# Geographical variation in autonomous self-pollination levels unrelated to pollinator service in *Helleborus foetidus* (Ranunculaceae)[1]

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Autonomous self-pollination may be considered as a mechanism enhancing plant reproductive success when plant access to pollen sources may limit seed production. We have studied the relationship between geographical patterns of variation in pollinator service to *Helleborus foetidus* and self-pollination ability in three widely spaced regions in the Iberian Peninsula. As could be expected from its early flowering period, pollinator visitation rates to both plants and flowers of *H. foetidus* were very low at all sites. Pollinator composition remained consistent among regions, but there was significant variation among regions in pollinator service. Despite the low visitation rates, fruit set did not appear to be pollen limited in any of the study areas, which may be explained by the long duration of flowers (up to 20 d). When pollinators were excluded experimentally, fruit set decreased significantly, but substantial levels of self-pollination occurred at all regions. Autonomous self-pollination levels were lowest in the two regions with lowest pollinator service and highest in the region with highest pollinator service. This disagreement between our results and the expectations derived from the reproductive assurance hypothesis may reflect a nonequilibrium situation of the northern *H. foetidus* populations in relation to their current pollinating environment.

**Key words:**    autonomous self-pollination; early flowering; geographical variation; *Helleborus foetidus*; pollen limitation; pollinator visitation rate; Ranunculaceae; reproductive assurance.

Hermaphroditic flowers of self-compatible plants have the intrinsic potential to produce seeds without the intervention of any pollinating agent. The extent to which this potential for autonomous self-pollination is realized in practice depends, among other factors, on morphological and functional features of flowers, including the extent of temporal or spatial separation of sexes (dichogamy and herkogamy, respectively), number of stamens, type of floral symmetry, and degree of self-compatibility (Lloyd and Schoen, 1992). Under conditions of reduced opportunities for outcrossing, like those derived from unfavorable weather, pollinator unreliability, or competition with other plants for pollinator service, the ability to autonomously self-pollinate may be selected for as a mechanism of reproductive assurance, an hypothesis originally proposed by Darwin (1876) to explain the frequent occurrence of autonomous self-fertilization in angiosperms. Since then, reproductive assurance has been traditionally considered as one central element of hypotheses attempting to explain the evolution of self-pollination in angiosperms in face of the well-known deleterious effects derived from inbreeding (e.g., Baker, 1955; Stebbins, 1970; Levin, 1972; Jain, 1976; Lloyd, 1980, 1992; Motten, 1982; Piper, Charlesworth, and Charlesworth, 1986; Jarne and Charlesworth, 1993; Holsinger, 1996; Schoen, Morgan, and Bataillon, 1996). Selfing will always be favored when ovules would otherwise remain unfertilized, even with strong inbreeding depression (Lloyd, 1979, 1992; Jarne and Charlesworth, 1993).

Although the advantages derived from autonomous self-pollination under unfavorable pollinating conditions seem undisputable, most of the supporting evidence available is based on the correlated variation across species between breeding system and some ecological parameters presumably related to the adversity of the pollinating conditions (but see, e.g., Piper, Charlesworth, and Charlesworth, 1986; Wyatt, 1986; Barrett, Morgan, and Husband, 1989; Rathcke and Real, 1993; Inoue, Maki, and Masuda, 1996; Fishman and Wyatt, 1999). For example, the observation that the incidence of autogamy is higher among annual herbs and colonizer species facing ephemeral or unpredictable ecological conditions than among related taxa living in more stable habitats has been interpreted as supporting the reproductive assurance hypothesis (Lloyd, 1980; Barrett, Morgan, and Husband, 1989; Barrett, Harder, and Worley, 1996). Early flowering, commonly associated with unpredictable weather and unreliable pollinator activity, seems to have been another ecological condition favoring autogamy as a way of reproductive assurance (Schemske et al., 1978; Motten, 1982). The higher selfing rates often found in peripheral populations of some species have been also interpreted as a mechanism of reproductive assurance evolved in response to the more severe ecological conditions generally prevailing near the boundaries of species distribution ranges (Wyatt 1986; Ramsey, Prakash, and Cairns, 1993; Ramsey, Cairns and Vaughton, 1994). These indirect observations have provided support for the wide acceptance of reproductive assurance as a central factor in the evolution of autogamous breeding systems. There have been, however, relatively few attempts at directly testing in the field whether autonomous self-pollination actually plays a role in reproductive assurance, and the

results of these studies do not always conform to expectations (Dole, 1992; Leclerc-Potvin and Ritland, 1994; Eckert and Schaefer, 1998). For example, the well-developed capacity for autonomous selfing exhibited by *Aquilegia canadensis* (Ranunculaceae) does not seem to have been selected because it provides reproductive assurance (Eckert and Schaefer, 1998).

