

## Spatio-temporal variation in the pollination mode of *Buxus balearica* (Buxaceae), an ambophilous and selfing species: mainland-island comparison

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Mixed-pollination systems may allow plants to achieve stable seed production when unpredictable conditions cause variation in the relative success of different pollination modes. We studied variation in time (two years) and space (in five populations, three from an island and two from mainland) in the pollination mode of *Buxus balearica*, an ambophilous (i.e. pollinated by wind and insects) and selfing species distributed in the Mediterranean Basin, by means of direct observations and experimental manipulations (bagging with different material). The relative importance of each pollination mode differed among populations; however, levels of selfing and wind pollination were similar between island and mainland. Flowers of *B. balearica* were visited only by generalist insects, and species composition and abundance of flower visitors varied both in space and time. Frequency of insect visits to plants were not higher in mainland than island populations, although insects on the mainland were more diverse, visited a proportionally greater number of flowers, and remained longer on the plants than insects on the island. Frequency of insect visits was negatively correlated with flowering synchrony (all populations pooled) and was found to increase seed set in one of the mainland populations (that with highest frequency of insect visits and highest flower visitation rate). Fruit and seed mass were found to be not affected by pollination mode. Scarcity of pollinators in the island seems to have an effect on the pollination mode, although the greatest variation in breeding system was found at a more local scale.

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Since pollinator visitation rates vary in space and time (Horvitz and Schemske 1990, Parker 1997, Herrera et al. 2001), alternative types of pollination (pollination modes) may sometimes evolve in angiosperms to achieve stable seed production (Barrett 1996, Gómez and Zamora 1996, Goodwillie 1999, Culley et al. 2002). Natural selection will favor a generalist system if reproductive success of combined pollination modes is greater than the success of each pollination vector alone over the lifetime of an individual (Waser et al. 1996, Culley et al. 2002). The existence of two or more

pollination modes in angiosperm species has increasingly received attention in the literature (Traveset and Sáez 1997, Culley et al. 2002, Valido et al. 2002, Schueller 2004). In such mixed-pollination systems, the relative importance of each pollination vector may vary among habitats (Faegri and van der Pijl 1979). For instance, the combination of insects and wind as pollination vectors (ambophily) is thought to be an adaptation to spatial and/or temporal variation in the local environments, which favor more one or another mode depending upon prevailing conditions (Vroege and Stelleman 1990,

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Gómez and Zamora 1996, Tamura and Kudo 2000, Totland and Sottocornola 2001).

Island-mainland comparisons are especially interesting for the study of mixed-pollination systems, since island populations differ not only in genetical (Frankman 1997, 1998) but also in ecological features (Barrett 1996) from mainland ones. However, there have been relatively few mainland-island comparisons of breeding systems and pollination modes (but see Larson and Barrett 1998, Schueller 2004). Almost all effort has focused on variation in selfing levels under the prediction that selection during colonization or establishment makes self-fertilization more common in islands (Barrett 1996, Anderson et al. 2000, Schueller 2004).

It is well-known that island pollinator faunas are often scarce and depauperate (Linhart and Feinsinger 1980, Barrett 1996, Anderson et al. 2001) and that insects tend to be more generalists and visit plant species with a lower frequency and a higher variability in time (Feinsinger et al. 1982, Inoue et al. 1996). Pollinator scarcity and reduced pollinator services are often associated both with high selfing rates (see references above) and a high frequency of anemophily, i.e. wind-pollination (Berry and Calvo 1989, Goodwillie 1999, Anderson et al. 2001). For ambophilous species, thus, we might expect that wind played a more important role in island than in mainland populations. Moreover, if the quantity or quality of seeds produced varies with pollination mode, parent fitness could be directly influenced by the balance of pollination modes fluctuating among sites or years (Berry and Calvo 1989, Gómez and Zamora 1996). Since different pollen donors to the same female flower may generate a greater seed mass variability (Marshall 1991, Bañuelos and Obeso 2003) we would also expect pollination modes generating more crosses among individuals to result in a greater variance in seed mass within a fruit.

The genus *Buxus* (Buxaceae) has been considered to be predominantly wind pollinated; however, at least the two European species of this genus are frequently visited by insects (Blanca et al. 1999, Von Balthazar and Endress 2002). Distribution of *Buxus balearica* includes both mainland and island populations around the Mediterranean Basin, most of them appearing fragmented and isolated (Benedí 1997). In the present study, we compared the pollination system of *B. balearica* among island (Balearic Islands) and mainland (southeastern Iberian Peninsula) populations. Our specific questions were: 1) what is the relative importance of each type of pollination vector, and does this vary spatially (between island and mainland and among different habitats) and/or temporally (between years)? 2) Does ambophily act as a successful reproductive assurance? 3) To what extent does the plant spontaneously self, and is there spatial variation in spontaneous selfing levels? 4) Do fruit and seed traits vary with pollination mode? 5) Are there

differences in insect species composition, flower visitation rates, and number of flowers visited between island and mainland populations? In addition, we wanted to determine if flowering phenology of *B. balearica* is related to pollination mode, as some studies have reported a greater flowering synchrony in wind-pollinated species compared to entomophilous ones (Ratheke and Lacey 1985, Bolmgren et al. 2003).

## Methods

### Study species

*Buxus balearica* (Buxaceae) is an evergreen shrub up to 6 m tall, although under harsh growing conditions it adopts a creeping habit. It is endemic to the Mediterranean Basin, growing in calcareous and rocky soils, under subhumid conditions, between sea level and 1000 m (Benedí 1997). Its distribution has drastically decreased due to both climate change and human activity (Bonafè 1979, Yll et al. 1997), and it currently consists of fragmented and isolated populations in southern Turkey, southern Sardinia, Morocco and Spain (Balearic Islands and east of Andalusia). The species is monoecious, and can produce over 30 000 inflorescences. Each inflorescence contains one female flower surrounded by a variable number of male flowers (1–6, mainly 4), each of them containing mainly 4 anthers (although can vary from 2 to 5); in addition, a variable number of male inflorescences can also be found in some individuals (Lázaro and Méndez unpubl.). The flowers last for a week (Von Balthazar and Endress 2002), and inflorescences can be protogynous or protandrous (although there is much overlap of the two sexes) bearing traits of typical anemophily (inconspicuous, absence of an attractive perianth, densely clustered small flowers with well-exposed anthers and stigmas, and high pollen: ovule ratio), but also of typical entomophily (faint sweet scent of male flowers, nectar secretion of both flower sexes, sculptured wall of the pollen grains). It is a masting species, alternating years with high and low flower and fruit production (Lázaro et al. unpubl.). Fruits (12–14 × 7–9 mm; Benedí 1997) are dehiscent and throw out its seeds (4.5–5.5 × ca 3 mm; Benedí 1997) when mature, from June to July. In some populations, ants can act as secondary dispersers. Fruits usually contain six ovules, although sometimes only four are found.

