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Spatial and temporal distribution patterns of nectar-inhabiting yeasts: how different floral microenvironments arise in winter-blooming *Helleborus foetidus*

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ABSTRACT

Yeasts frequently colonise floral nectar, where they can reach high densities. Recent investigations have further shown that yeast metabolism alters nectar properties by decreasing its total sugar content, modifying sugar composition, or raising nectar local temperature. However, the distribution patterns of nectar yeasts remain poorly investigated at multiple spatial and temporal scales. Here, we study natural variation of the nectar yeasts in a single host plant, *Helleborus foetidus*, in a mountainous region. We quantified spatio-temporal variation in the frequency and abundance of yeast species across six populations located along an altitudinal gradient. Variance partitioning techniques were used to estimate the relative magnitude of variation in yeast abundance between individual plants, flowers within plants, and nectaries within flowers. Although yeast frequency and abundance varied widely across sites and dates, the largest part of total variance occurred at the sub-individual level (i.e., flowers on the same plant). Pollinator composition and activity seemed the main factors explaining the observed patterns of yeast frequency and abundance across floral nectar samples.

Introduction

The presence of yeast in flowers has been repeatedly addressed by microbiologists, from the late nineteenth century onwards (Boutroux, 1884; Schuster and Ulehla, 1913; Grüss, 1917; Schoelhorn, 1919; Nadson and Krassilnikov, 1927; Capriotti, 1953; Vörös-Felkai, 1957; Sandhu and Waraich, 1985; Lachance et al., 2001; Brysch-Herzberg, 2004). Over the last decade it has become gradually more apparent that yeast metabolism alters the physicochemical properties of nectar, including the sugar concentration and composition (Canto et al., 2007, 2008; de Vega et al., 2009; de Vega and

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Herrera, 2013; Herrera et al., 2008), amino acid profile (Peay et al., 2012) and even flower temperature (Herrera and Pozo, 2010). Some of these changes occur due to the density-dependent action of a species-poor yeast community. Understanding the patterns of yeast prevalence and distribution in natural habitats has, therefore, become a topic of broad ecological interest (Belisle et al., 2012; de Vega et al., 2009; Herrera et al., 2009; Jacquemyn et al., 2013; Pozo et al., 2009).

Yeasts are widespread in floral nectar, occurring in 40–60 % of samples collected in North America, Europe and South Africa (Belisle et al., 2012; de Vega et al., 2009; Herrera et al., 2009). Despite the extensive occurrence of nectar yeasts, yeast frequency and abundance in floral nectar not only varies between regions, but also between different host plant species within a given region (de Vega et al., 2009; Herrera et al., 2009). Interestingly, the nectar yeast abundance data reported by Herrera et al. (2008, 2009) and de Vega et al. (2009) revealed considerable intraspecific variability as well (i.e., amongst individuals of the same plant species), but their sampling method, aimed at uncovering broad-scale patterns, did not explicitly address intraspecific variance in nectar yeast abundance. Further research was, therefore, necessary to determine the main factors contributing to observed variation in yeast abundance at different spatial scales.

The few currently available studies focussing on more detailed nectar yeast distribution patterns point out that yeast presence in a single plant population may be extremely patchy. The presence of yeast in nectar may vary as a result of the availability of nectar, but its occurrence is reliant on its dispersal either by pollinators or by air (Belisle et al., 2012; Golonka and Vilgalys, 2013; Pozo et al., 2009, 2012). As a result, several environmental factors may contribute directly or indirectly to generate heterogeneity in nectar yeast abundance in natural plant populations, including relative air humidity, air temperature, and precipitation (Belisle et al., 2012; Herrera et al., 2009; Lachance, 2006). Relative air humidity, for example, affects nectar secretion rates and concentrations (Corbet et al., 1979), whereas air temperature can have profound effects on plant phenology and floral density (Sánchez-Lafuente et al., 2005), in addition to nectar secretion and concentration (Freeman and Head, 1990). Temperature also influences pollinator composition and visitation rates (Herrera, 1995), as well as yeast growth and survival (Deak, 2006). The frequency of precipitation events can alter insect pollinator foraging patterns (Herrera, 1995), which in turn may affect yeast dispersal (Canto et al., 2008). Besides these abiotic variables, biotic variables such as floral density can also be expected to affect pollinator visitation rates (Belisle et al., 2012) and hence yeast frequency and abundance.