Comparing seed production of flowers that have been pollinated naturally with those of emasculated ones on the same plants was suggested by Schoen and Lloyd (1992) as the ideal sampling protocol to assess the reproductive assurance provided by the autogamous modes of selfing. This was the approach followed by Eckert and Schaefer (1998) in their study on *Aquilegia canadensis* mentioned above (see also Klips and Snow, 1997). It is possible, however, to gain indirect information on the importance of autonomous selfing as a way of reproductive assurance by studying the seed set or fruit set in plants that have been isolated from visitors (Lloyd and Schoen, 1992). Seed production in absence of pollinators (''autofertility,'' sensu Lloyd and Schoen, 1992) reflects the potential, rather than the actual, rate of autonomous self-fertilization, yet it can provide useful insights into some of the ecological and evolutionary correlates of autogamy (Lloyd and Schoen, 1992). Within some species, autofertility is negatively correlated with pollinator service across populations, which has been interpreted as supporting the role of reproductive assurance in the evolution of self-pollination (e.g., Wyatt, 1986; Barrett, Morgan, and Husband, 1989; Rathcke and Real, 1993; Inoue, Maki, and Masuda, 1996).

The present study was planned to assess patterns of variation at a broad geographical scale in levels of autonomous self-pollination in the bumble-bee-pollinated, perennial herb *Helleborus foetidus* (Ranunculaceae) and their possible relationship with variation in pollinator service. *Helleborus foetidus* is an early-blooming plant (flowering period from early winter through early spring) growing for the most part in mountain habitats, thus facing ecological conditions potentially conducive to unreliable pollination. The questions addressed thus were (1) Does pollinator service to *H. foetidus* (as evaluated by visitation rates to both whole plants and individual flowers) vary among regions and years? (2) If so, do differences in pollination service result in different degrees of pollen limitation among regions? (3) Do autonomous self-pollination levels vary among regions? (4) If so, is the variation in self-pollination ability related to pollinator service? These questions were addressed experimentally, by studying *H. foetidus* populations from three widely spaced regions in the Iberian Peninsula, southwestern Europe.

## MATERIALS AND METHODS

***Study system and sites***—*Helleborus foetidus* L. (Ranunculaceae) is a perennial herb widely distributed in western Europe (Werner and Ebel, 1994). In the Iberian Peninsula, the species typically grows in the understory of deciduous and mixed forests. Plants consist of one or a few ramets that develop a terminal inflorescence after several seasons of vegetative growth. Each inflorescence generally produces 25–100 flowers over its 1.5–2.5 mo flowering period, but these open gradually and only rarely are there >2–5 flowers simultaneously open in each inflorescence. Flowers are hermaphroditic, self-compatible, and are pollinated by medium- and large-sized bees, mainly bumble bees and anthophorid bees. Flowers are extremely long-lived (up to 20 d), apocarpous, and protogynous, with the extent of overlap between female and male stages varying geographically. The stigmas (born at the end of long, conspicuous styles) are receptive even before the corolla has fully opened, and the female stage lasts for ~6–15 d. The number of carpels per flower
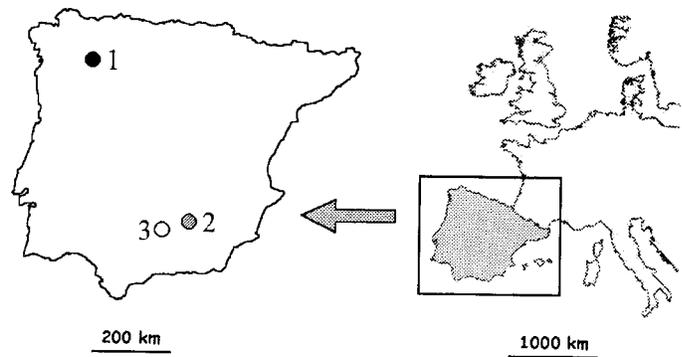


Fig. 1. The Iberian Peninsula (left), with the location of the three study regions: 1, Caurel (42°36′ N, 7°19′ W); 2, Cazorla (37°54′ N, 2°55′ W); 3, Mágina (37°44′ N, 3°15′ W).

ranges between 1 and 5, and the number of anthers between 25 and 60. Anthers start to dehisce centripetally when flowers are 6–8 d old, and the pollen presentation period lasts for ~2 wk. The floral nectaries, which are deeply hidden inside the globose or subcylindrical pendent corolla, provide abundant nectar to floral visitors (Herrera and Soriguer, 1983; Vesprini, Nepi, and Pacini, 1999).

This study was conducted in 1998 and 1999 at three different geographical regions in the Iberian Peninsula (Fig. 1). Study regions are located near the southwestern distributional limits of the species (Werner and Ebel, 1994). The two most distant regions (Caurel and Cazorla) were ~675 km apart, while the two nearest ones (Mágina and Cazorla) were only ~85 km away. At the northwestern region (Caurel), the *H. foetidus* populations chosen for study grew at 950–1350 m elevation in pine (*Pinus sylvestris*) plantations, *Castanea sativa* forests, open successional scrublands, and *Brachypodium rupestris* meadows. At the two southern regions (Cazorla and Mágina) the selected populations were in pine- (*Pinus nigra*) or oak- (*Quercus rotundifolia*) dominated forests at 900–1600 m elevation. Despite latitudinal and elevational differences, the flowering period was roughly similar at all the study areas, extending from late January to March.