### Study areas

All experiments and observations were carried out in spring of 2002 and 2003 in five Spanish populations of *B. balearica* (Fig. 1). Two of the study sites belong to the relict mainland populations in eastern Andalusia, separated by ca 90 km from each other. “Cerro Gordo” is at

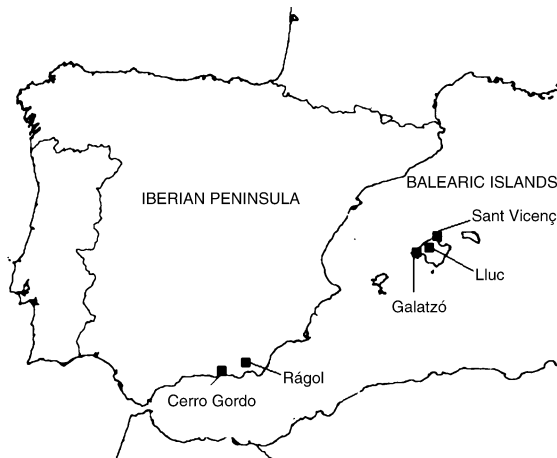


Fig. 1. Map of the Mediterranean area where the study populations are located.

sea level in Granada province, near a seasonal stream and the coast (mean precipitation,  $P_m = 461$  mm; mean maximum temperature  $T_{max} = 21^\circ\text{C}$ ; mean minimum temperature,  $T_{min} = 13^\circ\text{C}$ ). “Rágol” is located in the “Sierra de Gádor” mountains, in Almería province, at 460 m of elevation ( $P_m = 258$  mm;  $T_{max} = 22^\circ\text{C}$ ;  $T_{min} = 13^\circ\text{C}$ ). Both sites support a high diversity of flowering plant species, including >20 species of trees or shrubs, such as *Pinus halepensis*, *Juniperus oxicedrus*, *Coronilla juncea*, and *Ulex parviflorus*. The mean distance among *B. balearica* individuals is 1.2 m and 2.1 m at “Cerro Gordo” and “Rágol”, respectively. The three island populations are in the “Serra de Tramuntana” mountains (Mallorca Island), and species richness in their community (with <10 tree/shrub species) is much lower than at the two mainland sites. These three populations were chosen along an elevational gradient to include a high variability of habitats “Sant Vicenç” is at low-altitude (40 m a.s.l.), where *B. balearica* shrubs grow in a creeping form due to strong winds ( $P_m = 318$  mm;  $T_{max} = 23^\circ\text{C}$ ;  $T_{min} = 13^\circ\text{C}$ ); “Lluc” is at mid-elevation (320 m a.s.l.) ( $P_m = 1254$  mm;  $T_{max} = 20^\circ\text{C}$ ;  $T_{min} = 9^\circ\text{C}$ ); and “Galatzó” is at high-altitude (900 m a.s.l.), near the limit of the species in Mallorca Island (meteorological data from the nearest station at 535 m are:  $P_m = 746$  mm;  $T_{max} = 22^\circ\text{C}$ ;  $T_{min} = 14^\circ\text{C}$ ) (data provided by the National Meteorological Inst.). The surrounding vegetation consists mainly of *Chamaerops humilis*, *Pistacia lentiscus*, and *Ampelodesmos mauritanica*, at “Sant Vicenç”; *Pinus halepensis*, *Euphorbia dendroides*, *A. mauritanica*, *P. lentiscus*, and *Erica multiflora* at “Lluc” (the Mallorcan population with greatest plant diversity); and *A. mauritanica*, *P. lentiscus*, and *Smilax aspera* at “Galatzó”. The mean distance among individuals was 0.6, 1.5, and 3.5 m, for Sant Vicenç, Lluc, and Galatzó, respectively.

## Pollination experiments

We carried out pollination experiments in the five study areas during spring of 2002 and 2003. In 2002, we randomly selected and marked 6–12 individuals per population. In 2003, we included in the experiment those same individuals (except those not flowering) plus others that were again randomly selected (total chosen: 8–12 individuals). We applied three treatments to each individual: 1) spontaneous selfing (by means of cloth bags that prevented the passage of insects and airborne pollen; selfing hereafter), 2) anemogamy (fertilization by airborne pollen; by means of net bags which excluded insects (mesh size: 1 mm) placed on previously emasculated inflorescences to prevent spontaneous selfing); and 3) control (open pollination). We consistently used a minimum of 15 inflorescences, from 3 to 10 branches, per treatment and individual.

We performed all pollination experiments during March in mainland populations and between March and May in Mallorca, and recorded fruit initiation and maturation in mid-May and June on the mainland and about a month later on the island. Mature fruits were gathered before dehiscence to obtain fruit set (proportion of flowers that become fruits) and dissected in the laboratory to obtain seed set (number of seeds per number of total ovules). In order to correlate these two parameters with traits describing plant fecundity and plant size, for each experimental individual we estimated the total flower and fruit production, both in 2002 and 2003, and measured height and the two crown diameters (the longest and the shortest). Size was estimated as:  $\text{volume} = \frac{2}{3} \pi (h \times D/2 \times d/2)$ , where  $h$  is height,  $D$  is the longest diameter and  $d$  is the shortest diameter, measured to the nearest cm. Total production of inflorescences and fruits was estimated by counting them in a sector of the canopy and extrapolating to the entire shrub.

We further tested for differences in fruit and seed mass and in within fruit seed mass variation of fruits and seeds resulting from the various pollination modes. For this, we weighed (to the nearest 0.1 mg) all available fruits and seeds resulting from each treatment and population.

## Flower visitor censuses

To assess whether pollination composition, frequency of insect visits to plants (no. visits/h; FIV hereafter), flower visitation rates (flower/min; FVR hereafter), and behaviour of insect visitors differed between island and mainland populations, we performed censuses of pollinators to individuals at their flowering peak. In 2002, we could only make observations in one population from the mainland (Rágol) and one from the island (Galatzó) due to a low number of flowers available that year in the other populations. In 2003, however, observations were

made in all populations. Censuses of 15 min were randomly carried out at a total of 15 individuals during 3–5 d at each population; they were conducted from 10:30 to 16:30 h (period of maximum insect activity, determined from previous censuses during an entire day). The total amount of time spent censusing was 18–25 h per population and year. In each census, we recorded: 1) insect identity, 2) time each insect spent on the plant, 3) number of visited inflorescences, 4) parts of the inflorescences contacted, and, whenever possible, 5) the plant species to which the insect moved after visiting *B. balearica*. All these data are especially relevant for assessing the level of selfing (geitonogamy) that insects can cause. A number of specimens from each insect species were captured for identification. We also counted the number of inflorescences produced by each censused plant.

### Flowering phenology

From the beginning to the end of the flowering period of 2003, we monitored a 30–60 randomly chosen inflorescences (from 3 to 5 branches) from anthesis to senescence in a total of 10–20 individuals per population. We weekly recorded the number of buds and inflorescences at different stages of maturation: opening buds, open inflorescences and mature inflorescences (those with dehisced anthers). Due to the nature of our data, we followed Mahoro (2002) to calculate the flowering synchrony of an individual plant (defined as the degree to which that plant's blooming period overlapped with the blooming periods of all other plants in the population) as

$$S_i = 1/2(2 - \sum_{j=1}^n |y_{ij} - \bar{y}_{ij}|)$$

where  $y_{ij}$  is the ratio of flowers opened between consecutive pairs of censuses (i.e. between  $j-1$  and  $j$ ) to the total number of flowers opening during the season in the  $i$ -th individual;  $\bar{y}_{ij}$  is the mean  $y_{kj}$  ( $k \neq i$ ) (i.e. the mean including all members of the population except  $i$ ), and  $n$  is the total number of censusing days. A large  $S_i$  indicates a high individual synchrony with the other *B. balearica* individuals in the population. All branches in an individual were pooled to calculate  $S$ .

