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Contrasting inbreeding depression in early and late stages of the life cycle of a Mediterranean shrub, *Anagyris foetida* (Leguminosae)

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Abstract: *Anagyris foetida* L. has a Mediterranean distribution, but its populations are highly fragmented. This, together with its mixed mating system, puts it at clear risk of being strongly affected by inbreeding depression. To determine the effect of inbreeding depression in this species, hand pollinations were performed during the 2003/04 season in 2 populations from SW Spain to generate selfed and outcrossed seeds. These seeds were divided in 2 sample lots and seedlings were obtained in different years. During the first year of life the first lot showed no differences in vegetative parameters between the 2 populations, so that no differentiation was made between populations in analyzing the results. Reproductive parameters were determined during the first 2 flowering periods in the second sample lot. The results showed inbreeding depression to be manifest in the populations studied throughout the life cycle, adversely affecting the reproductive parameters more than the vegetative parameters, with the male capacity being strongly affected. This shows the need to study the entire life cycle to understand how it is affected by inbreeding depression, and to analyze more than one flowering period in long-lived species. The findings may have important implications for the long-term conservation management of these populations.

Key words: Fabaceae, fitness, flower production, reproductive capacity, seedling survival

1. Introduction

Inbreeding depression (ID) is defined as the fitness reduction of offspring after self-fertilization or fertilization between similar or closely related genotypes compared to outcrossed offspring. This phenomenon is a key factor in the evolution of plant breeding systems (Lloyd, 1980; Charlesworth and Charlesworth, 1987; Byers and Waller, 1999; Barret, 2002; Goodwillie et al., 2005). Fitness reduction by ID is a consequence of increased homozygosity, and is explained by 2 nonexclusive hypotheses: the partial dominance hypothesis and the overdominance hypothesis (Charlesworth and Willis, 2009). In the first hypothesis the loss of fitness is caused by an increase in recessive deleterious alleles and in the second hypothesis is caused by a reduction in the number of loci exhibiting heterozygote advantage. Most studies that have focused on ID suggest that it is mainly a consequence of partially recessive deleterious alleles (Charlesworth and Charlesworth, 1987; Willis, 1999; Carr and Dudash, 2003; Fox et al., 2008; Charlesworth and Willis, 2009).

The magnitude of ID is related to the mating system of the species involved, and can evolve with selfing

rate (Husband and Schemske, 1996). By definition, the effects of ID should be greater in preferentially selfing species or populations relative to outcrossed ones, but it has been demonstrated that ID caused by recessive deleterious alleles can be purged by natural selection in selfing populations (Lande and Schemske, 1985; Byers and Waller, 1999). This prompted some authors to consider only 2 extreme breeding systems to be evolutionarily stable: selfing with low ID rates (values less than 0.5) and outcrossing with high ID rates (values greater than 0.5). However, a significant proportion of plants have mixed mating systems (Goodwillie et al., 2005 and references therein; Goodwillie and Knight, 2006), and for them purging of recessive deleterious alleles is an impediment to their stability because it creates a positive feedback system in which increased inbreeding leads to a reduction in its disadvantages (Lande and Schemske, 1985).

ID can be manifested at different stages of the life cycle, and negatively affects various components of a plant's fitness such as seed formation (Agren and Schemske, 1993; Severns, 2003) and size (Hull-Sanders et al., 2005; Vaughton and Ramsey, 2006), germination (Sheridan and

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Karowe, 2000; Ishida, 2006; González-Varo and Traveset, 2010), seedling size and growth (Naito et al., 2005; Ishida, 2006), and reproductive capacity (Carr and Dudash, 1997; Ramsey et al., 2003; Ellmer and Andersson, 2004; Busch, 2005). In consequence, the numerous studies on the intensity of ID in early stages of development (Waser and Price, 1993; Trame et al., 1995; Hardner et al., 1998; Melsner et al., 1999) necessarily underestimate the total intensity of ID by not covering the entire life cycle. Indeed, in cross-pollinated species strong ID can be expressed both early and late in their life cycle, whereas in selfing species the magnitude of ID increases along the life cycle, with the greatest values in the late stages (Husband and Schemske, 1996).