***Pollinator service***—To evaluate geographical variation in pollinator service to flowering *H. foetidus* plants, pollinator censuses were simultaneously carried out at the three study regions during the 1998 and 1999 flowering seasons. Censuses started shortly after the first flowers opened and lasted for most of the flowering period. At each region, two *H. foetidus* populations were chosen in the same general area used in 1999 for the experimental study of autonomous self-pollination levels (see later), and 30–40 plants were marked in each population. At each of these individually marked plants, 20–25 3-min censuses were performed each year. For each census, we noted the number of open, functional flowers available to pollinators on each focal plant, the taxonomic identity of all floral visitors, and the total number of flowers visited. Two different estimates of pollinator service will be used here: the probability of a plant being visited by at least one pollinator during a 3-min census (''plant visitation rate,'' PVR hereafter), and the probability of one individual flower being visited at least once during a 3-min census (''flower visitation rate,'' FVR hereafter).

***Tests of pollen limitation***—Tests of pollen limitation to fruit set were conducted in 1998 (Cazorla and Mágina regions) and 1999 (Caurel and Mágina). In Cazorla and Mágina, a variable number of flowers were marked each year on each of 5–9 plants. All the flowers were left exposed to natural pollination. Roughly half of the flowers on each plant were not manipulated and received only natural pollination (''control'' flowers), while the other half received additional cross-pollen by rubbing freshly opened anthers from nearby flowering plants on the receptive stigmas (''experimental'' flowers). In Caurel, whole plants were used for either the control or experimental treatments ($N = 7$ and 13 plants, respectively). The proportion of carpels eventually maturing (''fruit set'') was used as a measure of reproductive success, and the

comparison between experimental and control flowers was used to assess pollen limitation of fruit set.

*Autonomous self-pollination levels*—An experiment was designed to test for differences among the three geographical regions (Caurel, Cazorla, and Mágina) in the degree of autonomous self-pollination in the absence of pollinators. Within each geographical region, replication was achieved by using a randomized complete block design. Before flowering started, five areas were selected at Mágina and Cazorla and six areas at Caurel that contained suitable populations of *H. foetidus*. Distances between nearest areas in the same region ranged between 300 and 5000 m. Each of these areas was considered a block. In each block, eight $1.5 \times 1.5$ m plots were selected, each containing 1–3 reproductive plants (overall mean $\pm$ 1 SD number of plants per plot was 1.1 $\pm$ 0.3; $N = 128$ plots). When several plants occurred in a plot, they were considered as a single individual for the purpose of analyses. Distance between nearest plots in the same block mostly ranged between 15 and 30 m. In each block and region, four plots were randomly assigned to a pollinator exclusion treatment (''excluded'' plots hereafter), and the remaining four were left as controls. In plots with several plants, all were assigned to the same treatment level. Plots assigned to the pollinator exclusion treatment were surrounded with a 0.5-m high wire cage, which was then covered with tulle (the same type used in all regions). Exclosures remained in place during the entire flowering period and were checked frequently for integrity. They were removed shortly after completion of the flowering season.

*Helleborus foetidus* flowers are apocarpous and, in flowers with two or more carpels, the development of individual carpels is largely independent of one another. Furthermore, as there was some variation in the mean number of carpels per flower among regions and among blocks within regions, it did not seem biologically reasonable to score individual flowers for fruit set without taking into consideration carpel number. For these reasons, the basic data for the study of geographical variation in autonomous self-pollination consisted of information on the fate of individual carpels in each plot, rather than on the fate of individual flowers. Just before carpels started to make dehiscence (late May–early June), we determined for each of the carpels initially present in each plot whether they matured or not.

In experimental plots, flowers and developing fruits of *H. foetidus* were sometimes eaten by mice (*Apodemus sylvaticus*) and lepidopteran larvae (*Noctua* spp. and *Trigonophora flammea*; Noctuidae). All carpels lost to these herbivores at any stage from flowering through maturation were subtracted from the initial estimates of carpel number per plot. Whenever a whole flower was completely consumed and the number of carpels lost was unknown, we considered the number of carpels lost to be equal to the mean number of carpels per flower for that particular plant. In the few cases where damage was extensive ($>10\%$ of flowers in a plot), the whole plot was eliminated from the analyses. A total of 11 plots, out of the initial 128, were omitted from the analyses (Caurel: four excluded plots; Mágina, three control and one excluded plot; Cazorla, three control plots). After these corrections, the initial number of carpels on experimental plants was $N = 21\,928$, with all regions and blocks pooled.