### Data analysis

The different variables measured in the pollination experiments were analyzed by means of Generalized Linear Modeling (GENMOD and GLIMMIX procedures of SAS ver. 6.12, Anon. 1997), since they did not fulfill the assumption of normality of residuals necessary to perform analysis of variance. In all cases, population

was included as a fixed factor, treatment as a within-subject (i.e. repeated measures) fixed factor and plant individual as a random factor (subject). We used the error distributions and link functions that best fitted the observed data (based on the whole-model deviances): 1) binomial distributions and logit links for initiated fruits, fruit and seed set, 2) gamma distributions and power ( $-1$ ) links for fruit and seed mass, and 3) a negative binomial distribution and power ( $-1$ ) link for the within-fruit variation in seed mass. For this last variable, the fitted model included also the number of seeds per fruit as a continuous covariate. All GLIMs were followed by a posteriori-comparisons tests (differences of least squares means), and were corrected using Sequential Bonferroni tests (Rice 1989).

A similar approach was followed to analyze the five variables measured in the flower visitor censuses. We performed GLIMs with population, year, and insect order or species (whenever appropriate) as fixed factors, and plant individual as a random factor. We used 1) a binomial distribution and logit link function for insect pollinator composition (0: dipterans, 1: hymenopterans), 2) a gamma distribution and logit link function for FIV and for FVR (including flower crop as a covariate), and 3) a negative binomial and logit link for the number of flowers contacted per insect visit. The duration of insect visits was analyzed by means of ANOVAs (GLM module of STATISTICA 6.0, Anon. 2001) followed by Tukey's tests, since log-transformed data satisfied the normality and homoscedasticity assumptions. Two tests were performed to compare the behavior of insect flower visitors among populations: one compared long versus short flights (expressed as binomial variable: 0- long flights, 1- short flights) while the other compared the next plant species visited (0- conspecific and 1- other plant species), considering only the data of short flights. For both analyses, we used a binomial distribution with logit link function (GLIM, GENMOD in SAS ver. 6.12, Anon. 1997). In addition, we used contingency tables and Fisher exact tests to compare insect orders within populations, since there were sampling zeros (STATISTICA 6.0, Anon. 2001).

Variation in flowering synchrony among populations was tested by means of one-way ANOVAs, since the assumptions of normality and homoscedasticity were met. In addition, we performed correlations between flowering synchrony, frequency of insect visits to plants, plant size and fecundity, and an anemogamy index, based on Pearson's tests. Such anemogamy index was calculated as  $A/C$ , where  $C$  and  $A$  represented seed set for the controls and the anemogamy treatment, respectively (modified from Tamura and Kudo 2000). We used seed set rather than fruit set as the first measure was found to be more affected by pollination mode.

## Results

### Pollination mode and breeding system

In 2002, the production of mature fruits was extremely low (1.6–3.3% in all populations, except in Rágol (19.7%)). Analyses comparing populations were thus done only with initiated fruits (and data from Cerro Gordo had to be excluded because fruit initiation was almost nil for all treatments). A higher proportion of fruits was initiated in Rágol and Sant Vicenç (46.8%) than in Lluç and Galatzó (7.6%) (Table 1). Fruit initiation differed among treatments (Table 1), being lower for selfing (4.5%) than either anemogamy (7.5%) or control (9.9%). There was a significant interaction between population and treatment (Table 1), as populations initiating more fruits were more likely to show differences among treatments. In Rágol, differences among treatments were also found in final fruit set (Table 1): selfing (14.4%) produced a significantly lower proportion of fruits than control (26.1%), although it was not statistically different from anemogamy (18.4%). Seed set varied consistently among treatments (Table 1), but in this case all three treatments were different from each other (36.9% for controls; 35.4% for anemogamy; and 13.3% for spontaneous selfing).

In 2003, fruit set (both initiated and mature fruits relative to flowers) also differed among populations (Table 1), being lowest in Cerro Gordo, highest in Lluç and intermediate in the other three populations (Fig. 2). Selfing produced a lower fruit set than anemogamy or control for both initiated and mature fruits, except at Sant Vicenç where treatments appeared to be similar (Table 1; Fig. 2). In Galatzó, fruit set was also found to

be significantly higher for the anemogamy than for the controls. Seed set, in contrast, differed only marginally among populations and did not vary significantly among treatments (Table 1); the effect of treatment again differed among populations: while all treatments resulted in similar seed sets at Sant Vicenç, selfing in the other populations produced a lower seed set than either anemogamy or controls, and anemogamy at Rágol produced a lower seed set than controls (Fig. 2).

In a separate analysis addressing temporal variation, all initiated fruits, final fruit set and seed set were similar between years in Rágol (the only population from which two years of data were available, Table 1). All treatments initiated significantly the same percentage of fruits (46.9%), but selfing produced a marginally significant lower final fruit set than the other treatments (14.4% for selfing, 36.3% for controls and 31.2% for anemogamy) and the interaction between year and treatment was not significant for both initiated and mature fruits (Table 1). The effect of treatment did not vary when we pooled data from two years (results not shown). Seed set varied significantly among treatments: selfing produced the lowest seed set (19.4%), control the highest (51.3%), and anemogamy intermediate (41.8%) and such differences were consistent between years (Table 1).

### Effect of pollination mode on fruit and seed mass

In 2002, data from Rágol showed a non-significant effect of treatment on fruit mass ( $\chi^2=1.92$ ,  $p=0.4$ ) after controlling for number of seeds per fruit, which was positively correlated with fruit mass ( $\chi^2=5.49$ ,  $p=0.019$ ). In 2003, fruits from Rágol, Cerro Gordo

Table 1. Results of the Generalized Linear Models analyzing the effect of pollination treatments, populations and years on initiated fruits, fruit set, and seed set. Significant p-values are marked in bold.

2002	df	Initiated fruits		Fruit set in Rágol		Seed set in Rágol	
		$\chi^2$	p	$\chi^2$	p	$\chi^2$	p
Population	3	14.81	<b>0.002</b>				
Treatment	2	16.18	<b>0.0003</b>	6.67	<b>0.036</b>	6.55	<b>0.038</b>
Population $\times$ Treatment	6	15.62	<b>0.016</b>				
2003	df	Initiated fruits		Fruit set		Seed set	
		$\chi^2$	p	$\chi^2$	p	$\chi^2$	p
Population	4	18.63	<b>0.0009</b>	22.10	<b>0.0002</b>	8.97	0.06
Treatment	2	16.95	<b>0.0002</b>	20.74	<b>&lt;0.0001</b>	4.61	0.1
Population $\times$ Treatment	8	23.72	<b>0.003</b>	20.19	<b>0.01</b>	18.10	<b>0.021</b>
Rágol 2002 and 2003	df	Initiated fruits		Fruit set		Seed set	
		$\chi^2$	p	$\chi^2$	p	$\chi^2$	p
Year	1	0.42	0.5	0.03	0.9	1.52	0.2
Treatment	2	4.64	0.1	5.37	0.068	8.71	<b>0.013</b>
Year $\times$ Treatment	2	4.46	0.1	3.26	0.2	0.12	0.9

