



Pollination consequences of simulated intrafloral microbial warming in an early-blooming herb[☆]



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ABSTRACT

In habitats where low ambient temperature limits entomophilous pollination of early-blooming plants, intrafloral warming facilitates pollinator visitation and enhances plant reproductive success. A novel mechanism for intrafloral warming was described recently for the early-blooming, bumblebee pollinated herb *Helleborus foetidus* (Ranunculaceae) where the warming agent was the metabolic heat dissipated by dense populations of the nectar-specialist yeast *Metschnikowia reukaufii*. This paper reports results of field experiments conducted over three consecutive years aimed at (1) ascertaining whether flowers of *H. foetidus* with warmed interiors differed in pollination success from flowers experiencing natural intrafloral microclimate; and (2) testing the hypothesis that the pollination effects of intrafloral warming should be stronger during the early stages of the *H. foetidus* flowering season, when inclement weather and low ambient temperatures often limit pollinator activity. Intrafloral warming resulted in increased number of pollen tubes per style and reduced probability of pollen limitation early in the flowering season, while these effects were reversed in late-season flowers. Since the number of pollen tubes was directly correlated with number of pollen grains on the stigma, fruit set and number of seeds produced by individual flowers, results of this study are interpreted as indicating that yeast-induced intrafloral warming has the potential to influence reproductive success in *H. foetidus* via effects on pollinator activity, although the nature of effects will vary depending on environmental conditions. Results also stress the importance of ambient and floral temperature for the pollination of early-blooming plants.

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1. Introduction

Ever since early ground-breaking work by Sprengel (1793) and others (Darwin, 1862; Müller, 1883), the significance of both biotic (e.g., flower features, animal visitors) and abiotic factors (e.g., light, rain, wind) for the sexual reproduction of plants has been unanimously acknowledged. Unbalanced attention, however, has been traditionally paid to these two major groups of agents, the extensive effort devoted to biotic factors standing in sharp contrast with the relatively modest effort dedicated to abiotic ones (Willmer, 2011). Despite this imbalance, however, it is well established that physical aspects of the environment can decisively influence plant sexual reproduction by conditioning the activity of pollinators (Stone et al., 1988; Herrera, 1995b; Totland and Matthews, 1998; Kilkenny and Galloway, 2008), influencing their behavior at flowers (Stanton and

Galen, 1989; Herrera, 1995a; Luzar and Gottsberger, 2001; Sapir et al., 2006), altering features of floral rewards (Corbet et al., 1979; Corbet and Willmer, 1981; Corbet and Delfosse, 1984), and affecting directly pollen germination and pollen tube growth (Jakobsen and Martens, 1994; Lankinen, 2001; Orueta, 2002; Seymour et al., 2009b; Distefano et al., 2012).

In markedly seasonal habitats of middle and high latitudes, flowering in early spring is a high-risk option in terms of insect-mediated sexual reproduction, and the importance of abiotic agents as drivers of plant-pollinator systems should be particularly high for the earliest-blooming plants, which often face adverse weather periods during which pollinator activity is precluded or severely restricted (Schemske et al., 1978; Motten, 1986; Herrera, 1995a; Totland and Matthews, 1998; Vesprini and Pacini, 2010). In arctic and mountain habitats, for example, where low ambient temperatures frequently impose stringent limits on the pollination of early-blooming entomophilous plants, floral warming mechanisms have been discovered that generate intrafloral thermal excesses relative to the ambient, which facilitate pollinator visitation and enhance plant reproductive success (Kevan, 1975; Stanton and Galen, 1989; Corbett et al., 1992; Herrera, 1995a; Kudo, 1995; Krannitz, 1996; McKee and Richards, 1998; Song et al., 2013). In

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Table 1

Dates and sample sizes for the floral warming experiments considered in this study. Each year, sample sizes were approximately equal for Control (flowers with unpowered resistor) and Warmed (flowers with powered resistor) factor levels.

Year	Experimental period							
	Early				Late			
	Dates	Sample sizes			Dates	Sample sizes		
		Plants	Flowers	Follicles		Plants	Flowers	Follicles
2010	5 Mar–9 Mar	12	47	95	19 Mar–3 Apr	17	127	280
2011	4 Mar–12 Mar	5	20	36	16 Mar–24 Mar	5	20	44
2012	28 Feb–3 Mar	13	28	55	18 Mar–23 Mar	13	31	68
Total		30	95	186		35	178	392

the vast majority of well-studied instances, floral warming either is the outcome of active, respiration-based thermogenesis by the flowers themselves (Knutson, 1974; Lamprecht et al., 2002; Seymour and Matthews, 2006; Seymour et al., 2009a) or arises from passive absorption of incident solar irradiance, often enhanced by floral heliotropism (sun-tracking), flower color and perianth architecture (Stanton and Galen, 1989; Herrera, 1995a; Kudo, 1995; Krannitz, 1996; Totland, 1996; McKee and Richards, 1998; Orueta, 2002; Sapir et al., 2006). One third, fundamentally different mechanism producing intrafloral warming was recently documented for the first time by Herrera and Pozo (2010) for the early-blooming herb *Helleborus foetidus*. In this understory plant, the metabolic heat dissipated by dense populations of the nectar-specialist yeast *Metschnikowia reukaufii* can increase the temperature of nectaries relative to the air outside flowers by as much as 3–5 °C (Herrera and Pozo, 2010). That earlier study, however, did not address the possible pollination consequences to *H. foetidus* plants of yeast-induced floral warming.

In this paper we report the results of manipulative field experiments conducted over three consecutive years primarily aimed at ascertaining whether, under natural field conditions, flowers of *H. foetidus* with warmed interiors mimicking the thermal effects of nectar-dwelling yeasts differed in pollination success from those experiencing natural intrafloral microclimates. A secondary objective of our study was to test the ancillary prediction that the putative pollination effects of intrafloral warming should be stronger during the early stages of the *H. foetidus* flowering season, when spells of severe weather limiting pollinator activity are most frequent. To this end, experiments were replicated each year during the early and late periods of the flowering season.