In this paper, we present a multiscale analysis of yeast distribution patterns in the nectar of a single species using a spatially nested design. This method enables variation to be investigated among populations at different altitudes, individuals within populations, flowers within plants, and distinct nectaries within individual flowers of the perennial, winter-flowering herb *Helleborus foetidus*. At the same time, it permits the study of variation at the sub-individual level (Herrera et al., 2006; Herrera, 2009). Nectar yeast prevalence was studied in six *H. foetidus* populations located at different elevations in a mountainous area in SE Spain. More specifically, the purpose of this study is to quantitatively assess the frequency and abundance of nectar yeasts in a single host plant at different locations where there is the likelihood of variation in both biotic (e.g., pollinator composition and activity, or floral density) and abiotic features (e.g., air temperature, rain, relative humidity of the air) linked to changes in altitude in Mediterranean mountainous areas (Giménez Benavides et al., 2006). Because *H. foetidus* has long-lasting flowers (Herrera et al., 2002), temporal variation in yeast prevalence during the flowering period was also investigated. Although the spatial and temporal scope of this study are relatively modest, results provide new insights into the role of biotic and abiotic factors potentially contributing to shape nectar yeast distribution patterns for a single host plant.

Materials and methods

Study species and sites

Helleborus foetidus is a winter blooming herb that is widely distributed in Western Europe. It is quite abundant at our study area (see below), where it is found at a wide range of elevations (Herrera et al., 2001). Each plant produces from one to a few inflorescences each year, and 20–75 flowers will open asynchronously throughout the 1-3 month-long flowering season. The flowers are protandrous and primarily visited by bumblebees (Herrera et al., 2001). Each individual flower lasts for 1–3 weeks, and usually bears five big, horn-shaped nectaries deeply hidden inside a globose, pendant corolla. Each individual nectary may contain up to 5 μl of nectar (see (Herrera et al., 2002) for further floral details). The presence of several nectaries within each flower enables the analysis of yeast variation patterns at the within-flower level.

This study was carried out in 2009 on six *H. foetidus* populations growing in well-preserved mountain forests in the Cazorla, Segura y las Villas Natural Park, Jaén province, SE Spain (see Herrera et al., 2009; for further details of the study region). Pairs of populations were selected along an altitudinal gradient, roughly covering the elevational range of *H. foetidus* in our study area. The two lowest elevation sites (denoted L1 and L2) were located 960 and 1 100 m above sea level (m a.s.l.); the two mid elevation sites (M1 and M2) were located 1 460 and 1 540 m a.s.l.; and the two highest elevation sites (H1 and H2) were 1790 and 1810 m a.s.l. Distances between populations of the same pair ranged from 0.8 to 2.5 km, and distances between pairs between 2 and 10 km (Fig S1). The site elevation, average abiotic conditions (temperature, air relative humidity), pollinator composition and activity, and floral density are given in Table S1 for the 2009 flowering season. In 2008 we detected slight differences in sunlight incidence, and hence in flowering time between the two low-altitude populations. Therefore, for spatial comparison purposes, we selected peak bloom in L2 according to the highest floral density estimates.

The flowering season tends to be shorter at high elevation sites, so we selected one of the low elevation sites (L2) for the temporal monitoring of nectar yeast before and after peak bloom.

Field and laboratory methods

Preliminary work and analysis in 2008 (M.I. Pozo, unpublished results) helped refine the methods for 2009, which are presented in the following paragraphs.

Each location was sampled once, at its local peak bloom. The sampling date was determined according to the proportion of plants with inflorescences bearing open flowers (=PIBOF), and sampling took place when PIBOF > 75 %. Ten randomly-distributed *H. foetidus* plants bearing at least two open flowers were randomly selected from each population at the beginning of the 2009 local flowering season. The flower age was standardised at the time of collection, and thus only middle-aged flowers were chosen, coinciding with an intermediate floral sexual stage (at least half of the anthers being mature). The sampling method resulted in a total of 120 nectaries being examined for nectar yeasts (one nectary from each of the two flowers per plant, $N = 10$ plants in each of the six populations). For the low elevation site, L2, the ten selected plants were sampled repeatedly on a bi-weekly basis, starting at the beginning of the flowering season. We monitored the L2 population from early March to mid April and collected 132 nectar samples (2 nectaries from 2 flowers per plant, and $N = 10$, 10, 9, and 4 plants, on the four collection dates, respectively).