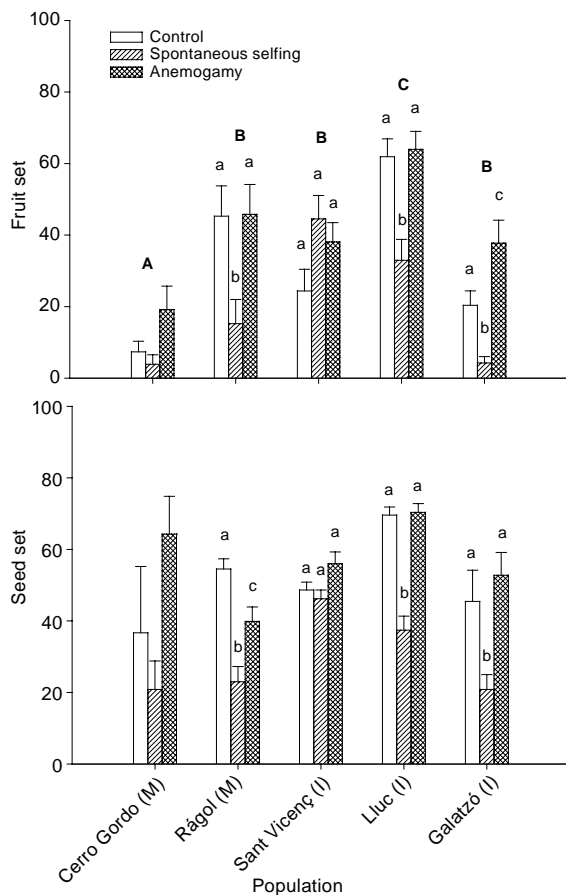


Fig. 2. Fruit (fruit/flower) and seed set (seeds/ovules) for the three experimental treatments. Data are from 2003. Anemogamy (but not control) included flower emasculation. Different letters indicate significant differences. Upper case: contrast among populations (M: mainland and I: island). Lower case: contrasts among treatments (within populations). Only contrasts among populations with  $N > 6$  are included.

and Sant Vicenç were heavier ( $0.14 \pm 0.05$  on average, no differences among them) than the rest ( $0.09 \pm 0.02$  g on average;  $\chi^2_4 = 14.87$ ,  $p = 0.005$ ). Fruits from selfed flowers tended to be lighter than fruits from wind-pollinated ones, although the effect of treatment was only marginally significant ( $\chi^2_2 = 4.92$ ,  $p = 0.085$ ). No interaction between treatment and population was found ( $\chi^2_8 = 11.94$ ,  $p = 0.15$ ). As in 2002, fruit mass increased with increasing number of seeds per fruit ( $\chi^2 = 18.13$ ,  $p < 0.0001$ ). Seed mass did not differ among treatments either in 2002 ( $\chi^2_2 = 1.99$ ,  $p = 0.37$ ) or 2003 (treatment:  $\chi^2_2 = 4.05$ ,  $p = 0.13$ ; interaction between population and treatment:  $\chi^2_6 = 7.57$ ,  $p = 0.27$ ). In 2003, seeds from Rágol were heavier than the rest ( $7.5 \pm 1.6$  vs  $5.7 \pm 1.1$  mg on average;  $\chi^2_3 = 13.76$ ,  $p = 0.003$ ). Variation in seed mass within a fruit was lower for anemogamy than for the other two treatments ( $\chi^2_2 = 7.40$ ,  $p = 0.025$ ; Fig. 3); there were no differences among populations ( $\chi^2_3 = 5.20$ ,  $p = 0.16$ ) and no interaction between population

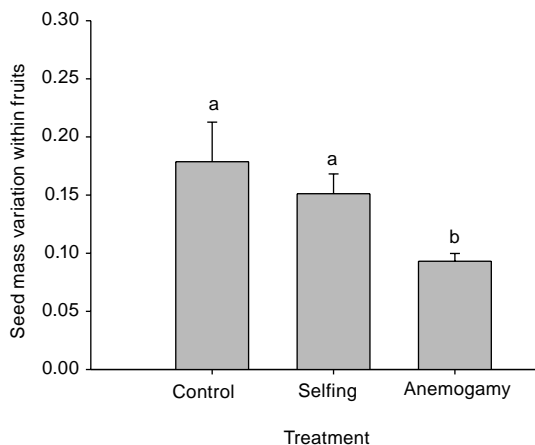


Fig. 3. Variation of seed mass within fruits (SD/mean) for each experimental treatment. Treatments with the same letter did not differ significantly.

and treatment was found ( $\chi^2_6 = 8.29$ ,  $p = 0.22$ ). The number of seeds per fruit had no effect on seed mass either ( $\chi^2 = 1.01$ ,  $p = 0.31$ ).

### Assemblage of flower insect visitors

At least 23 insect taxa, including unidentified ants, small beetles and thrips, visited the flowers of *B. balearica*, most of them being observed the year of greatest flower production (Table 2). A greater insect diversity was found in mainland (19 and 15 taxa at Rágol and Cerro Gordo, respectively) than in island (0, 11, and 4 taxa, at Sant Vicenç, Lluc, and Galatzó, respectively). The relative importance of the two main orders of pollinators (hymenopterans and dipterans) varied significantly between populations in 2002 ( $\chi^2 = 23.27$ ,  $p < 0.0001$ ), when dipterans were more frequent in Galatzó than in Rágol (Table 2), and in 2003 ( $\chi^2_3 = 154.74$ ,  $p < 0.0001$ ), when Cerro Gordo was dominated by hymenopterans, Lluc by dipterans, and both Rágol and Galatzó showed intermediate proportions of both orders (Table 2). In a separate analysis that compared data from Rágol and Galatzó for the two years, we found significant differences between populations ( $\chi^2 = 39.82$ ,  $p < 0.0001$ ), and years ( $\chi^2 = 10.65$ ,  $p = 0.001$ ). Dipterans became more important in 2003, especially in Rágol ( $\chi^2 = 14.96$ ,  $p = 0.0001$ ; Table 2).

### Frequency of insect visits to plants (FIV) and flower visitation rates (FVR)

FIV differed among populations, both in 2002 and 2003 ( $F_{1,59} = 16.12$ ,  $p = 0.0002$ ; and  $F_{3,101} = 6.48$ ,  $p = 0.0005$ , respectively). Rágol consistently showed the highest FIV (Fig. 4A). Flower crop (associated with plant size in all

Table 2. Insect species that visited the flowers of *B. balearica* in the mainland (M) and island (I) populations during the study period. V/H: visits per hour. %: percentage of visits (relative to the total number of visits) attributable to a given taxa (species or order) for each population and year. The missing percentage required to reach 100% consists of undetermined and/or sporadic visitors, not included in the table. N.a. =not available data.