ID is also influenced by environmental factors, because gene expression changes with environmental conditions (Armbruster and Reed, 2005; Fox and Reed, 2010). The magnitude of ID is positively correlated with the stressfulness of the environment, but this correlation is only manifest when the stress is severe (Fox and Reed, 2010). The effect of low intensities of stress on ID is small, and indeed may be completely masked in experiments with small sample sizes because of the typically large variance in ID among related individuals in a given population (Armbruster and Reed, 2005). Some studies have found a greater intensity of ID in stressful or natural environments compared to benign or controlled environments (Keller and Waller, 2002; Armbruster and Reed, 2005). However, in their meta-analysis, Angeloni et al. (2011) found no significant differences between field studies and greenhouse or common garden studies, even though the values of ID were generally reported to be greater in the field studies.

Finally, the phenomenon of ID needs especially to be taken into account in the conservation of species which are reduced to small or fragmented populations (Byers and Waller, 1999; Hedrick and Kalinowski, 2000; Keller and Waller, 2002; Işık, 2011). In such populations, the limited availability of cross-pollen may lead to increased ID as a result of breeding between related individuals (Ellstrand and Elam, 1993; Richards et al., 1999; Severns, 2003). On the other hand, ID should be lower in populations with a long history of inbreeding, where the selection may have purged the genome of its genetic load, whereas high levels of ID may arise after a sudden reduction in population size (Lande and Schemske, 1985; Keller and Waller, 2002; Angeloni et al., 2011).

The present work focuses on *Anagyris foetida* L., a self-compatible leguminous Mediterranean shrub with a mixed mating system (Valtueña et al., 2008a) and whose pollinators are 3 species of passerine birds (Ortega-Olivencia et al., 2005). Although with a wide distribution range, the species is constituted by numerous fragmented

populations. Our main objective was to determine the existence of ID over the life cycle of this species (from seed germination to fruit formation in mature individuals), and to quantify its intensity at each stage of that cycle, in 2 populations located in SW Spain.

2. Materials and methods

2.1. Study system

Anagyris foetida is a leguminous shrub, sometimes reaching arboreal habit, with trifoliate and alternate leaves, deciduous in summer, and with autumn-winter flowering. The flowers are papilionaceous, pendulous, odorless, yellowish, and borne directly on the trunks (cauliflory) (Ortega-Olivencia et al., 2005). They produce large amounts of very dilute, hexose-rich nectar (Valtueña et al., 2007), and are mainly pollinated by 3 species of passeriforms (Ortega-Olivencia et al., 2005). The fruit is a large pod (45–190 × 12.5–24 mm) with 1–4(8) seeds of large size (9–16 × 6.5–9.5 mm) (Valtueña et al., 2010). Seeds present physical dormancy and constitute important seed banks (Valtueña et al., 2008b) showing a great viability in time (Özgen et al., 2012). Physical dormancy is due to the presence of a hard seed coat very frequent in the family (López et al., 1999 and references therein; Büyükkartal et al., 2013). It is a self-compatible species, with a mixed mating system, showing no clear advantage of cross- over self-pollination in fruit-set, seed-set, and seed weight, and where autonomous self-pollination is avoided by the presence of a stigmatic cuticle, being the rupture of this cuticle necessary for pollen germination (Valtueña et al., 2008a). Its breeding success is very low due to the resource limitation it is subjected to (Valtueña et al., 2012) and because the initiation of fruit is limited by the availability of pollen (Valtueña et al., 2010).

Seeds used in the present work were obtained from 2 natural populations separated by 5 km, both in livestock range farms, in Extremadura (SW Spain). Population 1 (La Silveriña) is located on a small hill at 195–220 m a.s.l. and consists of ca. 90 individuals, most with an arboreal habit reaching almost 4 m in height. Population 2 (La Sancha) comprises more than 500 individuals of shrubby habit. Seedlings were monitored in these 2 natural populations and at the Experimental Botany Garden in the campus of the University of Extremadura (Badajoz, Spain) located about 20 km from the original populations. The sites of both populations and the Garden have a Mediterranean climate characterized by rainy, cool winters and dry, hot summers. For more details about the populations, see Ortega-Olivencia et al. (2005) and Valtueña et al. (2007, 2008a).