For each sampling, individually marked flowers were collected in the field, placed inside plastic containers and immediately taken to the laboratory, where they were kept in a refrigerator at 5 °C. In the lab, we then marked one nectary (L1, M1, M2, H1, H2) or two nectaries (temporal monitoring in L2) per flower prior to nectar extraction, which was done as soon as possible and no later than 24 hr after the field collection. We measured nectar volume using calibrated microcapillaries. From each nectary, 1–2 ml of nectar were extracted, diluted in a 40 % lactophenol cotton blue solution, and immediately examined under a microscope to determine yeast cell density. Sixteen replicated standard counts were conducted on each individual nectar sample using a Neubauer chamber, at a magnification of 400×. We assessed the presence of yeast in nectar by estimating yeast frequency (proportion of nectar samples with nectar yeasts) and abundance (mean number of cells μl^{-1} of nectar). Microscopic examination of nectar indicated that all microbes involved in the cell counts were unequivocally yeasts. One microlitre from each of the 120 nectaries examined was plated in YGC (Yeast-Extract Glucose Chloramphenicol, Fluka: 2 % glucose, 0.5 % yeast extract, 0.01 % chloramphenicol, pH 6.6) agar, and incubated at 25 °C for 7 d. After this, the representative morphotypes (based on the experience gained the previous year) were isolated and identified by terminal restriction fragment length polymorphism (TRFLP). After amplifying the D1/D2 DNA region using the primer pair NL1/NL4, the PCR product (about 500 bp) was digested adding the enzyme MseI. Unique, species-specific PCR products of 106 and 401 bp were obtained from all the isolates examined ($N = 245$). All the samples were unambiguously identified as *Metschnikowia reukaufii*. These identifications were confirmed by DNA sequence analysis from 45 isolates, the D1/D2 domain of the large subunit of rDNA being sequenced, as described in (Pozo et al., 2011). Forty sequences were identified as *M. reukaufii* according to their close similarity (>98 %) with the type strain, and 5 were identified as *Cryptococcus victoriae* (all of them belonging to the same population, LN, from the earliest collection date). The sequences were submitted to the GenBank database under accession numbers KJ128161 – KJ128205.

Temperature and relative air humidity records were obtained at each elevation by placing a Gemini data logger (Scientific house, Chichester, UK) at the north face of a tree trunk approximately 3 m above the ground. The data loggers were programmed to record data every 30 min for 4 months, between February and May 2009. We analysed the following meteorological variables: mean, maximum, and minimum air temperature, mean relative humidity, and the proportion of rainy days within the 15 before each collection date. We also determined the following three biotic factors: floral density, pollinator composition, and pollinator activity. The floral density at each locality was estimated along a 70 m transect by using 7 consecutive circular areas of 10 m diameter in which we counted the number of plants, and the number of open flowers on each plant. For each population, twenty five pollinator censuses of 3 min duration each were conducted on two non-consecutive sunny days around the nectar sample collection dates to determine pollinator composition (per cent of flower visits made by each of the two main visitors, *Apis mellifera* or *Bombus* spp.) and activity (flower visits/min), following the methods previously described (see Herrera et al., 2001; Herrera, 2005; for details). For the L2 population, the same procedure was followed on each sampling occasion ("early", "peak bloom" and "past peak bloom"), with the exception of the latest collection date, as not enough plants bearing open flowers were available.

Statistical analyses

All the statistical analyses were conducted using the SAS package (SAS Institute, Cary, USA), unless otherwise indicated. Although yeast prevalence comprises both frequency and abundance in samples, yeast frequency, the relative frequency of yeast presence (all values being distinct to zero), is less useful for detecting fine-scale differences in yeast prevalence. Therefore, the statistical tests were computed by using yeast abundance, as yeast frequency closely correlated with abundance in all cases examined ($r_s = 0.73$; $p < 0.05$; $N = 9$ samplings).