2. Materials and methods

2.1. Study system

Helleborus foetidus L. (Ranunculaceae) is a perennial herb widely distributed in western and southwestern Europe, where it can be found from sea level to 2100 m elevation in diverse habitat types ranging from open scrub to conifer and broad-leaved forests (Mathew, 1989). Plants produce one or a few inflorescences in early winter, and in our southern Spanish study area (see below) flowering mostly takes place during February–April. Individual flowers are self-compatible, protogynous, last for up to four weeks, and are mostly pollinated by bumblebees (*Bombus terrestris* and *B. pratorum*). Insufficient pollination limits seed production in some years and locations. During the early flowering period, spells of inclement weather including freezing temperatures and snowfalls are frequent (Fig. 1), which preclude pollinator activity and result in pollen limitation. As weather improves from early to late stages over the long flowering period, pollen limitation likewise tends to decline as a consequence of increased pollinator visitation. Flowers are apocarpous, generally bearing 1–3 carpels. For convenience,

the term ‘follicle’ will be used here to designate either flower carpels (= gynoecium) or true follicles (= developing fruits). The large globular perianth (length = 15–17 mm, width = 13–15 mm) consists exclusively of five large, overlapping green sepals, since the petals have become modified into nectaries. Each flower generally contains five nectaries shaped like flattened horns and hidden deeply inside the perianth. These form a distinct ring between the stamens and the sepals and, if unvisited, each nectary may contain up to 5 µL of sucrose-dominated nectar. The flower-specialist yeast *Metschnikowia reukaufii* (Metschnikowiaceae, Ascomycota) is a regular inhabitant of the floral nectar of *H. foetidus*, its populations often reaching densities >10⁵ cells/mm³. The internal structure of *H. foetidus* flowers is depicted in Plate 1a of Herrera et al. (2008) and Fig. 1a of Herrera and Pozo (2010). Extensive information on the floral biology, pollination ecology and floral microbiology of *H. foetidus* is provided, among other, by Vesprini et al. (1999), Vesprini and Pacini (2000, 2010), Herrera (2002), Herrera et al. (2001, 2008, 2013), Brysch-Herzberg (2004), Herrera and Pozo (2010), Canto et al. (2011), and Pozo et al. (2014).

2.2. Field and laboratory methods

This study was carried out at a large, essentially continuous population of *H. foetidus* in the understory of mature *Pinus nigra* forests of mountain habitats in the Sierra de Cazorla, Jaén province, southeastern Spain (‘Las Navillas’ site of Herrera and Pozo, 2010; Herrera et al., 2013). Experiments were conducted there between late February–early April of 2010, 2011 and 2012 (see Table 1 for experimental dates), which encompassed the main part of the local flowering season of *H. foetidus* and was largely coincident with the period of previous experiments on floral warming by yeasts (Herrera and Pozo, 2010).

Each year, between 5 and 17 widely-spaced *H. foetidus* plants bearing inflorescences were randomly chosen in the study population (Table 1). Different plants were chosen on different years, since not all individuals flowered every year. Two experimental series (‘Early’ and ‘Late’ hereafter; to avoid ambiguity, factor level designations will always be capitalized) were run every year on each plant. Dates chosen roughly corresponded to the initial and final third of the local flowering period. In each series, newly-opened, female-stage flowers without dehiscent anthers were chosen in each plant for experimentation (see Table 1 for sample sizes). The purpose of conducting experiments only on female-stage flowers was (i) to avoid unintentional self-pollination during flower manipulation; and (ii) to minimize any possible interference of naturally occurring yeasts on experimental results, since yeasts are absent from nectar at early floral stages or, if present, occur at negligible densities (Herrera et al., 2008). One axial carbon film resistor (150 Ω, 3.5 mm long, 1.85 mm wide; reference RS 135–796, RS Amidata, Madrid) was carefully inserted into each flower. Resistor wires (each 28 mm long, 0.45 mm diameter) were bent 90° until they were parallel to each other, and the base of sepals was then gently



Fig. 1. Spells of inclement weather with snowfalls and freezing temperatures are frequent at our study site during the early stages of the blooming period of *Helleborus foetidus*. The plant at the right was one of our 2010 experimental individuals (photograph taken on 9 March 2010).

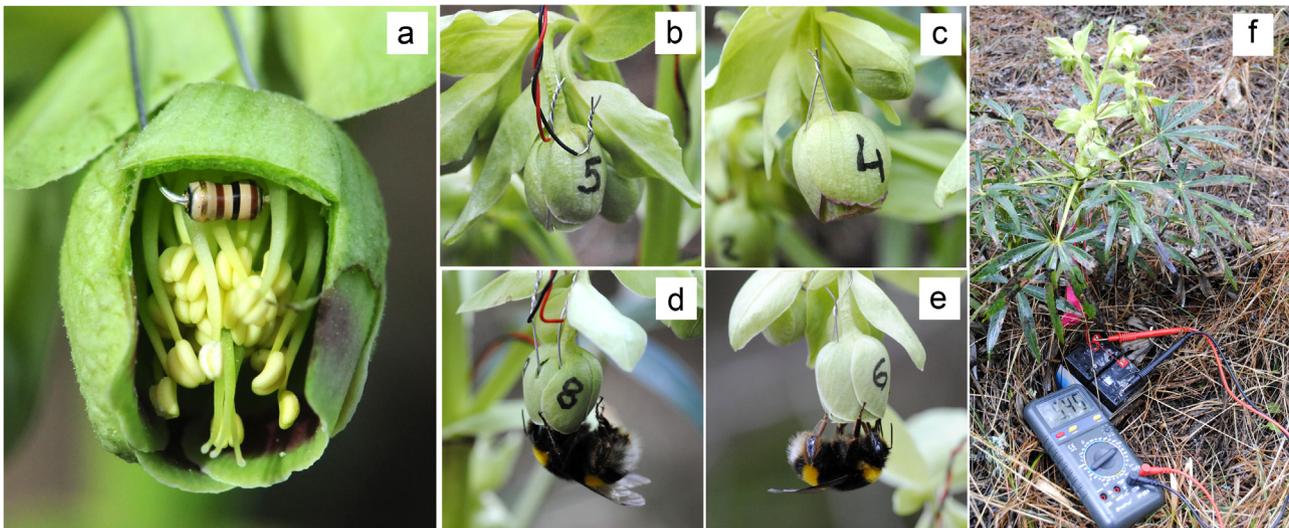


Fig. 2. Experimental setup used in this study. Location of resistor within an experimental flower (a); sepals partly removed to show flower interior); external view of Warmed (b) and Control (c) flowers; bumblebees (*Bombus terrestris*) visiting Warmed (d) and Control (e) flowers; and experimental plant being tested for circuitry continuity and battery voltage (f).

pierced from inside with the tips of resistor wires. Pulling from the externally protruding wire tips using a fine pliers, resistor wires were taken out of the flower as far as possible until the resistor rested firmly stuck at the flower interior's bottom with its main axis perpendicular to the longitudinal floral axis (Fig. 2a). Special attention was paid to avoid damaging flower parts or contacting stigmas during manipulation. As a protection against short-circuiting by snow, rain drops or floral tissue, resistor wires were coated prior to installation with fast-drying, isolating acrylic lac (reference RS 569-290, RS Amidata, Madrid). Within each plant, a similar number of experimental flowers were randomly assigned to either Warmed or Control (unwarmed) treatment levels. Resistors in the first group were powered by wiring to sealed lead-acid batteries (6 V, 4 Ah; reference 537-5422, RS Amidata, Madrid), while resistors in Control flowers remained unpowered (Fig. 2b,c). Batteries were laid on the ground close to the plant, and each experimental run on a given set of Warmed plus Control flowers lasted for 5–6 days, throughout which most flowers remained in female stage. Circuitry continuity and battery voltage were checked daily over experimental runs, and batteries replaced whenever voltage fell under ~4 V (Fig. 2f). Casual

observations revealed that foraging bumblebees visited Warmed and Control flowers normally during experiments (Fig. 2d,e).