	Cerro Gordo (M)		Rágol (M)				Lluc (I)		Galatzó (I)			
	2003		2002		2003		2003		2002		2003	
	V/H	%	V/H	%	V/H	%	V/H	%	V/H	%	V/H	%
Hymenoptera	9.0	84.5	18.3	85.2	24.3	65.0	1.3	18.6	1.1	60.4	0.3	50.0
<i>Apis mellifera</i>	8.6	81.1	18.1	83.4	20.9	55.6	1	11.0	1	52.1	0.1	20.0
<i>Andrena</i> sp.	0.1	0.8			1.8	4.7						
<i>Ceratina cucurbitina</i>	0.1	0.8			0.3	0.7	0.1	1.4				
<i>Polistes omisus</i>	0.2	1.6	0.2	0.7	1.2	3.2	0.1	1.4	0.1	4.2	0.2	30.0
<i>Polistes gallicus</i>					0.1	0.4	0.1	0.7				
Diptera	<b>1.3</b>	<b>11.4</b>	<b>2.9</b>	<b>13.8</b>	<b>13.1</b>	<b>34.8</b>	<b>6.7</b>	<b>80.0</b>	<b>0.7</b>	<b>39.6</b>	<b>0.3</b>	<b>50.0</b>
<i>Bibio</i> sp.							0.7	8.2				
<i>Bombylius medius</i>			0.1	0.6	0.5	1.4						
<i>Calliphora vicina</i>	0.1	0.8			6.3	16.6	1.8	19.9				
<i>Ephesirphus balteatus</i>	0.3	2.5	0.3	1.5	5.1	13.5	1.5	17.1	0.4	22.9	0.2	30.0
<i>Helina evecta</i>	0.6	5.7	0.1	0.4	0.1	0.4	1.9	21.9	0.2	12.5		
<i>Sarcophaga</i> sp.	0.3	2.4	2.4	11.3	1.1	2.8	0.8	9.6	0.1	4.2	0.1	10.0
Lepidoptera	<b>0.4</b>	<b>4.1</b>	<b>0.2</b>	<b>1.0</b>	<b>0.1</b>	<b>0.2</b>	<b>0.0</b>	<b>0.0</b>	<b>0.0</b>	<b>0.0</b>	<b>0.0</b>	<b>0.0</b>
<i>Cynthia cardui</i>			0.2	0.9	0.1	0.2						
Lepidoptera indet.	0.4	4.1										
<i>Brachygalea albolineata</i>			0.04	0.1								
Coleoptera							0.1	1.4				
<i>Oxythreia funesta</i>	N.a.	N.a.	N.a.	N.a.	N.a.	N.a.	0.1	1.4				

populations; all  $p < 0.005$ ) had no significant effect on FIV in 2002 ( $F_{1,59} = 1.38$ ,  $p = 0.25$ ); in 2003, however, there was a significant interaction between population and flower crop ( $F_{1,101} = 6.47$ ,  $p = 0.0001$ ), as FIV increased with flower crop only in Rágol ( $r = 0.72$ ,  $p = 0.002$ ,  $N = 15$ ; for all other populations,  $p > 0.05$ ).

FVR also varied among populations, both in 2002 and 2003 ( $F_{1,62} = 10.79$ ,  $p = 0.002$ ; and  $F_{3,115} = 3.86$ ,  $p = 0.011$ , respectively). Rágol consistently showed the highest values whilst Galatzó presented the lowest (Fig. 4B). Significant population  $\times$  flower crop interactions were found both years ( $F_{2,62} = 3.18$ ,  $p = 0.048$ , and  $F_{4,115} = 4.79$ ,  $p = 0.001$ ), as FVR increased with flower crop only in Rágol ( $r = 0.27$ ,  $p < 0.0001$ ,  $N = 63$  and  $r = 0.52$ ,  $p < 0.0001$ ,  $N = 60$ , for 2002 and 2003 respectively; for all other populations,  $p > 0.05$ ).

A separate analyses showed no differences in FIV and FVR between years and populations ( $\chi^2 = 5.61$ ,  $p = 0.018$  and  $\chi^2 = 3.76$ ,  $p = 0.053$ , for FIV and FVR, respectively), and also a significant population  $\times$  year interaction ( $\chi^2 = 3.97$ ,  $p = 0.046$  and  $\chi^2 = 6.29$ ,  $p = 0.012$ , for FIV and FVR, respectively): in Galatzó, both variables were higher in 2002 than in 2003, whereas the opposite occurred in Rágol (Fig. 4).

#### Differences among insect taxa in visit duration and number of contacted flowers

Data from 2002 showed that hymenopterans remained longer than dipterans on plants ( $F_{1,489} = 26.66$ ,  $p < 0.0001$ ; Table 3), and visited a greater number of

flowers ( $\chi^2 = 51.52$ ,  $p < 0.0001$ ; Table 3). Both variables also differed between populations ( $F_{1,489} = 11.39$ ,  $p = 0.0008$ ; and  $\chi^2 = 18.99$ ,  $p < 0.0001$ ): insects in Rágol spent more time on the plant and visited more flowers than those in Galatzó. The interaction between insect order and population was not significant for duration of insect visits ( $F_{1,489} = 0.72$ ,  $p = 0.4$ ; Table 3), but it was for the number of contacted flowers ( $\chi^2 = 10.89$ ,  $p = 0.001$ ): differences between orders were significant in Rágol, but not in Galatzó (Table 3). In a separate analysis that only included pollinator species shared by the two populations (*Apis mellifera*, *Polistes omisus*, *Sarcophaga* sp., *Helina evecta* and *Ephesirphus balteatus*), differences in duration of insect visits between populations became non-significant ( $F_{1,469} = 0.045$ ,  $p = 0.83$ ), indicating that differences between populations were due to the different proportion of insect orders in each. However, differences among insect species remained significant ( $F_{4,469} = 5.97$ ,  $p = 0.0001$ ). Of these five pollinator species, *A. mellifera* made the longest visits per plant ( $48.7 \pm 43.9$  s) compared to *P. omisus* ( $12.7 \pm 4.8$  s), *Sarcophaga* sp. ( $7.1 \pm 0.8$  s), *H. evecta* ( $8.4 \pm 2.7$  s) and *E. balteatus* ( $22.7 \pm 15.1$  s). A significant interaction between population and insect species was found ( $F_{4,469} = 2.63$ ,  $p = 0.034$ ), since all species except flies spent more time on the flowers at Rágol than at Galatzó. Honeybees also visited significantly more flowers than the other species ( $9.1 \pm 7.8$  vs  $2.0 \pm 0.7$ ,  $1.8 \pm 0.3$ ,  $1.1 \pm 0.2$ , and  $1.6 \pm 0.8$ , for *P. omisus*, *Sarcophaga* sp., *H. evecta* and *E. balteatus* respectively;  $\chi^2_4 = 38.87$ ,  $p < 0.0001$ ). There were no differences