Yeast abundance distribution tended to be sharply bimodal, reaching either extraordinarily high values or falling on or near zero, thus this variable was log-transformed for the analyses. Even so, yeast abundance distribution was not well-normalised in all cases, and variance was heterogeneously distributed among the groups. For this reason we used non-parametric Kruskal–Wallis tests (NPAR1WAY Procedure) to estimate significance of differences in the mean log-transformed yeast abundance between elevations, populations, and collection dates, respectively. We also used Kruskal Wallis tests to compare differences in nectar yeast mean abundance between paired populations from the same elevations and between the different collection dates for the LN2 population.

To assess variation in yeast abundance between populations, among the plants within a population, and between

flowers from individual plants, variance partitions and tests on the statistical significance of variance components were conducted using restricted maximum likelihood (REML) as implemented in the procedure MIXED. We followed a fully-nested sampling method, with populations, plants, and flowers as the variance levels examined; all of them were declared as random. The replicate counting obtained for each single-nectary sample allowed us to estimate measurement error and thus assess the variance component and statistical significance of this component between individual plants. An additional variance component was added to the analysis when we measured two individual nectaries from the same flowers, allowing variance partitioning between plants, flowers and nectaries as nested levels of variance, again all of them declared as random and with replicate counts included in the error term.

Finally, a multivariate analysis of Partial Least Squares (PLS; SIMCA-P, v.12, *Umetrics Inc.*) was used to explore the relationships between environmental variables and yeast abundance. The rank-transformed mean yeast abundance in each sampling (Wilcoxon mean scores (Conover, 1981)), and the relative yeast frequency (% of nectar samples with yeasts) were the response variables. The explanatory variables included the biotic and abiotic parameters mentioned above. We then represented the contribution of each predictor in fitting the PLS model for both predictors and response, based on the *Variable Importance for Projection* (VIP) statistic as described in Wold et al. (2001), which summarises the contribution a variable makes to the model. Independent variables with VIP values below 0.8 were considered to be not relevant for the VIP (Tenenhaus, 1998). For the purposes of PLS analyses, we considered the three different samplings in L2 as statistically independent units, due to the fact that different flowers were surveyed in each sampling.

Results

Spatio-temporal distribution patterns of nectar yeasts

Mean yeast abundance differed significantly ($\chi^2 = 6.61$, d.f. = 2, $p = 0.03$) between populations at different elevations, but no gradual changes in mean yeast abundance were found according to the altitudinal pattern investigated (Fig 1). Furthermore, replicate populations at the same altitudinal level did not show a consistent pattern, and statistically significant differences in yeast abundance were found between populations at low and high elevation sites (Fig 1). Consequently, when we considered populations irrespective of its elevation category, we also found statistically significant differences among populations in abundance of yeasts in floral nectar ($\chi^2 = 17.22$, d.f. = 5, $p = 0.004$).

Abundance of nectar yeasts varied significantly ($\chi^2 = 26.27$, d.f. = 3, $p < 0.0001$) between collection dates (Fig 2). Yeast abundance was very low in the beginning of the sampling period, increased rapidly in the next weeks, but decreased again at the end of the flowering period. At the individual plant level, the same pattern emerged and, for those individuals that bloomed along the complete sampling period, yeast abundance first increased reaching the maximum around

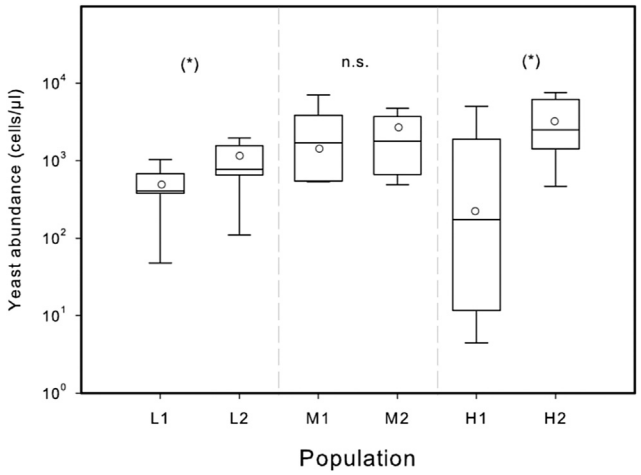

Fig 1 – Yeast abundance (number of cells μl^{-1}) in the six *H. foetidus* populations studied, arranged by elevation. Horizontal lines represent mean values, and vertical segments denote ±1 SE. Means from populations located at the same elevation level were also compared and statistical significance ($p < 0.05$) was indicated with *.

population peak bloom and afterwards decreased (results not shown).