*Statistical analyses*—Differences among regions in pollinator service were tested by fitting generalized linear models to the pollinator census data using SAS procedure GENMOD (SAS, 1996a). Separate fits were repeated using plant visitation rate (PVR) and flower visitation rate (FVR) as response variables. Individual 3-min censuses were the sampling units used for the analyses ($N = 7562$). Both PVR and FVR were modeled as binomial response functions, using logits as the link function. Region (Caurel, Cazorla, and Mágina), Year (1998 and 1999), and their interaction were included as independent variables in the model. Census results from the same plant in the same year were expected to be correlated, thus models were fitted using the generalized estimating equations (GEE) method, with Plant treated as the SUBJECT effect and an ''unstructured'' covariance structure (Stokes, Davis, and Koch, 1995; SAS, 1996b).

The significance of the effects of geographical region (region) and pollinator exclusion (treatment) on the fruit set in experimental plots was analyzed using generalized linear mixed models and SAS macro GLIMMIX (Littell et

al., 1996; see Herrera, 2000, for applications of this method in the context of plant reproduction studies). The response variable (fruit set, i.e., the probability of an individual carpel eventually maturing) was modeled as a binomial response. Treatment and region were both considered as fixed effects in the model, while block within region was considered as a random effect, as these were true within-region random replicates. The region factor was treated as fixed because we were not interested in extrapolating the results of the study to an inference space broader than the three regions considered. From a statistical viewpoint, a possible drawback of using carpels rather than flowers as replicates is that carpels from the same flower possibly are not completely independent, thus inflating degrees of freedom to an undetermined extent. In the present study, however, the possible non-independence of carpels was not influential on results. All the conclusions remained unaltered when analyses were conducted using flowers as replicates, and flower fate as the response variable (scoring a flower as successful if at least one carpel matured and as unsuccessful if none of the carpels matured, irrespective of the number of initial carpels).

The general prediction implicitly tested in this study, namely that differential levels of autonomous self-pollination among regions should be related to variation in pollinator service, would be supported if (1) Regions differed significantly in the fruit set of excluded plots and/or there was a significant region $\times$ treatment interaction effect, thus denoting that the effect of pollinator exclusion on reproductive success had not remained consistent across regions; and (2) regional variations in patterns of autonomous self-pollination, if they exist, are congruent with observed among-region variation in pollinator service. Expectations in (1) were first tested by assessing the statistical significance of the region $\times$ treatment effect and then performing comparisons between treatment levels separately for each region using the SLICE option in the LSMEANS statement of the MIXED procedure (Littell et al., 1996). This option provides tests of ''simple main effects'' (sensu Pedhazur, 1982) in factorial designs, corresponding to the effect of a given factor tested at the different levels of the other factors.

## RESULTS

*Pollinator service*—Census sample sizes and a summary of flower visitation by pollinators at the three study regions are presented in the Appendix. Pollinator visits to flowering *H. foetidus* plants were extremely infrequent during this study, being recorded in only 7.8% of the $N = 7562$ 3-min censuses conducted (all regions and years combined).

There were only slight variations among regions, sites within regions, and years in the gross taxonomic composition of *H. foetidus* pollinators, with two bumble bee species (*Bombus pratorum* and *B. terrestris*) consistently accounting for most flower visits (Appendix). Bumble bees, honeybees, and *Anthophora acervorum* almost invariably contacted anthers and stigmas of visited flowers and thus must be considered true pollinators. *Andrena* spp., *Osmia* spp., and halictid bees, in contrast, surely played a much less important role in the pollination of *H. foetidus* flowers, as these small-sized bees only rarely contacted the stigmas. Adequate quantitative data on differential pollinating effectiveness are lacking, and combined visitation data from all species will be used in the statistical analyses. The proportional importance of the presumably least efficient pollinators (*Andrena, Osmia*, halictids) was so small (5.5% of total flower visits recorded) that their inclusion in the analyses was inconsequential for the results.

The two components of pollinator service considered here (plant and flower visitation rate) were similar with regard to the statistical significance of the effects of region, year, and their interaction (Table 1). There was a highly significant effect of region, a significant effect of year, and a nonsignificant interaction effect on PVR and FVR. The magnitude of the $\chi^2$ values is much greater for the region than for the year effect,

TABLE 1. Summary of significance tests for the effects of region (Caurel, Cazorla, and Mágina), Year (1998, 1999), and their interaction (region × year) on the two components of pollinator service to *Helleborus foetidus* considered in this study. "Plant visitation rate" refers to the probability of a focal plant being visited at least once during a 3-min census period, while "Flower visitation rate" refers to the probability of one individual flower being visited at least once during the census period.

| Dependent variable | Effect in model | Significance test[a] | | |
|---|---|---|---|---|
| | | df | $\chi^2$ | P |
| Plant visitation rate | Region | 2 | 72.67 | <<0.0001 |
| | Year | 1 | 8.14 | 0.004 |
| | Region × Year | 2 | 0.69 | 0.71 |
| Flower visitation rate | Region | 2 | 77.85 | <<0.0001 |
| | Year | 1 | 7.31 | 0.007 |
| | Region × Year | 2 | 3.77 | 0.15 |

[a] Type 3 tests conducted on generalized linear models fitted to the data using the generalized estimating equations (GEE) method.

thus indicating that during the study period regional differences were more important than those associated with annual variation. Further, the nonsignificance of the interaction effect denotes that regional differences in pollinator service remained consistent between years in spite of significant interannual variation in PVR and FVR.