To verify the efficacy of the manipulation of intrafloral temperature in Warmed flowers and quantify temperature increase, we measured air temperature outside (T_{outside} ; ~25 mm away from the flower opening) and within (T_{within}) Warmed (with powered resistors) and Control (with unpowered resistors) flowers at haphazardly-chosen times during daytime on different dates of the 2010 experimental period. Ultra fast (time constant = 0.005 s), fine (tip diameter = 0.22 mm) Type T thermocouples (model IT-23, Physitemp Instruments, Clifton, New Jersey) connected to a digital thermometer were used for air temperature measurements. To avoid possible effects of direct solar radiation, air temperatures were always recorded after experimental plants had been in the shade for at least one hour.

The number of pollen tubes in the style of experimental flowers was used in this study as a proxy for maternal pollination success (Alonso et al., 2012; Arceo-Gómez et al., 2016) and seed fecundity (Herrera, 2002). These assumptions were validated during the experiments of 2012 (see below). Every year, all styles were

Table 2

Summary of selected weather variables during Early and Late experimental periods, the three study years combined (see Table 1 for dates), for the four nearest weather stations around the study site (see text). Means shown are model-adjusted cell means obtained from linear mixed models having Period (Early vs. Late) as main factor and year as random effect.

	Experimental period (mean ± SE)		Difference between periods	
	Early	Late	Chi-squared	P-value
Daily maximum temperature (°C)	12.1 ± 0.6	15.7 ± 0.5	31.73	<0.0001
Daily mean temperature (°C)	6.9 ± 0.3	9.5 ± 0.3	37.48	<0.0001
Daily total irradiance (mj/m ²)	11.3 ± 1.0	17.3 ± 0.9	45.98	<0.0001

collected from withered experimental flowers 20–25 days after completion of experimental runs, and stored in microcentrifuge tubes filled with 2.5–2.5–95% formaldehyde-acetic acid-ethyl alcohol solution. The number of fully developed pollen tubes in collected styles that had penetrated the stigma and were in the stylar canal were counted using epifluorescence microscopy, following methods detailed in Herrera (2002). Style preparation involved treating at 65 °C for 20 min in 5 mol/L NaOH for softening, rinsing in distilled water, and staining for 20 min at 65 °C in decolorized aniline blue.

The presumed relationships between pollen tube numbers, on one side, and pollination success and maternal fecundity, on the other, were verified in 2012 for experimental flowers. The number of pollen grains on the stigma of each collected style was counted using a differential staining technique. Individual aniline blue-stained styles were placed on a microscope slide with a drop of 0.05% aqueous toluidine blue for less than 1 min and immediately rinsed with one or two drops of tap water. Liquid excess around each style was carefully removed with absorbent paper, and styles finally mounted under a coverslip with a drop of decolorized aniline blue solution containing 10% glycerin. Pollen grains were then counted using ordinary bright-field microscopy. In this way, paired pollen grain and pollen tube counts were obtained for all individual styles of experimental flowers in 2012. The relationship between number of pollen tubes and realized maternal fecundity was also evaluated that year by collecting all follicles from experimental flowers in mid June, shortly before they would have dehisced naturally. Follicles were dissected individually and the number of seeds counted.

2.3. Data analysis

All statistical analyses were carried out using the R environment (R Development Core Team, 2015). Two complementary analyses were applied to evaluate the effects of warming (Warmed vs. Control flowers), experimental period (Early vs. Late), and their interaction on pollination success. Mixed models were fitted to data, where individual styles of experimental flowers were treated as the sampling units, and study year, plant, and flower were included as hierarchically nested random effects. In one analysis, the response variable was the number of pollen tubes in the style, and a linear mixed model was fitted using function lmer in the lme4 package (Bates et al., 2015). In the second analysis, the response was a binary variable indicating whether the number of pollen tubes in the style was <11 (=mean number of ovules per follicle; Herrera, 2002), which provided a conservative estimate of pollen limitation at the individual follicle level. A generalized linear mixed model was fitted to data using function glmer in lme4 package. Distribution of residuals revealed that the two models fitted the data satisfactorily. Analysis of deviance (Type III Wald chi-square tests) were used to test for the statistical significance of main effects and their interaction. Least-squares, model-adjusted means and associated standard errors for the different factor level combinations were obtained with the lsmeans function in the lsmeans package (Lenth, 2016).

The seasonal dynamics of environmental conditions from Early to Late experimental periods was assessed using meteorological data (daily mean and maximum temperature, daily total radiation) from the four nearest weather stations around the study site (Pozo Alcón, Huesa, Villacarrillo and Puebla de Don Fadrique; mean distance = 29 km) from Red de Información Agroclimática de Andalucía (<https://www.juntadeandalucia.es/agriculturaypesca/jifapa/ria>; accessed 7 October 2016). Separate linear mixed models for each weather variable were fitted to the combined data set for all experimental dates. Experimental period (Early vs. Late), weather station, and their interaction were treated as fixed factors, and year was included as a random effect. Differences between experimental periods were tested using analyses of deviance.

3. Results

Weather conditions improved significantly as the *H. foetidus* flowering season progressed. In 2010, mean air temperature (\pm SE) outside flowers at the study site risen from 3.4 ± 0.2 °C (range = 1.3–7.0 °C) to 8.3 ± 0.3 °C (range = 2.2–15.3 °C) from Early to Late periods (chi-squared = 78.5, $P < 0.0001$, Kruskal-Wallis rank sum test). Meteorological data for 2010–2012 from neighboring weather stations likewise revealed a consistent weather improvement from Early to Late periods at regional level. Separate mixed-model analyses showed significant effects of experimental period (Early vs. Late) on every weather variable considered (Table 2). Daily maximum temperature, mean daily temperature, and daily solar irradiance all increased from Early to Late periods. The weather station \times period interaction was not statistically significant for any weather variable ($P \geq 0.91$), thus denoting a consistent weather improvement through the *H. foetidus* flowering season across locations.

