sugar proportions observed in the present study in nectar from nectaries in pollinator-exposed field plants. Herrera et al. (2008) reported decreases in total sugar concentration related to increases in yeast density. This is supported by the present results, which show that decreases in sugar concentration in pollinator-exposed field plants were greater than those in pollinator-unexposed field plants. It is noteworthy that flowers of *H. foetidus* are bell-shaped and pendant, thus microorganisms are transmitted to nectaries by pollinators and do not derive from those dispersed in the air. Additionally, nectar contaminated by yeasts vectored by pollinators may become toxic and influence pollinator visitation dynamics (Ehlers and Olesen, 1997; Herrera et al., 2008; Wiens et al., 2008). In conclusion, the above data substantiate the notion that when pollinators visit the nectaries of *H. foetidus* flowers they may contaminate the nectar, causing changes in sugar composition, lowering sugar concentration and consequently degrading nectar energy value.

Intraplant variation in nectar traits

Environmental differences produced different levels of nectar trait variability at the intraplant level in *H. foetidus*. The observed intraplant variation in nectar sugar composition, concentration and volume between female and male flower phases may reflect two possible intraplant mechanisms: protogyny and holocrine secretion. Protogyny is maturation of the female reproductive organs before the male organs, and nectar production within this mechanism is linked to flower phase and nectary age. Holocrine secretion is a nectar production mechanism in which nectar contained within nectary cells, the multiple cell layers that give structure to the nectary gland, is released into the nectary cavity by rupture of whole cells (Vesprini et al., 2008). In this mechanism, nectar production is linked to a gradient of nectar-secreting tissue layers arranged from the proximal to the distal layers of the nectary cavity (Vesprini et al., 1999). If these mechanisms operate in *H. foetidus*, then it is to be expected that the nectar underwent observable changes in sugar composition, concentration and volume as flowers changed from the female to the male phase and nectar-secreting cells were sloughed off from the proximal to distal layers. Under these circumstances, the differences in nectar traits would be limited to the among-flower and among-nectary levels. Further cytological study would help to better understand the influence of these two mechanisms on nectar traits in *H. foetidus*.

Variability in nectar concentration and volume between glasshouse and field plants originated principally at the among-plant level, and to a lesser extent at the among-flower level. Environment can be expected to have a strong influence on plant performance and will be initially reflected in variation at the individual (among-plant) level (Lambers et al., 1998). At this level, plants adjust their resource stock and then allocate it differentially to sexual functions, emphasizing responsiveness to environmental changes and a consequent plasticity in nectar traits such as sugar concentration and volume. Nectar sugar composition did not respond to different growing conditions, but pollinator exposure produced significant changes, particularly in the hexose balance. These changes were observed at the among-flower level, which is probably due to flower visits of pollinators. During foraging forays, pollinators maximize the food resources (i.e., flowers) they encounter, using their glossa to touch and probe all the nectaries in a given flower (Waddington, 2001). Changes in nectar sugar composition, concentration and volume produced by pollinator nectary-probing can therefore be expected to appear at the flower level. In conclusion, the results indicate that nectar traits in *H. foetidus* can vary between floral sexual phase, between environmental growing conditions and between levels of flower exposure to pollinator visits. Future nectar ecology and nectar chemistry research will need to consider that *H. foetidus* nectar traits exhibit different kinds of variation at intraplant levels under different environmental conditions.

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