Patterns of variation among regions and years for the two measures of pollinator service are depicted in Fig. 2. For PVR (Fig. 2A), there was a distinct regional gradient in the direction Caurel > Cazorla > Mágina, and all pairwise contrasts between regions were statistically significant ($\chi^2 \geq 29.5$ in the three pairwise contrasts, df = 1, $P << 0.001$ in all cases). The pattern differed slightly for FVR (Fig. 2B), with Caurel ~ Cazorla > Mágina. Caurel and Cazorla did not differ significantly in FVR ($\chi^2 = 0.17$, df = 1, $P = 0.68$), while both of them differed significantly from Mágina ($\chi^2 \geq 97.9$ in the two pairwise contrasts, df = 1, $P << 0.001$).

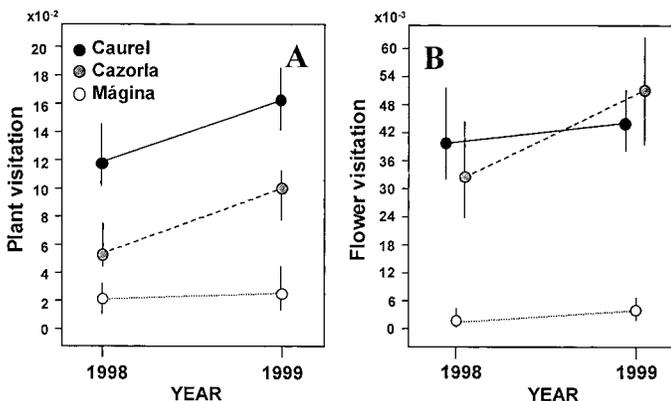*Pollen limitation*—Experimental pollen addition slightly in-



Fig. 2. Variation among regions and years in pollinator service to *Helleborus foetidus* plants and flowers. Pollinator service was assessed by means of the estimated probability of individual plants (A) and individual flowers (B) being visited at least once during a 3-min census period ("plant visitation" and "flower visitation," respectively). Values plotted are model-adjusted means obtained from fitting generalized linear models to the census data and modeling plant and flower visitation data as binomial processes. Vertical lines denote the 95% parametric confidence intervals around the means. See also Table 1.

TABLE 2. Results of tests of pollen limitation of fruit set (percentage of carpels maturing at least one seed) of *Helleborus foetidus* at the three study regions. Comparisons involve fruit set of flowers exposed to natural pollination ("control") and flowers subjected to additional hand-pollination ("additional pollen").

| Population | Year | Control | | Additional pollen | | |
|---|---|---|---|---|---|---|
| | | N | Fruit set (%) | N | Fruit set (%) | Difference[a] P |
| Caurel | 1999 | 387 | 79.6 | 589 | 82.2 | 0.32 |
| Cazorla | 1998 | 95 | 80.0 | 91 | 91.2 | 0.037 |
| Mágina, Site 1 | 1998 + 1999[b] | 51 | 76.5 | 59 | 81.4 | 0.64 |
| Mágina, Site 2 | 1998 + 1999[b] | 60 | 66.7 | 62 | 77.4 | 0.23 |

[a] P values obtained using Fisher exact probability tests.
[b] A considerable number of experimental carpels were destroyed by herbivores at the Mágina region, so data from two years for the same population were combined into a single sample to increase sample size.

creased fruit set of experimental flowers at all sites and years, but the difference with control flowers was statistically significant only for Cazorla in 1998, and even in this case the *P* value was only barely significant (Table 2). Combining the results of the four experiments using the simple meta-analysis procedure suggested by Sokal and Rohlf (1981, p. 779) does not support any overall significant effect of pollen addition on fruit set in the *H. foetidus* populations studied ($\chi^2 = 12.70$, df = 8, $P = 0.12$).

*Autonomous self-pollination*—Pollinator exclusion experiments revealed highly significant effects of treatment (natural pollination vs. pollinator exclusion), region (Caurel, Cazorla, and Mágina), and their interaction on fruit set of experimental *H. foetidus* plants (Table 3A). Fruit set was significantly higher in control than in excluded plots in every region (Table 3B), although some fruits were set in excluded plots at the three regions (Fig. 3). Regions differed significantly with regard to fruit set in excluded plots (Table 3B). Fruit set in excluded plots was highest in Caurel (model-adjusted mean fruit set = 34.4%) and considerably lower in Cazorla (7.2%) and Mágina (10.8%). These results not only demonstrate the existence of substantial levels of autonomous self-pollination in some pop-

TABLE 3. Summary of significance tests for the effects of pollination Treatment (natural pollination vs. pollinators excluded), Region (Caurel, Cazorla, and Mágina), and their interaction, on fruit set (probability of carpel maturation, modeled as a binomial process). (A) Tests of main effects and their interaction. (B) Tests of simple main effects for treatment (separately for the three regions) and region (separately for treatment levels). See also Fig. 3 for a graphical presentation of the significant Treatment × Region interaction.