Air temperature measurements taken in 2010 within (T_{within}) and outside (T_{outside}) Warmed (with powered resistors) and Control flowers (with unpowered resistors) demonstrated that heat dissipated from powered resistors produced a measurable increase of intrafloral air temperature relative to both the external air immediately surrounding flowers and the interior of Control flowers. Air temperature excess of flower interior relative to the surrounding air outside flower ($T_{\text{excess}} = T_{\text{within}} - T_{\text{outside}}$) was significantly higher for Warmed than for Control flowers ($F = 593.3$, $df = 1, 229$, $P < 0.0001$). T_{within} was significantly higher than T_{outside} in Warmed flowers ($t = 25.89$, $df = 115$, $P < 0.0001$; paired t -test), while it did not differ significantly from T_{outside} in Control ones ($t = 0.66$, $df = 114$, $P = 0.51$; paired t -test). Mean T_{excess} (\pm SE) of Warmed flowers was 5.4 ± 0.2 °C, and no significant difference in T_{excess} was found between Early ($T_{\text{excess}} = 5.7 \pm 0.3$ °C) and Late ($T_{\text{excess}} = 5.2 \pm 0.3$ °C) flowers ($F = 1.26$, $df = 1, 114$, $P = 0.26$).

The number of pollen tubes in individual styles was a valid proxy for both female pollination success and maternal fecundity of *H. foetidus* in the 2012 sample of experimental flowers. At the level of individual styles, there existed a close linear correlation between number of pollen grains and number of pollen tubes ($r = 0.798$, $df = 115$, $t = 14.21$, $P < 0.0001$). Linear regressions of pollen tube number against pollen grain number fitted separately

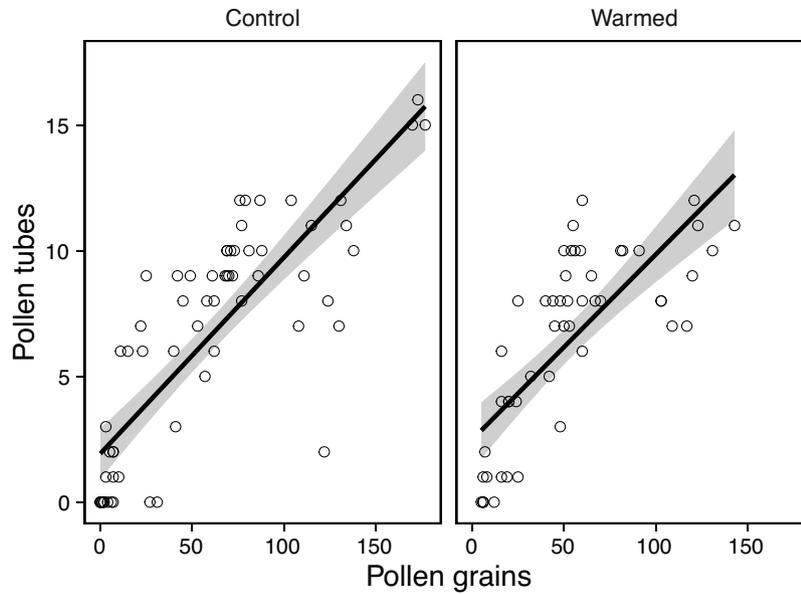


Fig. 3. Least-squares fitted linear regressions of pollen tubes against pollen grains in the styles of individual follicles of Warmed and Control (unwarmed) experimental flowers in 2012 (shaded areas depict 95% confidence intervals). Each symbol corresponds to a different follicle ($N = 69$ and 54 for Control and Warmed, respectively). See text for results of slope and intercept homogeneity tests.

Table 3

Analysis of deviance tables (Type III Wald chi-square tests; $df = 1$ in all cases) for mixed models testing for the effects of floral warming (Warmed vs. Control), experimental period (Early vs. Late), and their interaction on number of pollen tubes and probability of pollen limitation (number of pollen tubes < mean number of ovules per follicle) in the style of experimental flowers of *Helleborus foetidus*. Year, plant within year, and flower within plant and year, were included in models as hierarchically nested random effects.

Source	Response variable			
	Number of pollen tubes		Probability of pollen limitation	
	Chi-square	P-value	Chi-square	P-value
Warming (W)	1.97	0.16	2.83	0.092
Period (P)	12.52	0.0004	8.47	0.004
W x P	5.71	0.016	5.88	0.015

for Warmed and Control flowers (Fig. 3) were statistically indistinguishable with regard to both slope ($\chi^2 = 0.0034$, $df = 1$, $P = 0.95$) and intercept ($\chi^2 = 0.027$, $df = 1$, $P = 0.87$). Close linear relationships were likewise found at the individual flower level between total pollen tube numbers per flower, on one side, and total pollen grains per flower ($r = 0.844$, $df = 53$, $t = 11.48$, $P < 0.0001$), proportion of initial follicles in a flower eventually setting seeds ($r = 0.703$, $df = 54$, $t = 7.27$, $P < 0.0001$), and total number of seeds produced per flower ($r = 0.745$, $df = 51$, $t = 7.98$, $P < 0.0001$).

After statistically accounting for variance in pollen tube numbers due to differences between years, plants and flowers (i.e., by including these variables as random effects in the mixed models), the main effects of experimental warming on number of pollen tubes in individual styles and follicle-level probability of pollen limitation were both statistically nonsignificant (Table 3). Most importantly, however, the corresponding Warming x Period interaction effects were significant (Table 3), which precludes a simple interpretation of main effects and reflects the contrasting effects of intrafloral warming for Early and Late flowers. Seasonal dependence of the nature of warming effects is illustrated by the interaction plots in Fig. 4. Warming increased pollen tube numbers and reduced the probability of pollen limitation among Early flowers, while the reverse was true for Late flowers. This seasonal shift in the pollination consequences of intrafloral warming, from