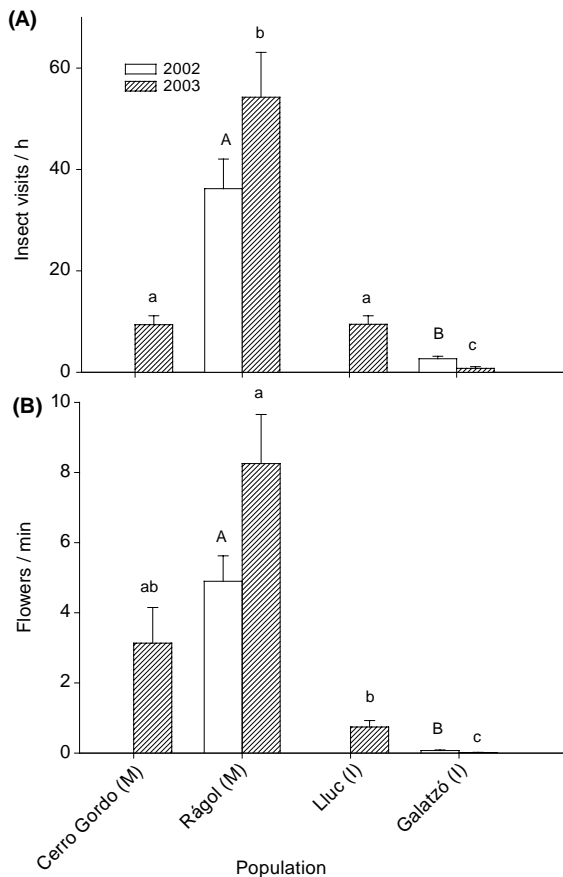


Fig. 4. Frequency of insect visits to plants and flower visitation rates for each mainland (M) and island (I) population and year. Bars with the same letter do not differ within a year (upper case: within 2002; lower case: within 2003). For 2002, only data from Rágol and Galatzó were available.

between populations ( $\chi^2=0.70$ ,  $p=0.4$ ) and the interaction between population and insect species was only marginally significant ( $\chi^2_4=9.26$ ,  $p=0.055$ ).

Table 3. Mean ( $\pm$ SD) of the number of contacted flowers per visit to a plant (F) and of the total visitation time in seconds (T) for the different orders of insects found visiting *B. balearica* flowers in the mainland (M) and island (I) populations. Empty cells mean that the insect order was not observed in the population and year. N.a. means that occasional observations were made during the study period but quantitative data are not available. Orders representing <5% of visits were not included in the analyses. Numbers in parenthesis represent samples sizes (N). Within each row, different letters indicate significant differences between Hymenoptera and Diptera.

			Hymenoptera	Diptera	Lepidoptera	Coleoptera
Cerro Gordo (M)	2003	F	(84) 26.5 $\pm$ 58.8 a	(11) 1.8 $\pm$ 1.5 b	(6) 1.7 $\pm$ 0.8	
		T	(94) 138.4 $\pm$ 232.8 a	(13) 90.6 $\pm$ 151.1 b	(5) 144.8 $\pm$ 144.7	
Rágol (M)	2002	F	(360) 14.4 $\pm$ 17.4 a	(76) 2.0 $\pm$ 2.3 b	(3) 5.3 $\pm$ 4.9	N.a.
		T	(369) 78.7 $\pm$ 118.0 a	(76) 31.2 $\pm$ 39.2 b	(6) 146.0 $\pm$ 225.8	N.a.
	2003	F	(341) 20.5 $\pm$ 31.0 a	(263) 3.3 $\pm$ 4.1 b	(2) 1.0 $\pm$ 0.0	N.a.
		T	(335) 79.3 $\pm$ 114.3 a	(255) 27.2 $\pm$ 38.7 b	(1) 22.31	N.a.
Lluc (I)	2003	F	(26) 12.6 $\pm$ 18.6 a	(117) 2.2 $\pm$ 2.6 b		(2) 1.0 $\pm$ 0.0
		T	(26) 54.9 $\pm$ 71.5 a	(117) 34.1 $\pm$ 56.6 b		(2) 27.0 $\pm$ 33.5
Galatzó (I)	2002	F	(28) 3.5 $\pm$ 4.6 a	(20) 1.6 $\pm$ 1.1 a		
		T	(28) 17.2 $\pm$ 18.1 a	(20) 8.1 $\pm$ 6.4 b		
	2003	F	(5) 1.4 $\pm$ 0.9 a	(5) 1.0 $\pm$ 0.0 a		
		T	(5) 5.0 $\pm$ 1.8 a	(5) 10.7 $\pm$ 16.5 b		

Data from 2003 were consistent with those from 2002. Hymenoptera remained longer in the plants and contacted more flowers per visit than dipterans ( $F_{1,832}=8.07$ ,  $p=0.005$  and  $\chi^2=36.36$ ,  $p<0.0001$ , respectively). Visitation time was longer and more flowers were contacted per visit in mainland than island populations ( $F_{3,832}=8.01$ ,  $p<0.0001$  and  $\chi^2_3=26.66$ ,  $p<0.0001$ ; Table 3). The interaction between insect order and population was not significant for visitation time ( $F_{3,832}=0.75$ ,  $p=0.52$ ) but it was for the number of contacted flowers ( $\chi^2_3=7.24$ ,  $p<0.0001$ ) (Table 3), as hymenoptera and dipterans contacted the same number of flowers in Galatzó. Considering only the three shared species (honeybees: *A. mellifera*, wasps: *P. omisus* and flies: *E. balteatus*), we still found differences among the four populations, Galatzó being that where insect spent the shortest time and contacted the least number of flowers ( $F_{3,520}=2.77$ ,  $p=0.041$  and  $\chi^2_3=10.36$ ,  $p=0.016$ , respectively), and among insect species ( $F_{2,520}=7.80$ ,  $p=0.0005$  and  $\chi^2_2=41.29$ ,  $p<0.0001$ ). Honeybees remained longer in the plant and visited more flowers ( $77.1 \pm 47.8$  s and  $18.4 \pm 10.9$  flowers) than wasps ( $44.1 \pm 47.7$  s and  $4.7 \pm 2.5$  flowers) and flies ( $17.8 \pm 3.4$  s and  $2.2 \pm 2.1$  flowers), and there was no interaction between population and insect species for either variable ( $F_{6,520}=0.84$ ,  $p=0.54$  and  $\chi^2_6=3.28$ ,  $p=0.77$ , respectively). A separate analysis using two-years data from Rágol and Galatzó showed that insect visit duration and number of contacted flowers were not related to annual variation in flower crop ( $F_{1,1083}=1.44$ ,  $p=0.23$  and  $\chi^2=0.30$ ;  $p=0.59$ , respectively).

### Insect movement among flowering individuals

In 2003, we recorded the movements of individual insects after visiting a flowering individual of



*B. balearica* in three populations. Visitors in Galatzó could not be followed because they always flew long distances (and were thus lost) after visiting a flowering individual. In the other populations, there were significant differences in the proportion of insects that flew long (>5 m) and short distances (<5 m) after visiting an individual ( $\chi^2_2=65.16$ ,  $p<0.0001$ ): long flights in Cerro Gordo (2.63%) were less frequent than in the other two populations (72.44%). When we only considered those insects that flew short distances, we also found differences among populations ( $\chi^2_2=11.43$ ,  $p=0.003$ ) in insect movements between plants: a higher percentage of flower visitors moved to flowering conspecifics in Cerro Gordo (18% of the total visits) than in Lluc and Rágol ( $\leq 2\%$ ). Those moving to other species were 12% in Cerro Gordo, 5% in Lluc and <1% in Rágol. No differences among insect orders were found in their movements between plants in the two mainland populations (Cerro Gordo,  $p=0.67$ ; and Rágol,  $p=0.83$ ). However, in Lluc, dipterans went more frequently to other species than to conspecifics ( $p=0.048$ ).