A)

| Effect in model | Significance test | | |
|---|---|---|---|
| | df | F | P |
| Treatment | 1,21908 | 3394.5 | <0.0001 |
| Region | 2,13 | 37.9 | <0.0001 |
| Treatment × Region | 2,21908 | 90.3 | <0.0001 |

B)

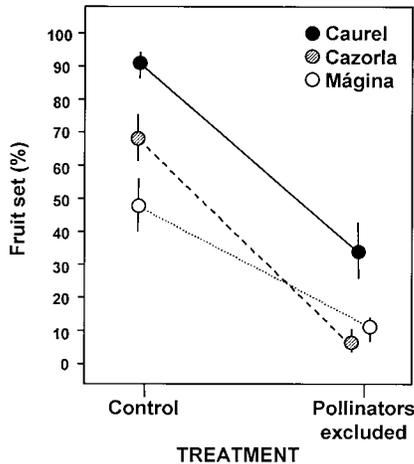| Source | | | |
|---|---|---|---|
| Among regions for control plants | 2,21908 | 46.9 | <0.0001 |
| Among regions for excluded plants | 2,21908 | 31.1 | <0.0001 |
| Between treatments: Caurel | 1,21908 | 1275.9 | <0.0001 |
| Between treatments: Cazorla | 1,21908 | 974.3 | <0.0001 |
| Between treatments: Mágina | 1,21908 | 1879.4 | <0.0001 |

Fig. 3. Interaction graph for the effects of Treatment (pollinators excluded vs. natural pollination) and Region on fruit set of *Helleborus foetidus* in experimental plots. Values plotted are model-adjusted means obtained by fitting a generalized linear mixed model to the data and modeling fruit set as a binomial process. Vertical lines denote the 95% parametric confidence intervals around the means. See also Table 3.

ulations of *H. foetidus*, but also that the extent of autonomous self-pollination varies among regions. These differences cannot solely be attributed to the existence of intrinsic differences between regions in fruit set, which are revealed by the significant effect of region after statistically accounting for the treatment and interaction effects (Table 3A). The reduction in fruit set caused by pollinator exclusion did not occur at the same rate at the three populations, as demonstrated by the significant region × treatment interaction effect (Table 3A, Fig. 3). On average, fruit set in excluded plots represented 37.7%, 10.4%, and 22.2% of the fruit set in control plots in Caurel, Cazorla and Mágina, respectively.

## DISCUSSION

***Pollinator service and pollen limitation***—By conducting many short-duration pollinator censuses on focal plants and modeling the results as a binomial process, we have assessed pollinator service to *H. foetidus* in terms of plant- and flower-level visitation probabilities. These visitation probabilities were extremely low at all sites. This scarcity of pollinator visits confirmed our a priori expectations, based on the unfavorable weather prevailing during the winter–early spring flowering season of *H. foetidus* in the mountain habitats studied here and effectively suggests that *H. foetidus* is a candidate species to exhibit some reproductive assurance based on autonomous self-pollination.

There was significant regional variation in pollinator service. Plant and flower visitation rates, however, differed slightly in their patterns of variation among regions. The lowest values of both PVR and FVR occurred in Mágina. Plant visitation rate was significantly higher in Caurel than in Cazorla, yet the two regions were similar with regard to FVR, which indicates that plants, but not individual flowers, are more frequently visited in Caurel than in Cazorla. This contrast between the two measurements of pollinator service reflects the fact that, once a individual plant received a pollinator visit, a greater proportion of the available flowers were visited in Ca-

zorla (mean ± 1 SD = 61.7 ± 33.6%, N = 186 "positive" censuses) than in either Caurel (27.9 ± 23.0%, N = 338) or Mágina (23.9 ± 27.7%, N = 63). These differences closely reflect, in turn, that despite broad regional differences in the mean number of simultaneously open flowers per plant (16.2 ± 8.8 flowers, 5.7 ± 4.6 flowers, and 24.9 ± 22.2 flowers, in Caurel, Cazorla, and Mágina, respectively), pollinators tended to visit roughly the same number of flowers per plant per visit (3.8 ± 3.2, 2.6 ± 1.6, and 2.6 ± 1.9 flowers in Caurel, Cazorla, and Mágina, respectively). Observed regional variation in FVR thus reflects plant architectural differences as well as inherent variation in pollinator service, hence PVR figures probably are a better measure of region-specific pollinator service levels than is FVR.