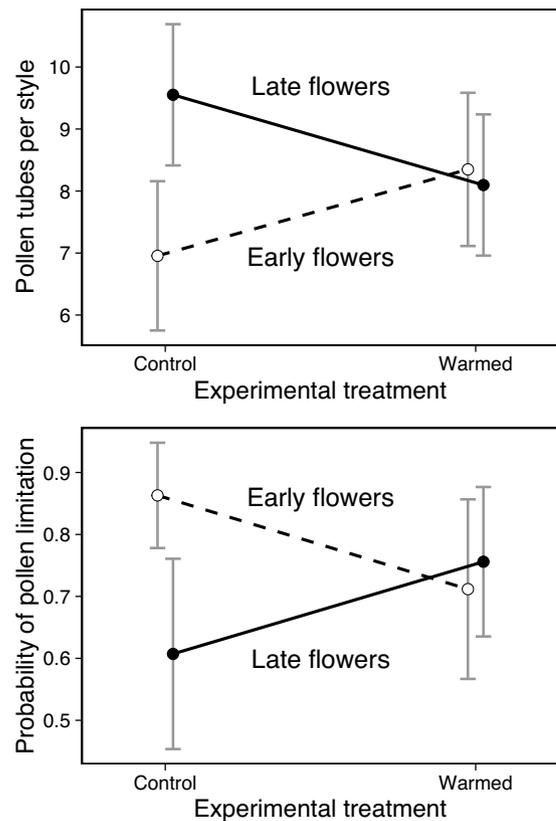


Fig. 4. Interaction graphs showing the joint effects of experimental period (Early vs. Late in the flowering season) and intrafloral warming (Warmed vs. Control) on number of pollen tubes and probability of pollen limitation (number of tubes < mean number of ovules per follicle) in individual styles of experimental flowers of *Helleborus foetidus*. Circles denote model-adjusted, least-squares cell means for Early (open dots, dashed line) and Late (filled dots, continuous line) flowers, and vertical segments extend over ± 1 SE. Significance tests of main effects and interaction shown in Table 3.

enhancement to reduction, ran parallel to a seasonal improvement in the pollination success of Control flowers, as shown by their

increased pollen tube numbers and reduced probability of pollen limitation from Early to Late dates (Fig. 4).

4. Discussion

By means of manipulative and observational field experiments, Herrera and Pozo (2010) tested and verified the hypothesis that heat produced by the extremely fast sugar catabolism of *Metschnikowia reukaufii* populations inhabiting floral nectar of *H. foetidus* increased the temperature excess of nectaries (ΔT_{nect}) in relation to the air just outside the flowers. In these experiments, some of which were conducted in the same *H. foetidus* population used in the present study, exclusion of yeasts from the nectaries reduced, and addition of yeasts to virgin flowers increased ΔT_{nect} . In unmanipulated flowers exposed in the field to natural pollinator visitation, ΔT_{nect} was found to be linearly related to log yeast cell density in nectar, often reaching +3.5–6°C in nectaries with the densest *M. reukaufii* populations ($>10^4$ cells/mm³; Fig. 2b in Herrera and Pozo, 2010). This yeast-induced temperature excess is roughly similar to the mean T_{excess} obtained in the present study for artificially Warmed flowers (+5.4°C). This indicates that heat dissipation by powered resistors in our experiments provided a realistic imitation of metabolic heat dissipation by *M. reukaufii* in flowers with yeast populations $>10^4$ cells/mm³ of nectar, which in our study area may represent ~40–90% of all flowers depending on year, site and period of the blooming season (Herrera et al., 2008; Herrera and Pozo, 2010; Pozo et al., 2014). Insofar as the intrafloral thermal environment is concerned, therefore, this provides justification for discussing results of the present study in the context of the ecological interaction between *H. foetidus* flowers and its nectarivorous yeast inhabitants. It must be noted, however, that our finding that intrafloral warming was consequential for pollination success and fecundity in *H. foetidus* is also relevant in the broader context of the role played by abiotic factors in the floral biology and pollination of early-blooming plants.

Experiments revealed an effect of intrafloral warming on the pollination success of *H. foetidus* flowers. Statistical significance of the Warming x Period interaction on pollen tube numbers and probability of pollen limitation, however, indicated that the effect of Warming was not simple. Early in the flowering season Warming enhanced the number of pollen tubes per style and reduced the probability of pollen limitation, while these effects were reversed in late-season flowers. These results support the hypothesis that, everything else being equal, yeast-induced floral warming should have a definite effect on the pollination success of *H. foetidus* flowers (Herrera and Pozo, 2010), but also reveal a context-dependence of the phenomenon. This latter finding is consistent with the expectation formulated in the Introduction that, among early-blooming plants, positive pollination effects of intrafloral warming should be most apparent during the early phase of the flowering season, when pollen limitation should be most frequent due to longer and more frequent spells of inclement weather precluding pollinator activity (Schemske et al., 1978; Vesprini and Pacini, 2010). Our results support these premises for *H. foetidus*. The higher ambient temperature and nearly doubled daily solar irradiance during the Late experimental period represented a substantial, predictable weather improvement along the *H. foetidus* flowering season. Furthermore, Control flowers exhibited increased pollen tube numbers and reduced pollen limitation probabilities from Early to Late periods. In addition to the biological aspects discussed below, one obvious practical implication arising from our results is that selection of experimental dates is bound to acquire particular importance in pollination studies of early-flowering plants (see also Schemske et al., 1978). In these cases, yielding to the natural inclination to make field work coincide with periods of predictably finer

weather may increase the risk of obtaining partial, and possibly distorted, views of the system under study.

The close correlation found here between the number of pollen grains on the stigma and pollen tubes in the style of *H. foetidus* flowers is similar to that reported frequently for other animal-pollinated plants (Alonso et al., 2012; Arceo-Gómez et al., 2016; Wagner et al., 2016), and further highlights the practical value of pollen tube counts as a convenient proxy for assessing maternal pollination and reproductive success, particularly in self-compatible species or in situations where pollen grain counts are impractical or unreliable (Herrera, 2002, 2004). The finding that the tubes-grains relationships for Warmed and Control flowers were statistically undistinguishable rules out the possibility that differences between Warmed and Control flowers in pollen tube numbers were due to pollen grain germination being enhanced by higher intrafloral temperature. Consequently, the most parsimonious explanation for our finding that early in the flowering season intrafloral warming increased pollen tube numbers and reduced pollen limitation is that during that period Warmed flowers received more pollen grains than Control ones. This pollination advantage eventually resulted also in a fecundity advantage in terms of fruit set and seed production, as shown by the close linear correlations found between these two variables and number of pollen tubes.