The relative importance of the within-plant component of variance

Spatial differences mainly occurred at the within-population level (Fig 3A, left panel) or the within-collection date level (Fig 3B, left panel). Within populations, the highest proportion of the total variance in mean yeast abundance was found between flowers of the same plant ($Z = 4.70$, $p < 0.0001$,

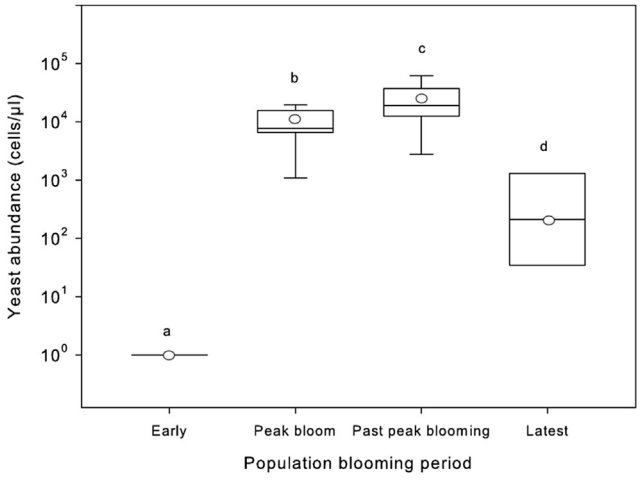

Fig 2 – Yeast abundance (number of cells μl^{-1}) in nectar samples according to population phenology stage as followed in one *H. foetidus* population. Horizontal lines represent mean values, and vertical segments denote ±1 SE. Means with different letter differ significantly ($p < 0.05$).