2.2. Experimental pollinations

To obtain the seeds used in the experiments, 10 individuals per population were selected during the 2003/04 season. All

the individuals were mature and similar in size. Hereafter, mature individual means it produces flowers. For each individual, a minimum of 50 hand self-pollinations and 25 hand cross pollinations were made at the same time in different branches following the procedure described in Valtueña et al. (2008a). Before cross-pollinations, flowers at preanthesis stage were emasculated to avoid the deposition of self-pollen in the stigma because it is located at the same level as the anthers when the flowers are in anthesis. In both kinds of pollinations flowers were bagged to avoid visits of pollinators. A total of 1063 hand self-pollinations (500 in population 1 and 563 in population 2) and 612 hand cross-pollinations (251 in population 1 and 361 in population 2) were performed, using as pollen donors at least 2 individuals 5 m away from the receptor individual. In this experiment, we quantified both the seed-set as the mean number of seeds per fruit and the mean seed weight after weighing every individual seed with a precision scale (± 1 mg).

Despite the effort put into these pollinations, very few individuals developed more than 10 seeds per treatment (4 individuals in population 1, and 3 in population 2) or did not produce seeds after 1 or both treatments. This made it impossible to study the variation among maternal plants and so discriminate the maternal effect on the analyzed parameters. This obliged us to consider not the individuals but the total of seeds obtained per treatment in our analyses. The seeds obtained were divided into the following 2 sample lots (neither treatment used more than 25 seeds from any given individual): (a) first lot (hereafter Sa1, seeds put to germinate the same year when they were collected) consisting of 199 selfed seeds (119 from population 1 and 80 from population 2) and 166 crossed seeds (105 from population 1 and 61 from population 2); and (b) second lot (Sa2, seeds put to germinate 1 year later) consisting of 147 selfed seeds and 147 crossed seeds. In this second sample lot, no distinction was made between populations due to the lack of significant differences between both populations for all the parameters previously studied in Sa1.

2.3. Vegetative traits

After mechanical scarification with a sterile sharp hunting knife blade, seeds were germinated in petri dishes (7–10 seeds/dish) according to Valtueña et al. (2008b), using a germinator (Percival I36LLVL) simulating the photoperiod, temperature, and relative humidity conditions existing in the natural populations during September (date of seed germination in wild populations; day 12.5 h, 29.4 °C, 45% relative humidity and night 11.5 h, 16.7 °C, 75% relative humidity) (Valtueña et al., 2008b). Daily for a month we counted the number of seeds germinated considered as such those whose radicle protruded more than 3 mm. For Sa1, the germination experiment was initiated on October

2004, whereas, for Sa2, it was delayed to January 2006 because of the high mortality observed in the seedlings from the first sample of seeds during the winter (mainly by excess of humidity or freezing in the field).

Each germinated seed was sown in a labeled pot (6-cm diameter, 6.5-cm depth) containing a mixture of peat moss, sand, and vermiculite in a 3:1:1 ratio. The pots were placed in plastic trays and put into the germinator under the same conditions as in the germination experiment for a period of 30 days counted from planting the first germinated seed. They were monitored daily, watering as needed, and the date of emergence of each seedling was recorded. Subsequently, the seedlings were subjected for 2 weeks to a period of acclimation to natural conditions. During this period the pots with the seedlings were placed in an experimental garden, under environmental conditions similar to the natural populations, and the survival, height, and number of leaves were recorded weekly.

After the period of acclimation, the seedlings were transplanted to their final locations for the rest of the study (Sa1 close to natural populations in December 2004 and Sa2 in the experimental garden in February 2006). Transplanting was in rows, alternating outcrossed and selfed individuals, with a separation between plants of 60 cm.