The greater pollination success of Warmed flowers early in the season most likely reflected a positive foraging response to raised intrafloral temperature by bumblebees, the main pollinators of *H. foetidus*. Such positive foraging responses towards Warmed flowers could reflect increased visitation rate, longer visit durations, or some combination of these. Bumblebees are well-known for their endothermic abilities, which allow them to forage in overcast weather and under relatively low ambient temperatures (Heinrich, 1979; Goulson, 2003). In our study area, queens and workers of *B. terrestris* and *B. pratorum* visited *H. foetidus* flowers throughout the long flowering season of the species, including periods of harsh weather insofar as ambient temperature did not fall below ~6°C (see also Vesprini and Pacini, 2010). Despite its endothermic ability, however, controlled experiments have shown that *B. terrestris* prefers to land on warmer flowers when given a choice between artificial flowers that differ slightly in temperature but are otherwise similar in nectar rewards (Dyer et al., 2006; Whitney et al., 2008). A similar preference for warmer nectar and/or warmer flowers has been also documented for other endothermic (Nicolson et al., 2013; Tan et al., 2013) and ectothermic insect pollinators (Herrera, 1995a; Krannitz, 1996). Increased duration of pollinator visits to individual flowers, which generally results in increased pollen deposition per visit (Thomson and Plowright, 1980; Thøstesen and Olesen, 1996), has been also found to increase with increasing intrafloral temperature (Luzar and Gottsberger, 2001). A combination of bumblebee preference and longer visit durations can therefore parsimoniously account for the improved pollination success of Warmed *H. foetidus* flowers early in the season.

By the same token, the seasonal shift from positive to negative effects of floral warming on pollination success from Early to Late *H. foetidus* flowers found here could be accounted for by a seasonal reversal in the response of bumblebees to warmer flowers. Under controlled laboratory conditions, foraging bees may exhibit complex context-dependent behavioral shifts in response to variations in flower and ambient temperature, nectar sugar concentration and nectar viscosity (Whitney et al., 2008; Norgate et al., 2010; Nicolson et al., 2013; Tan et al., 2013). In particular, bee foraging responses to warmer food rewards may shift from positive to negative with increasing ambient temperature (Norgate et al., 2010), such as that occurring from Early to Late experimental periods in our study. With due reservations, we tentatively argue that a similar context-dependent shift in the response of bumblebees to warmer

flowers may have contributed to the contrasting effects of floral warming for Early and Late *H. foetidus* flowers. Regardless of the actual mechanisms involved, seasonal dependence of floral warming effects provides another instance of a simple biological feature having context-dependent effects on plant reproduction (Kalisz et al., 2004; Yu and Huang, 2006; Herrera, 2011). Furthermore, context-dependence of pollinator responses to intrafloral warmth (i.e., floral warming being advantageous only under a relatively narrow range of weather conditions) could help to explain the failure of some experiments to detect floral warming effects on pollination success (Totland, 1996).

Results of the present study are consistent with theoretical expectations that, under certain environmental conditions, floral heat may act as a reward for pollinators (Rands and Whitney, 2008). In contrast to previous investigations, however, the natural agent of intrafloral warming in *H. foetidus* is neither the plant itself (via metabolic thermogenesis) nor an external abiotic factor (solar irradiance), but it is instead a microbial agent occurring at high densities as noted in the Introduction. Some yeasts produce significant amounts of metabolic heat during the exponential growth phase, particularly when exposed to high carbon:nitrogen ratios (Gustafsson, 1991; Cooney et al., 1996; Lamprecht, 2003) such as those typically found in sugar-rich floral nectars (Lievens et al., 2015). Beyond certain densities, yeasts can thus become a significant nectar warming agent for early-blooming plants that, as *H. foetidus*, produce copious sugar-rich nectar and live in shady forest understories, where direct sunshine can hardly be used for warming (Herrera and Pozo, 2010).

By using electric power to heat flowers, and conducting experiments on young female-phase flowers free of yeasts, we have mimicked microbial floral warming without the confounding effects of other factors that are associated with yeasts' presence. In natural conditions, however, flowers of *H. foetidus* warmed by dense *M. reukaufii* populations will also have other floral features modified by yeast metabolic activity, such as sugar composition and concentration of nectar (Canto et al., 2008, 2011; Herrera et al., 2008; see also Vannette et al., 2013; Schaeffer et al., 2015). Floral thermal excess relates directly to yeast cell density, which in turn correlates directly with flower age and inversely with percent sugar in nectar (Herrera et al., 2008; Herrera and Pozo, 2010). The presumed early-season preferences of bumblebees for warmer flowers might therefore vanish beyond some yeast abundance and/or nectar sugar degradation threshold (Whitney et al., 2008; Herrera and Pozo, 2010; Tan et al., 2013). To date, experimentation on the *H. foetidus*-*M. reukaufii*-pollinators system has considered only one factor at a time (temperature, nectar composition; Canto et al., 2008; Herrera et al., 2008; Herrera and Pozo, 2010; present study). In addition, nectar yeast effects on floral functionality are also expected during the male stage of *H. foetidus* flowers, which could influence pollen transfer dynamics (Schaeffer et al., 2014), an aspect which was not addressed in the present study. Consequently, we suggest that the next steps to improve our understanding of the mechanisms underlying pollinator responses to yeast presence in *H. foetidus* flowers (Herrera et al., 2013; see also Vannette et al., 2013; Schaeffer et al., 2014, 2015) should involve multifactorial experiments capable of dissecting possible synergies and antagonisms between the various physical and chemical sequels of the presence of yeasts in flowers, along with their comparative effects on male and female components of reproductive success.