Contrary to expectations was the finding that fruit set did not appear to be consistently pollen-limited in the populations of *H. foetidus* studied. Given the low plant and flower pollinator visitation probabilities observed, this result can only be explained by consideration of the long duration of *H. foetidus* flowers. Even with flower visitation probabilities as low as those found here, the probability of each of these long-lived flowers receiving at least one effective pollinator visit during the long female receptive stage is far from negligible. This is supported by the observation that virtually all newly withered, naturally pollinated flowers of *H. foetidus* at the three study regions have pollen tubes down the style (99.6%, 100%, and 98.6% in Cazorla, Caurel and Mágina, respectively; C. M. Herrera, personal observations). For species that produce relatively few flowers and face unreliable pollinator services, long duration of flowers will increase the probability of being visited at least once (Primack, 1985; Motten, 1986; Ashman and Schoen, 1994). Long floral durations and positive correlations (across species) between floral longevity and fruit set have been reported for some early-flowering plants (Schemske et al., 1978; Motten, 1986; Ishii and Sakai, 2000). *Helleborus foetidus* fits this pattern well. Compared with reported flower longevities (e.g., Primack, 1985; Motten, 1986; Stratton, 1989), *H. foetidus* flowers are extraordinarily long-lived, and this trait most likely contributed decisively to observed reproductive success. In *Narcissus longispathus* (Amaryllidaceae), another early-blooming species from the Cazorla region, Herrera (1995) likewise found high fruit set levels and weak pollen limitation in spite of infrequent pollinator visitation and also attributed these results to the long duration of its flowers (16 d).

***Autonomous self-pollination and reproductive assurance***— When pollinators were excluded experimentally, fruit set of *H. foetidus* plants decreased significantly, but it was still substantial at the three study regions. Regions did differ, however, both in mean fruit set in absence of pollinators and in the proportional reduction in fruit set brought about by experimental pollinator exclusion. Autonomous self-pollination was highest and proportional reduction in fruit set derived from pollinator exclusion was lowest at the northern Caurel region, while the two southern regions were roughly similar in having rather low self-pollination levels and high proportional reductions in fruit set. According to conventional interpretations, these regional differences in the ability of *H. foetidus* to autonomously self-pollinate should reflect differential adaptations to variation in pollinator service. Our results, however, do not support this view, as variation among regions in self-pollinating ability was not congruent with dif-

ferences found in pollinator service. Most remarkably, *H. foetidus* plants exhibited the greatest ability to self-pollinate precisely in Caurel, where pollinator service was greatest. Observed population differences in fruit set in absence of pollinators may reflect not only differential rates of autonomous pollination, as implied here, but also differences in early inbreeding depression. Differences in fruit set could arise from differential success of self-pollinated carpels even if self-pollination rates were the same at all populations. This interpretation can be ruled out in the present instance, as preliminary evidence indicates that early inbreeding depression is not greater at the two southern populations (C. M. Herrera et al., unpublished data).

According to the reproductive assurance hypothesis, autonomous self-pollination must be considered as a mechanism aimed to assure a minimum threshold of reproductive success when successful pollination by other, presumably more beneficial, agents has been reduced or suppressed. It is most advantageous when there is no ovule discounting, i.e., when self-pollination occurs after the chances for cross-pollination ("delayed selfing" sensu Lloyd and Schoen, 1992), as will most likely be the case in protogynous species like *H. foetidus*. The ability to self-pollinate may have evolved as a response to the paucity of pollinators (e.g., Wyatt, 1983; Erhardt and Jäggi, 1995; Vaughton and Ramsey, 1995; Ramsey and Vaughton, 1996), since selection should favor traits that facilitate pollination if seed production is consistently pollen limited. Therefore, a precondition for reproductive assurance becoming a major selective force favoring the development of self-fertilization is that seed production should be consistently pollen limited. However, we did not find evidence for a consistent pollen limitation to fruit set in the study regions.

Despite significant differences between years and regions in pollinator service at both the plant and flower levels, the composition of the pollinator assemblage remained remarkably constant, with bumble bees tending to account for most flower visits in all sites and years. Bumble bees are efficient pollinators of *H. foetidus* flowers, as supported by the finding that fruit set of naturally pollinated flowers was relatively high even in the region where bumble bee visits to flowers were most infrequent (Mágina). Taken together, therefore, the results of this study are not consistent with the interpretation that differences between regions in the ability of *H. foetidus* to autonomously self-pollinate are an adaptive response to differences in pollinator abundance and/or in the ability of the main visitors to act as efficient pollinators. Our results thus do not support the reproductive assurance hypothesis as the current selective force responsible for the differences among regions in the ability to self-pollinate.