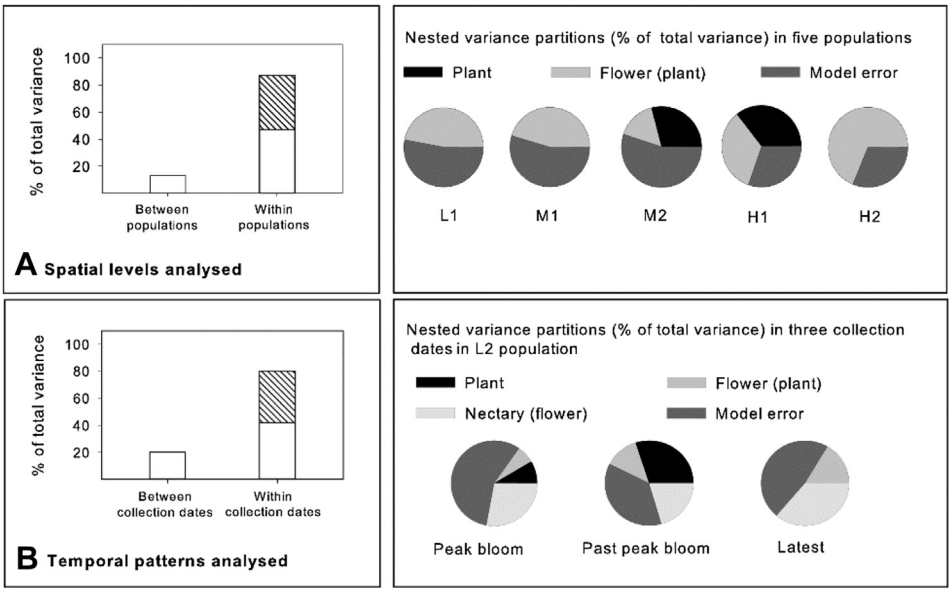

Fig 3 – Dissection of variance components of yeast abundance in nectar samples of *H. foetidus* in a spatially nested scheme. (A) Spatial patterns: left panel, all populations pooled, with the contribution of populations and within populations level to the total variance in mean yeast abundance in the five populations considered. Stacked bars in the within populations level comprises plant within population and flower within plant (white bar) plus model error (patterned bar) as the hierarchical levels of variance analysed. Right panel, dissection of the within population hierarchical variance by population. (B) Temporal patterns: left panel, contribution of collection date and within collection date levels to the total variance in mean yeast abundance for L2 population. Stacked bars in the within populations level comprises plant within population, flower within plant and nectary within flower (white bar), plus model error (patterned bar) as the hierarchical levels of variance analysed. Right panel, dissection of the within collection date hierarchical variance by collection date.

39.7 % of the total variance). However, variance estimates for the different spatial scales considered varied substantially between populations (Fig 3A, right panel). With the exception of population H1, differences among individual plants reached low or even null significance. A consistent trend at all populations was that differences between flowers of the same plant explained a high proportion of the total variance in yeast abundance (16–70 %), but the highest proportion of variance still remained unexplained by the model when flowers within plants were the smallest sampling scale (model error, 30–55 %).

Focusing on the single population (L2) for which replicate measurements of yeast abundance were also available for nectaries within flowers (Fig 3B), a significant part of the total variance in nectar yeast mean abundance was due to differences among nectaries of the same flower (21 % of the total variance, $Z = 4.26$, $p < 0.0001$), although model error was also significant and accounted for the highest component of the total variance (38 %, $Z = 25.98$, $p < 0.0001$). The relative importance of the different hierarchical levels of variance considered varied along the blooming period. At collection dates corresponding to the mid blooming stage, a high percentage of the nectar-wide variance in yeast abundance was due to variation among nectaries of the same flower, followed by variation among flowers of the same plant. However, differences among nectaries of the same flower were statistically significant at the three collection dates available ($Z = 2.8$, 2.7 and 1.7; respectively, $p < 0.005$) and accounted for >21 % of the population-wide variance in all cases. Differences among individual plants increased at collection dates nearest the peak bloom.

Biotic and abiotic factors affecting local distribution patterns

Results of the PLS analysis showed that the first three factors accounted for 75.1 % of the total variation in explanatory variables and for 81.5 % of the total variation in response variables. The Variable Importance in Projection (VIP) scores plot indicated that biotic variables had higher absolute coefficients and higher VIP values than the abiotic variables (Fig 4B). In particular, the pollinator composition variables appeared as the best predictors of yeast prevalence. Both the proportion of flower visits by *Bombus* sp. and *A. mellifera* had high VIP values (1.76 and 1.58, respectively). Honeybee visits had a strong negative effect on both yeast abundance and frequency in nectar samples (Fig 4A), whereas mean bumblebee abundance per census had uncertain effects on yeast prevalence. On the one hand, a high proportion of flower visits by bumblebees caused a positive effect on nectar yeast frequency, but on the other hand it caused a negative effect on yeast abundance. Floral density in the population had a very small value of VIP. Regarding abiotic predictors, only mean air temperature over the 15d preceding nectar sampling had a relevant effect on nectar yeast prevalence (VIP value of 0.97), and this effect was mainly caused by a positive effect on nectar yeast frequency.