### Flowering phenology and synchrony

The flowering period lasted between two and four weeks within each population, but since mainland populations flowered about a month earlier than island ones, and the flowering period decreased with increasing elevation in Mallorca, our observation period lasted for two and a half months (Fig. 5). Flowering synchrony varied significantly among populations ( $F_{4,44}=681.57$ ,  $p<0.0001$ ): it was highest in Galatzó, lowest in Rágol, and intermediate in the other three populations (Fig. 5).

Pooling data from all populations, we found that flowering synchrony and FIV were negative correlated ( $r=-0.62$ ,  $p=0.002$ ,  $N=23$ ; Fig. 6), i.e. frequency of insect visits increased with asynchrony. On the other hand, the index of anemogamy (A/C) was neither correlated to flowering synchrony nor to visitation rate ( $r=-0.004$ ,  $p=0.98$ ,  $N=31$  and  $r=0.008$ ,  $p=0.97$ ,  $N=20$  respectively). The low number of observations precluded an analysis within populations.

Flower crop varied among populations and years (data not presented here), but no relationship between

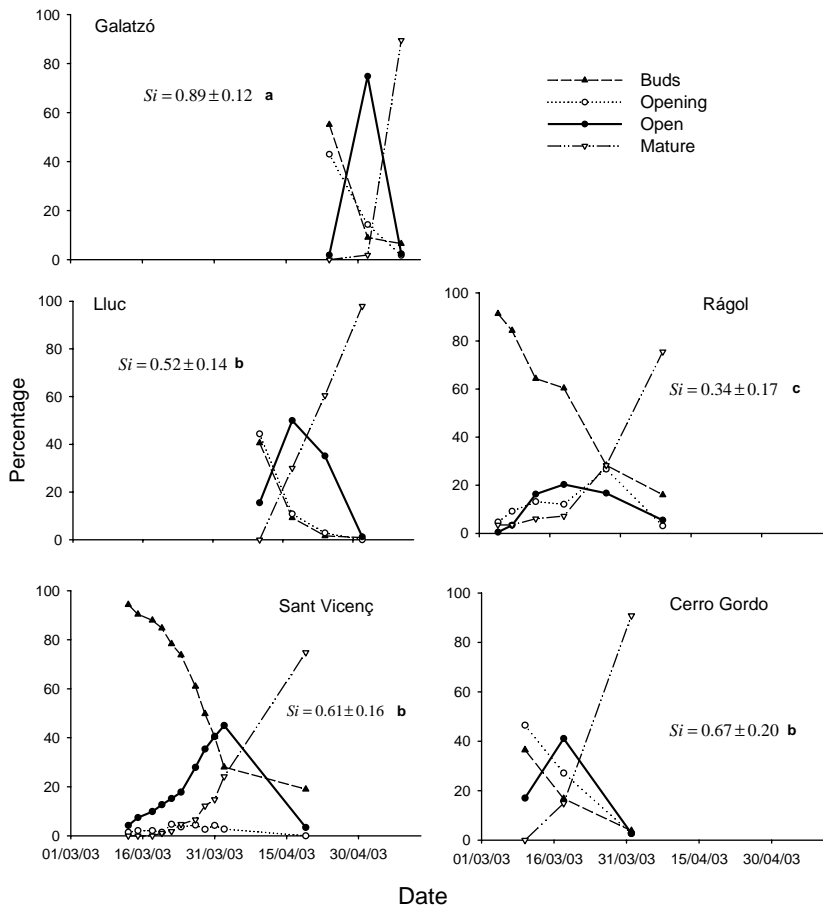


Fig. 5. Flowering phenology and synchrony indexes for the study populations. Different lines represent the states of the inflorescences. Values of Flowering Synchrony Index with the same letter are not significantly different.

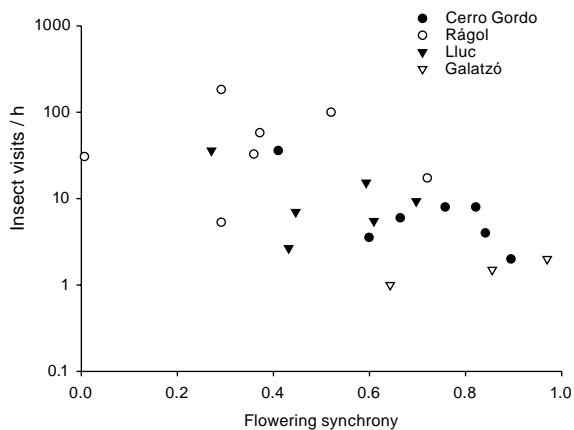


Fig. 6. Relationship between flowering synchrony and frequency of insect visits in *B. balearica*. Each data point represents a single individual shrub (all populations pooled).

individual flowering synchrony index and number of flower per individual was detected, either when all populations were pooled or when each population was considered separately (all  $p \gg 0.05$ ).

## Discussion

### Spatio-temporal variation in pollination mode

In angiosperms, wind pollination has probably evolved in response to environmental conditions that make biotic pollination less advantageous (Whitehead 1983, Cox 1991). Such conditions can be diverse, but since wind pollination is a passive process that depends on wind intensity, humidity, rainfall and temperature (Whitehead 1983, Culley et al. 2002), it is probably favored in open and arid habitats (Stebbins 1970, Whitehead 1983, Vroeghe and Stelleman 1990). Other community traits like high density of compatible mates, low filtration of aerial pollen by dense plant canopies, deciduousness, and infrequent or unreliable pollinators can also make anemophily more effective than entomophily (Regal 1982, Whitehead 1983). Moreover, island floras have been reported as being frequently wind-pollinated (Regal 1982, Whitehead 1983, Anderson et al. 2001).

Our results show that wind alone may significantly contribute to fruit/seed set in *B. balearica*, and suggest that insects might play only a minor role as pollinator vectors in most populations, at least for the two years examined during the study. Wind-pollinated flowers showed similar or even higher levels of fruit/seed set compared to controls in all populations except Ràgol. The importance of anemogamy did not differ between mainland and island regions and exhibited very limited variation at a more local scale (i.e. among populations). Such high reliance of *B. balearica* on anemophilous pollination in most populations might probably be

related to the unreliability of insect pollination service. In fact, most flower visitors were generalist pollinators, and composition of the pollinator assemblage, frequency of visits and flower visitation rates varied on both spatial and temporal scales, as found in other studies (Horvitz and Schemske 1990, Parker 1997, Herrera et al. 2001, Sánchez-Lafuente 2002, among others). Insect pollination service was even less reliable in island sites, as the diversity of flower visitors, flower visitation rates, time spent on the plants and number of flowers contacted per visit were consistently higher in the mainland. Moreover, the three main pollinators in island populations were flies (*Helina evecta*, *Calliphora vicina* and *Ephesirphus balteatus*) which contacted few flowers per visit and moved afterwards to other species more frequently than to conspecifics; in contrast, the main pollinator in the mainland was *Apis mellifera*, which contacted much more flowers in each visit. A decrease in animal pollination in island compared to mainland populations has also been reported in other studies (see Linhart and Feinsinger 1980, Feinsinger et al. 1982, Barrett 1996, Inoue et al. 1996, Anderson et al. 2001). Insect species diversity and flower visitation rates tend to decrease with elevation for other species, either wind or insect-pollinated (Berry and Calvo 1989, Gómez and Zamora 1996, Bingham and Orthner 1998, Blionis and Vokou 2001, Totland and Sottocornola 2001), which is usually attributed to the harsher (especially windy and with lower temperatures) conditions at high elevation. However, this pattern was not found in *B. balearica*, as conditions are not necessarily harsher with increasing elevation in Mallorca island.