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References

- Alonso, C., Herrera, C.M., Ashman, T.-L., 2012. A piece of the puzzle: a method for comparing pollination quality and quantity across multiple species and reproductive events. *New Phytol.* 193, 532–542.
- Arceo-Gómez, G., Alonso, C., Abdala-Roberts, L., Parra-Tabla, V., 2016. Patterns and sources of variation in pollen deposition and pollen tube formation in flowers of the endemic monoecious shrub *Cnidioscolus souzae* (Euphorbiaceae). *Plant Biol.* <http://dx.doi.org/10.1111/plb.12445>.
- Bates, D., Maechler, M., Bolker, B., Walker, S., 2015. Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* 67, <http://dx.doi.org/10.18637/jss.v067.i01>.
- Brysch-Herzberg, M., 2004. Ecology of yeasts in plant-bumblebee mutualism in Central Europe. *FEMS Microbiol. Ecol.* 50, 87–100.
- Canto, A., Herrera, C.M., Medrano, M., Pérez, R., García, I.M., 2008. Pollinator foraging modifies nectar sugar composition in *Helleborus foetidus* L. (Ranunculaceae): an experimental test. *Am. J. Bot.* 95, 315–320.
- Canto, A., Herrera, C.M., García, I.M., Pérez, R., Vaz, M., 2011. Intraplant variation in nectar traits in *Helleborus foetidus* (Ranunculaceae) as related to floral phase, environmental conditions and pollinator exposure. *Flora* 206, 668–675.
- Cooney, M.J., Marison, I.W., van Gulik, W.M., von Stockar, U., 1996. Calorimetric and stoichiometric analysis of growth of *Kluyveromyces fragilis* in continuous culture: nitrogen limitation imposed upon carbon-limited growth. *Appl. Microbiol. Biotechnol.* 44, 643–653.
- Corbet, S.A., Delfosse, E.S., 1984. Honeybees and the nectar of *Echium plantagineum* L. in southeastern Australia. *Aust. J. Ecol.* 9, 125–139.
- Corbet, S.A., Willmer, P.G., 1981. The nectar of *Justicia* and *Columnnea*: composition and concentration in a humid tropical climate. *Oecologia* 51, 412–418.
- Corbet, S.A., Willmer, P.G., Beament, J.W.L., Unwin, D.M., Prys-Jones, O.E., 1979. Post-secretory determinants of sugar concentration in nectar. *Plant Cell Environ.* 2, 293–308.
- Corbett, A.L., Krannitz, P.G., Aarssen, L.W., 1992. The influence of petals on reproductive success in the arctic poppy (*Papaver radicum*). *Can. J. Bot.* 70, 200–204.
- Darwin, C., 1862. On the Various Contrivances by Which British and Foreign Orchids Are Fertilised by Insects. John Murray, London.
- Distefano, G., Hedhly, A., Casas, G.L., La Malfa, S., Herrero, M., Gentile, A., 2012. Male-female interaction and temperature variation affect pollen performance in *Citrus*. *Sci. Hortic.* 140, 1–7.
- Dyer, A.G., Whitney, H.M., Arnold, S.E.J., Glover, B.J., Chittka, L., 2006. Bees associate warmth with floral colour. *Nature* 442, 525.
- Goulson, D., 2003. Bumblebees. Behaviour Ecology and Conservation. Oxford Univ. Press, Oxford.
- Gustafsson, L., 1991. Microbiological calorimetry. *Thermochim. Acta* 193, 145–171.
- Heinrich, B., 1979. Bumblebee Economics. Harvard University Press, Cambridge, Massachusetts, USA.
- Herrera, C.M., Pozo, M.I., 2010. Nectar yeasts warm the flowers of a winter-blooming plant. *Proc. R. Soc. B* 277, 1827–1834.
- Herrera, C.M., Sánchez-Lafuente, A.M., Medrano, M., Guitián, J., Cerdá, X., Rey, P.J., 2001. Geographical variation in autonomous self-pollination levels unrelated to pollinator service in *Helleborus foetidus* (Ranunculaceae). *Am. J. Bot.* 88, 1025–1032.
- Herrera, C.M., García, I.M., Pérez, R., 2008. Invisible floral larcenies: microbial communities degrade floral nectar of bumblebee-pollinated plants. *Ecology* 89, 2369–2376.
- Herrera, C.M., Pozo, M.I., Medrano, M., 2013. Yeasts in nectar of an early-blooming herb: sought by bumble bees, detrimental to plant fecundity. *Ecology* 94, 273–279.
- Herrera, C.M., 1995a. Floral biology, microclimate, and pollination by ectothermic bees in an early-blooming herb. *Ecology* 76, 218–228.
- Herrera, C.M., 1995b. Microclimate and individual variation in pollinators: flowering plants are more than their flowers. *Ecology* 76, 1516–1524.
- Herrera, C.M., 2002. Censusing natural microgametophyte populations: variable spatial mosaics and extreme fine-graininess in winter-flowering *Helleborus foetidus* (Ranunculaceae). *Am. J. Bot.* 89, 1570–1578.
- Herrera, C.M., 2004. Distribution ecology of pollen tubes: fine-grained, labile spatial mosaics in southern Spanish Lamiaceae. *New Phytol.* 161, 473–484.
- Herrera, C.M., 2011. Complex implications around a simple trait: ecological context determines the fecundity effects of corolla marcescence. *Am. J. Bot.* 98, 812–818.
- Jakobsen, H.B., Martens, H., 1994. Influence of temperature and ageing of ovules and pollen on reproductive success in *Trifolium repens* L. *Ann. Bot.* 74, 493–501.
- Kalisz, S., Vogler, D.W., Hanley, K.M., 2004. Context-dependent autonomous self-fertilization yields reproductive assurance and mixed mating. *Nature* 430, 884–887.
- Kevan, P.G., 1975. Sun-tracking solar furnaces in high arctic flowers: significance for pollination and insects. *Science* 189, 723–726.