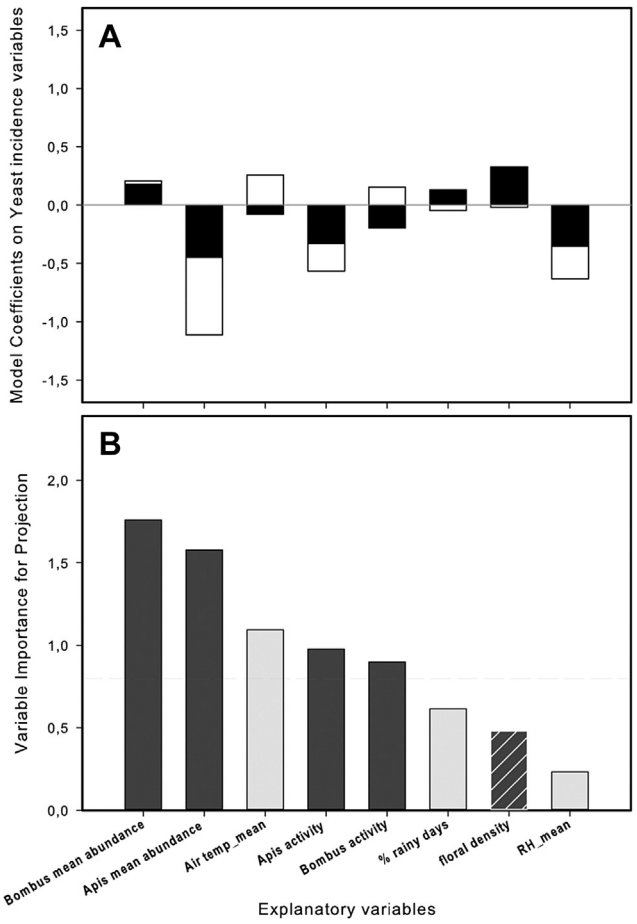

Fig 4 – Variable importance plot. (A) Model coefficients for explanatory variables in our three-latent factors PLS model (stacked bars, white for variable contribution on mean yeast frequency and black for contribution on mean yeast abundance). (B) Variable importance in projection (VIP) coefficients obtained from the PLS regression procedure. Explanatory variables ordered along the X-axis according to their explanatory power of Y (mean yeast frequency and rank transformed mean yeast abundance in nectar samples). Different colours used to indicate the type of variable: light gray for abiotic variables; dark gray for pollinator composition and activity; and striped bar for floral density.

Discussion

We found considerable spatio-temporal variation in the prevalence of nectar-inhabiting yeasts in floral nectar of the winter blooming plant species *H. foetidus*. Spatial variation was mainly accounted for by differences at the intra-plant level and thus did not produce clear patterns in relation to the broad environmental gradient associated with elevation. It is possible that some of the negative results concerning spatial patterns may be the consequence of insufficient statistical power due to the relatively small number of spatial replicates. However, the current evidence points out that, if such effects do actually exist, they probably are neither strong, nor pervasive. Seasonal variation within a site was an important source of variation in yeast abundance, suggesting that temporal changes in the pollinator environment may be an important factor affecting the distribution of nectar yeasts.

The present study has documented the existence of broad intraspecific variability in the frequency of occurrence of yeasts inhabiting *H. foetidus* nectar. Even for a single study region and year, yeast frequency estimates ranged from 0 to 100 %, depending on population and collection date. Two previous studies have estimated the frequency of nectar yeast contamination in *H. foetidus* (Brysch-Herzberg, 2004; Herrera et al., 2008). In a German population, Brysch-Herzberg (2004) found that frequency of nectar samples with yeasts was 4 % at the beginning of the blooming period and before the appearance of bumblebees, and 11 % after first appearance of bumblebees. Further, in one SE Spanish location (Herrera et al., 2008), the frequency of yeast-containing nectar samples varied between 60 % (female phase) to 100 % for intermediate and male sexual stage in 20 *H. foetidus* flowers.

To our knowledge, the present study is the first attempt at elucidating the relative importance of between and within population components of variance in mean nectar yeast abundance, and results have revealed a distinct mosaic of yeast density occurring at the within-population level. Within populations, large differences in yeast abundance occurred at the sub-individual level, here represented by flowers within plants and nectaries within flowers.

The variation in nectar yeast abundance at the within plant level is likely to be produced by factors operating at this scale, and, according to our initial expectations, nectar yeast distribution patterns are intertwined with yeast dispersal by certain types of pollinators. Our results corroborate the findings of Herrera et al. (2009) and de Vega et al. (2009), showing that yeast abundance in nectar of several plant species was significantly related to pollinator composition. Moreover, although it has been shown that bumblebees carried yeast effectively from one flower to another, our results indicate that not all pollinators are equally effective at vectoring yeasts to floral nectar. In particular, nectar yeast frequency tended to decrease with increasing proportion of flower visits by honeybees, suggesting that they lick nectar without transferring yeast to the remaining volume. Interestingly, experimental inoculation of unvisited *H. foetidus* nectaries using the glossae of wild-caught honeybees did not induce "yeast-mediated" modification of nectar sugars (Canto et al., 2008). Although there is no direct evidence of bacteria presence in the glossae of honeybees, their presence in honeybee gut (Good et al., 2014) and in the bee hive and bee food resources (Gilliam, 1997) might indicate that some bacterial species might be transferred to nectar, and therefore they could prevent yeast growth, due to competitive and other negative interactions between bacteria and yeast in nectar (Tucker and Fukami, 2014). Furthermore, the effect of bumblebee visits on nectar yeast abundance was frequency-dependent. Nectaries are initially sterile, implying that the frequency of effective yeast vectors, such as bumblebees, is crucial to increase the frequency of yeast-containing nectar samples. However, if the period between insect visits is not long enough to permit cell proliferation inside the nectary, a negative effect of pollinator visit frequency would have been detected. In a winter