### Ambophily as successful reproductive assurance

The mixture of both wind and insect pollination is more common than previously thought (see review in Culley et al. 2002), although it is still controversial whether it represents a stable system or it is a transient stage towards either absolute anemophily or absolute entomophily (Stebbins 1970, Stelleman 1984, Cox 1991, Culley et al. 2002). Indeed, Karrenberg et al. (2002) claimed that ambophily is the ancestral stage for some *Salix* species. Whenever the relative importance of both pollinator vectors varies spatially and/or temporally, such a mixed system may be maintained without any strong selection for one or another pollination mode. For a large number of species, wind pollination may well compensate for a low frequency of insect visits, caused for instance by an early flowering period in a particular population, or by a low insect abundance in a given year (Berry and Calvo 1989, Vroeghe and Stelleman 1990, Gómez and Zamora 1996, Mahy et al. 1998, Goodwillie 1999, Totland and Sottocornola 2001). On the other hand, insects might also compensate for variability in

wind and/or weather (e.g. rainfall, humidity; Tamura and Kudo 2000). In most cases, the contribution of both pollination modes to reproductive assurance and success and its influence on the maintenance of ambophily are still unclear.

Except in the case of the Sant Vicenç population, where the absence of insect flower visitors is probably related to the strong winds usually blowing at that north-oriented site, the flower visitation rates we found fall within the ranges reported in other studies on entomophilous species, either in the mainland (Herrera 1989) or in island (Traveset and Sáez 1997). Insects appeared to play a relevant role as pollinators in Ràgol, where they increased seed set by 15% and where frequency of insect visits and flower visitation rates were much higher than in all other populations. Insect pollination might thus be playing an assurance role for seed production in this population, as has been reported for *Salix miyabeana* and *Salix sachalinensis* (Tamura and Kudo 2000). In the other populations, although anemogamy showed to notably contribute to seed set – compared to controls – we cannot discard insects as a relevant effective pollination mode. Future works with ambophilous species should include the study of interactions between pollination vectors (related for instance to time of pollen arrival or pollen quality), when assessing their reproductive success, instead of just adding up the successes of each pollination mode when considered in isolation.

### Levels of spontaneous selfing

For some species, facultative self-pollination may serve to assure a successful reproduction and may be selected for (Stebbins 1970, Berry and Calvo 1989). Such species can maintain levels of genetic variability that can be as high as those found in related obligate outcrossers (Stebbins 1970). Wind-pollinated plants appear to have a bimodal distribution of selfing rates, while animal-pollinated plants do not (Aide 1986, Vogler and Kalisz 2001), but there is a lack of studies recognizing the combination of both pollination modes within taxa (i.e. on ambophilous species) to clarify that relationship (Culley et al. 2002). In *B. balearica*, large differences in levels of spontaneous selfing (4–45% for fruit set, and 20–46% for seed set) were found among populations. The most “selfed” population was Sant Vicenç, the site in which winds are strongest and where insect flower visitors were nearly absent; the reason why seed production in the selfing treatment was higher than in the other populations may be related to a higher pollen dispersal within bags, although we cannot exclude the possibility that it is the result of a large history of inbreeding in that population.

Selfing is expected to be greater in islands than in continental areas due to lower pollinator availability

and/or to an ancient scarcity of mates (Linhart and Feinsinger 1980, Barrett 1996, Inoue et al. 1996, Anderson et al. 2000, Pérez-Bañón et al. 2003, Schueller 2004). Although actual selfing rates in the natural populations cannot be determined with our data, our results suggest that spontaneous selfing levels in *B. balearica* are not higher in island populations, and instead that they vary at a more local scale. Deposition of self-pollen both by wind and insects is likely to play a significant role in determining the number of seeds per fruit in *B. balearica*, as it has been suggested for other species (Mahy et al. 1998, for *Calluna vulgaris*). For some self-compatible species, it has been suggested that insects may lead to higher outcrossing rates than wind, especially when individual plants are highly dispersed (Regal 1982, and references therein; Norman et al. 1997). Nevertheless, insects may sometimes promote high levels of geitonogamy, in which case wind may be a more effective pollinator vector (Gómez and Zamora 1996). In the case of *B. balearica*, *Apis mellifera* is the most frequent visitor in mainland populations and probably promotes the highest levels of geitonogamy as it contacts more flowers per visit than the rest of insects. On the other hand, morphological features associated with wind pollination have the potential to increase rates of self-pollination (Culley et al. 2002), and in tall shrubs like *B. balearica*, with hundreds to thousands of simultaneously open flowers, the probability of geitonogamy appears to be rather high.

In order to understand the evolution and maintenance of ambophily the effectiveness of each pollination vector should be considered both from the quantitative (quantity of pollen grains transferred, and the qualitative (origin of these pollen grains) points of view. The use of molecular techniques to determine if wind and insect pollinated seeds come from self- or cross-pollination would certainly contribute to such understanding.

### Pollination mode: effects on fruit/seed mass and on flowering synchrony

Wind and insect-transported pollen appeared to be similarly efficient regarding the mass of fruits and seeds produced. Selfed fruits tended to be lighter than the other treatments, possibly due to inbreeding depression. Data on inbreeding and outbreeding depression in this species are presented elsewhere (Lázaro and Traveset unpubl.). We expected a greater within-fruit variation in seed mass in the anemogamy treatment, as pollen deposited on a flower is more likely to arrive from different “fathers”. This has been found, for instance, with experimental hand-pollinations in *Rhamnus alpina* (Bañuelos and Obeso 2003). However, wind-pollinated seeds of *B. balearica* were more homogeneous in size than controls or even selfed seeds, perhaps because wind-

pollinated flowers receive less self-pollen, and the effect of inbreeding depression promoted by geitonogamy varies much among seeds within fruits.

Flowering period of *B. balearica* was found to be shorter in the island than in the mainland and to decrease with altitude among island populations. Flowering is expected to be more synchronous for anemophilous than for biotically pollinated species (Rabinowitz et al. 1981, Whitehead 1983, Rathcke and Lacey 1985, Bolmgren et al. 2003). An extended duration of flowering may be advantageous for spreading the risk of uncertain biotic pollination (Rathcke and Lacey 1985, Proctor et al. 1996), although asynchrony is also seen as the result of a relaxation of natural selection (Ollerton and Lack 1992). In *B. balearica*, flowering synchrony was highest in Galatzo and lowest in Rágol and was not related to the index of anemogamy, although more asynchronous populations received higher frequencies of insect visits. This negative association had previously been reported for the crucifer *Hormathophylla spinosa* (Gómez 1993). More research on this topic is certainly needed.

In short, our study shows that although *Buxus balearica* is frequently visited by putative pollinators, such visits do not result in an increased reproductive success (as compared to wind-pollination). We only found indications of reproductive assurance by entomophily in one mainland population, which indeed had higher diversity and efficiency of insect visitors than all other populations. In contrast to expectations, the levels of selfing did not differ between island and mainland populations, but they were highest in the population without entomophilous pollination.

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