***Proximate and ultimate explanations***—This investigation was not designed to elucidate the mechanisms of self-pollination in *H. foetidus*, thus we cannot provide conclusive evidence of the proximate causes giving rise to observed between-region differences in levels of autonomous self-pollination. Preliminary evidence, however, does allow for ruling out two possible explanations and suggesting what seems the most likely one. The first explanation to be ruled out is any possible effect of differential abundance among regions of some very small, cryptic pollinating agent that could go through tulle and actively pollinate flowers inside excluded plots (Lloyd and Schoen, 1992). Small-sized thrips (Thysanoptera), for example, are inconspicuous pollen vectors bringing about self-pollination in some species (Baker and Cruden, 1991). Neither thrips nor any other small insect that could have performed pollination were ever observed at any region inside *H. foetidus* flowers during the flowering period. The second explanation that can also be safely ruled out is one based on geographical differences in morphological or structural characteristics of flowers. Although a detailed study of the floral morphology of *H. foetidus* over the Iberian Peninsula has revealed significant geographical variation in a number of metric and meristic traits, no evidence exists linking the greatest autonomous self-pollination of Caurel flowers with either reduced levels of herkogamy or increased number of stamens per flowers (C. M. Herrera et al., unpublished data). Rather than to structural features, differences found in this study in autonomous self-pollination are most likely attributable to regional differences in functional features of flowers, specifically in the nature and extent of dichogamy. Even though all *H. foetidus* populations studied to date are unequivocally protogynous, the stigmas of Caurel flowers remain receptive for almost the whole duration of the flower, which means that there is extensive overlap between the female receptive stage and the long period of anther dehiscence. In the southern regions, in contrast, stigmas generally are no longer receptive at the time when anthers start to dehisce, and there is virtually no overlap between the male and female stages (M. Medrano and C. M. Herrera, personal observations). This regional variation in the extent of intrafloral overlap between the female and the male stages is the most parsimonious explanation for patterns of differential self-pollination found in this study.

Regardless of the proximate mechanism(s) involved, however, the unrelatedness of autonomous self-pollination levels and pollinator service found in this study demands some evolutionary explanation. At present, two tentative hypotheses can be suggested to guide future studies on this species in the Iberian Peninsula. Firstly, reproductive assurance is a reasonable evolutionary option only if the inbreeding depression that accompanies increased selfing in an originally outcrossing populations diminishes over generations (Harder and Barrett, 1996; Holsinger, 1996). Observed regional differences in autonomous self-pollination could thus perhaps be the consequence of differential levels of inbreeding depression having selected for differential extent of dichogamy. As noted earlier, preliminary observations are not consistent with this hypothesis, as early inbreeding depression was not higher in the southern regions. The second hypothesis is related to the possibility that pollinator conditions revealed by this study are not representative of long-term conditions. Due merely to latitudinal differences, long-term average weather conditions faced by northern *H. foetidus* must be less favorable, and pollination probabilities consequently lower, than those faced by southern populations. This has probably molded the species' floral biology and favored the evolution of self-pollination as a reproductive assurance mechanism. The present short-term study might have hit a period of unusual pollinator abundance and/or activity in Caurel caused, for example, by some transitory amelioration of climatic conditions in the northwestern Iberian Peninsula. In this hypothesized scenario, prevailing selection regimes on *H. foetidus* could not be safely inferred from the present short-term investigation alone (Herrera, 1996; Waser et al., 1996). The disagreement between our results and the expectations derived from reproductive assurance considerations might thus be explained by a nonequilibrium situation

of northern *H. foetidus* populations in relation to their current pollinating environments. Studies are currently underway to test these two hypotheses.

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APPENDIX. Cumulative number of flowers visited by the different pollinator taxa of *Helleborus foetidus* at each of the six sites where censuses were conducted ($N$ = number of 3-min censuses conducted per population).

| | | Caurel | | | | Cazorla | | | | Mágina | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | | Site 1 | | Site 2 | | Site 1 | | Site 2 | | Site 1 | | Site 2 | |
| Bee species | $N$ = | 1998 451 | 1999 770 | 1998 534 | 1999 626 | 1998 520 | 1999 693 | 1998 545 | 1999 772 | 1998 626 | 1999 700 | 1998 569 | 1999 756 |
| *Andrena* spp. | | 0 | 3 | 7 | 23 | 2 | 0 | 8 | 2 | 0 | 3 | 9 | 16 |
| *Anthophora acervorum* | | 89 | 103 | 2 | 0 | 3 | 6 | 8 | 0 | 0 | 0 | 8 | 0 |
| *Apis mellifera* | | 45 | 125 | 0 | 68 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Bombus pascuorum* | | 20 | 4 | 8 | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Bombus pratorum* | | 88 | 272 | 113 | 158 | 31 | 0 | 41 | 0 | 0 | 0 | 0 | 0 |
| *Bombus terrestris* | | 2 | 33 | 3 | 102 | 36 | 131 | 61 | 215 | 12 | 0 | 33 | 67 |
| *Bombus* sp. | | 0 | 0 | 0 | 0 | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Halictidae gen. sp. | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 15 | 3 |
| *Osmia* spp. | | 0 | 0 | 0 | 0 | 0 | 10 | 1 | 7 | 0 | 0 | 0 | 0 |