- Kilkenny, F.F., Galloway, L.F., 2008. Reproductive success in varying light environments: direct and indirect effects of light on plants and pollinators. *Oecologia* 155, 247–255.
- Knutson, R.M., 1974. Heat production and temperature regulation in eastern skunk cabbage. *Science* 186, 746–747.
- Krannitz, P.G., 1996. Reproductive ecology of *Dryas integrifolia* in the high Arctic semi-desert. *Can. J. Bot.* 74, 1451–1460.
- Kudo, G., 1995. Ecological significance of flower heliotropism in the spring ephemeral *Adonis ramosa* (Ranunculaceae). *Oikos* 72, 14–20.
- Lamprecht, I., Schmolz, E., Blanco, L., Romero, C.M., 2002. Flower ovens: thermal investigations on heat producing plants. *Thermochim. Acta* 391, 107–118.
- Lamprecht, I., 2003. Calorimetry and thermodynamics of living systems. *Thermochim. Acta* 405, 1–13.
- Lankinen, Å., 2001. In vitro pollen competitive ability in *Viola tricolor*: temperature and pollen donor effects. *Oecologia* 128, 492–498.
- Lenth, R., 2016. Least-squares means: the R package lsmeans. *J. Stat. Softw.* 69, <http://dx.doi.org/10.18637/jss.v069.i01>.
- Lievens, B., Hallsworth, J.E., Pozo, M.I., Ben Belgacem, Z., Stevenson, A., Willems, K.A., Jacquemyn, H., 2015. Microbiology of sugar-rich environments: diversity, ecology and system constraints. *Environ. Microbiol.* 17, 278–298.
- Luzar, N., Gottsberger, G., 2001. Flower heliotropism and floral heating of five alpine plant species and the effect on flower visiting in *Ranunculus montanus* in the Austrian Alps. *Arct. Antarct. Alp. Res.* 33, 93–99.
- Müller, H., 1883 *The Fertilisation of Flowers* (Thompson, D'Arcy W., Trans) MacMillan, London.
- Mathew, B., 1989. *Hellebores*. Alpine Garden Society. St. John's Woking, Surrey.
- McKee, J., Richards, A.J., 1998. Effect of flower structure and flower colour on intrafloral warming and pollen germination and pollen-tube growth in winter flowering *Crocus l.* (Iridaceae). *Bot. J. Linn. Soc.* 128, 369–384.
- Motten, A.F., 1986. Pollination ecology of the spring wildflower community of a temperate deciduous forest. *Ecol. Monogr.* 56, 21–42.
- Nicolson, S.W., de Veer, L., Kohler, A., Pirk, C.W.W., 2013. Honeybees prefer warmer nectar and less viscous nectar, regardless of sugar concentration. *Proc. R. Soc. B* 280, 20131597.
- Norgate, M., Boyd-Gerny, S., Simonov, V., Rosa, M.G.P., Heard, T.A., Dyer, A.G., 2010. Ambient temperature influences Australian native stingless bee (*Trigona carbonaria*) preference for warm nectar. *PLoS One* 5, e12000.
- Orueta, D., 2002. Thermal relationships between *Calendula arvensis* inflorescences and *Uzia aurata* bombyliid flies. *Ecology* 83, 3073–3085.
- Pozo, M.I., Herrera, C.M., Alonso, C., 2014. Spatial and temporal distribution patterns of nectar-inhabiting yeasts: how different floral microenvironments arise in winter-blooming *Helleborus foetidus*. *Fungal Ecol.* 11, 173–180.
- R Development Core Team, 2015. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna.
- Rands, S.A., Whitney, H.M., 2008. Floral temperature and optimal foraging: is heat a feasible floral reward for pollinators? *PLoS One* 3, e2007.
- Sapir, Y., Shmida, A., Ne'eman, G., 2006. Morning floral heat as a reward to the pollinators of the *Oncoclytus* irises. *Oecologia* 147, 53–59.
- Schaeffer, R.N., Phillips, C.R., Duryea, M.C., Andicoechea, J., Irwin, R.E., 2014. Nectar yeasts in the tall larkspur *Delphinium barbeyi* (Ranunculaceae) and effects on components of pollinator foraging behavior. *PLoS One* 9, e108214.
- Schaeffer, R.N., Vannette, R.L., Irwin, R.E., 2015. Nectar yeasts in *Delphinium nuttallianum* (Ranunculaceae) and their effects on nectar quality. *Fungal Ecol.* 18, 100–106.
- Schemske, D.W., Willson, M.F., Melampy, M.N., Miller, L.J., Verner, L., Schemske, K.M., Best, L.B., 1978. Flowering ecology of some spring woodland herbs. *Ecology* 59, 351–366.
- Seymour, R.S., Matthews, P.G.D., 2006. The role of thermogenesis in the pollination biology of the Amazon waterlily *Victoria amazonica*. *Ann. Bot.* 98, 1129–1135.
- Seymour, R.S., Maass, E., Bolin, J.F., 2009a. Floral thermogenesis of three species of *Hydnora* (Hydnoraceae) in Africa. *Ann. Bot.* 104, 823–832.
- Seymour, R.S., Ito, Y., Onda, Y., Ito, K., 2009b. Effects of floral thermogenesis on pollen function in Asian skunk cabbage *Symplocarpus renifolius*. *Biol. Lett.* 5, 568–570.
- Song, B., Zhang, Z.Q., Stocklin, J., Yang, Y., Niu, Y., Chen, J.G., Sun, H., 2013. Multifunctional bracts enhance plant fitness during flowering and seed development in *Rheum nobile* (Polygonaceae), a giant herb endemic to the high Himalayas. *Oecologia* 172, 359–370.
- Sprengel, C.K., 1793. *Das entdeckte Geheimniss der Natur im Bau und in der Befruchtung der Blumen*. English translation by Haase. In: Lloyd, D.G., Barrett, S.C.H. (Eds.), *Floral Biology*, 1996. Chapman and Hall, New York, pp. 3–43.
- Stanton, M.L., Galen, C., 1989. Consequences of flower heliotropism for reproduction in an alpine buttercup (*Ranunculus adoneus*). *Oecologia* 78, 477–485.
- Stone, G.N., Amos, J.N., Stone, T.F., Knight, R.L., Gay, H., Parrott, F., 1988. Thermal effects on activity patterns and behavioural switching in a concourse of foragers on *Stachytarpheta mutabilis* (Verbenaceae) in Papua New Guinea. *Oecologia* 77, 56–63.
- Tan, K., Latty, T., Hu, Z.W.N., Wang, Z.W., Yang, S., Chen, W.W., Oldroyd, B.P., 2013. Preferences and tradeoffs in nectar temperature and nectar concentration in the Asian hive bee *Apis cerana*. *Behav. Ecol. Sociobiol.* 68, 13–20.
- Thøstesen, A.M., Olesen, J.M., 1996. Pollen removal and deposition by specialist and generalist bumblebees in *Aconitum septentrionale*. *Oikos* 77, 77–84.
- Thomson, J.D., Plowright, R.C., 1980. Pollen carryover, nectar rewards, and pollinator behavior with special reference to *Diervilla lonicera*. *Oecologia* 46, 68–74.
- Totland, Ø., Matthews, I., 1998. Determinants of pollinator activity and flower preference in the early spring blooming *Crocus vernus*. *Acta Oecol.* 19, 155–165.
- Totland, Ø., 1996. Flower heliotropism in an alpine population of *Ranunculus acris* (Ranunculaceae): effects on flower temperature, insect visitation, and seed production. *Am. J. Bot.* 83, 452–458.
- Vannette, R.L., Gauthier, M.P.L., Fukami, T., 2013. Nectar bacteria, but not yeast, weaken a plant-pollinator mutualism. *Proc. R. Soc. B* 280, 20122601.
- Vesprini, J.L., Pacini, E., 2000. Breeding systems in two species of the genus *Helleborus* (Ranunculaceae). *Plant Biosyst.* 134, 193–197.
- Vesprini, J.L., Pacini, E., 2010. Pollination ecology in sympatric winter flowering *Helleborus* (Ranunculaceae). *Flora* 205, 627–632.
- Vesprini, J.L., Nepi, M., Pacini, E., 1999. Nectary structure, nectar secretion patterns and nectar composition in two *Helleborus* species. *Plant Biol.* 1, 560–568.
- Wagner, J., Lechleitner, M., Hosp, D., 2016. Pollen limitation is not the rule in nival plants: a study from the European Central Alps. *Am. J. Bot.* 103, 375–387.
- Whitney, H.M., Dyer, A., Chittka, L., Rands, S.A., Glover, B.J., 2008. The interaction of temperature and sucrose concentration on foraging preferences in bumblebees. *Naturwiss.* 95, 845–850.
- Willmer, P., 2011. *Pollination and Floral Ecology*. Princeton Univ. Press, Princeton.
- Yu, Q., Huang, S.Q., 2006. Flexible stigma presentation assists context-dependent pollination in a wild columbine. *New Phytol.* 169, 237–241.