blooming species such as *H. foetidus*, pollinators may be scarce or absent during extended periods of inclement weather, and very long periods may elapse between consecutive pollinator visits to a given flower (Pozo, *pers. obs.*). Nevertheless, the implementation of cross-correlational analyses, and analyses of factors may provide initial suggestions about the explanatory mechanisms for the studied season, but the reliability of these results need additional years of sampling, and their causality should be finally established by experimental manipulation (e.g., see Canto et al., 2008).

Population blooming stage affected yeast frequency and abundance in nectar samples, as demonstrated by the yeast temporal monitoring in one *H. foetidus* population. Nectar yeast incidence and density varied significantly along the flowering period, and it seems that nectar yeasts tend to be more frequent and abundant at collection dates around the peak bloom. This pattern was closely related to changing meteorological conditions along the flowering season, which in turn affected pollinator activity. We may infer some consequences about the absolute lack of yeast for the first collection date, as *H. foetidus* is a winter-flowering herb, and one of the earliest blooming species in our study site. It is hypothesized that nectar-specialised yeasts, after overwintering in a way that remains largely unknown, are indispensably transferred to nectar by emergent bee queens probing flowers in the early spring (Brysch-Herzberg, 2004; Canto et al., 2008). In this context, our temporal monitoring of nectar yeasts, carried out simultaneously with pollinator censuses, supports these suggestions about the beginning of the yeast annual cycle in floral nectar of *H. foetidus*. Population phenology may help to explain altitudinal variation in yeast prevalence in nectar samples. Although a gradual change in nectar yeast prevalence according to the altitudinal gradient was not detected, higher levels of yeast frequency and abundance in nectar samples at high elevation sites were consistently observed. Plants growing at higher elevations exhibited shorter blooming periods, but that was compensated for by showing higher floral density at the population's peak bloom period. Increased floral density potentially affects pollinator activity; in fact, the highest elevation site, H2, was one with highest records of floral density and bumblebee abundance and activity. This increase in floral density may raise the probability of yeast dispersal and proliferation of cells inside the nectary of plants in higher elevation populations.

Implications

The magnitude of intraspecific variation in nectar yeast frequency found in the present study is similar, or even greater, than variation found in interspecific comparisons for the same study region (Herrera et al., 2009). As a result, intraspecific variation should be taken into consideration when designing sampling schemes for nectar yeast studies, since inaccurate assessment of nectar yeast presence in a given plant species may arise from insufficient sampling.

This finding entails both methodological and ecological implications. From a methodological point of view, further attempts to assess the causative role of biotic and abiotic factors in nectar yeast abundance should include monitoring biotic and abiotic variables at smaller scales within populations. As for the study of plant–pollinator relationship, we may expect wide variation in nectar conditions (sugar composition and concentration, or nectar temperature) to occur at the within-plant level, as mediated by a density-dependent action of yeast metabolism (Herrera et al., 2008; Herrera and Pozo, 2010; Canto and Herrera, 2012). Differences between contiguous nectaries in the same flower represent the smallest spatial scale perceived by foraging pollinators, so pollinator choice is likely to be affected by yeast patchy distribution across nectaries (Herrera et al., 2013). On the other hand, the wide variation that takes place at the subindividual level decreases the probability of selective forces in plants to successfully act against nectar yeasts (Herrera, 2009), in the case that those were effectively deterrent for pollinators (Herrera et al., 2013; Vannette et al., 2013).

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Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funeco.2014.06.007.

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