In both sample lots (Sa1 and Sa2) survival and vegetative parameters were recorded weekly for the first 2 weeks, fortnightly for the next 45 days, and finally monthly. In Sa1, these parameters were recorded until natural leaf-fall in early June 2005. This experiment was expected to continue during the following autumn, but all individuals were lost due to human intervention in the area. In Sa2, during the summer of 2006, the plants were watered twice a week to favor their survival, with the result that in this period the normal leaf-fall did not take place. Survival and vegetative parameters were recorded as indicated previously but after August 2006 they were recorded bi-monthly in view of the small variation between monthly data. The vegetative parameters studied (height, stem diameter, number of leaves, and leaf volume) were those that in a previous test were correlated with the biomass (taken to be the dry weight) and then they could be used to calculate it. After the summer of 2007, only survival and height were monitored. Biomass was only estimated in the seedlings during the first year of life to avoid pulling up established individuals from the natural populations.

2.4. Reproductive capacity

The reproductive capacity study was carried out only with Sa2 that reached maturity (flowered) during 2007–08 and 2008–09. In both flowering seasons, we recorded the percentage of flowering plants, and on each plant the number of inflorescences, flowers/inflorescence, and total flowers produced.

In all individuals that flowered, at the beginning of the flowering period we collected 2 flower buds close to anthesis and stored them in 70% ethanol at 4 °C. In the laboratory, the number of ovules per ovary was recorded, with this value being taken as the potential female reproductive capacity. We also estimated the total number of pollen grains and aborted pollen grains per flower by the dilution method (Cruden, 1977) and after staining them with lactophenol blue. Aborted pollen grains were distinguished by being collapsed, with abnormal shape or weakly stained. Moreover, for 2 flowers of each individual, we quantified the volume and concentration of nectar on the first day of anthesis as described in Ortega-Olivencia et al. (2005), and later the dry weights of the calyx and corolla were measured separately on a precision balance (± 0.1 mg) after oven-drying for 48 h at 80 °C.

At the end of the flowering season, we counted the number of mature fruits per plant following open-pollination, which allowed us to calculate their fruit-set. For all fruits produced in 2007–08, we quantified the number and weight of the seeds as described in Valtueña et al. (2008a). In the 2008–09 season, some individuals had a very large production of fruits, so that we determined the number and weight of seeds in a maximum of 20 randomly chosen fruits per individual. It was made in 42 crossed and 21 selfed individuals.

2.5. Relative fitness, cumulative relative fitness, and value of the ID

The relative fitness (F_s) for each parameter is the ratio between the fitnesses of the selfed (w_s) and the outcrossed (w_o) progeny: $F_s = w_s/w_o$ (Husband and Schemske, 1996). The cumulative relative fitness (F_p) takes into account the entire study period and all the parameters considered, being defined as the product of each parameter's relative fitness.

The ID (δ) for each parameter studied is defined as 1 minus the relative fitness ($\delta = 1 - F_s$) and for overall ($\delta = 1 - F_p$). Positive values indicate the existence of ID, values close to 0 its absence, and negative values the existence of outbreeding depression.

ID could affect vegetative ($ID_{\text{vegetative}}$) and/or reproductive parameters ($ID_{\text{reproductive}}$) and, in this case, both male and female parameters. In $ID_{\text{reproductive}}$, the effect on female reproductive capacity could be estimated in terms either of the number of ovules per ovary or of the fruiting parameters (fruit-set, seeds per fruit, and mean seed weight). In the former case one would be estimating the potential ID effect ($ID_{\text{rep_potential}}$), and in the latter the real reproductive ID effect ($ID_{\text{rep_real}}$). The ID effect on male reproductive capacity was measured taking into account the total amount of pollen produced by the flower and the percentage that was viable.

Floral parameters with an unknown real impact on the species' reproductive fitness were not used in the calculations. These parameters were the volume of nectar produced, the amount of sugars in the nectar, and the weight of the corolla and of the calyx.

2.6. Statistical analyses

Statistical analyses were performed using SPSS 15.0.1. The normality of the variables was determined using the nonparametric Kolmogorov–Smirnov test with Lilliefors correction, and homoscedasticity with the Levene test. Fruit-set and seed-set were analyzed using generalized linear models (GZLM), fitting to a negative binomial distribution with a logarithmic link function. The logarithms of the flower and fruit numbers were used as the respective offset variables. Applicability of an analysis of variance for the seed weight was ensured by the small deviation of the residuals from normality.

For each sample lot, comparison of success against failure of germination, emergence, and seedling survival was performed using the Pearson chi-squared test by means of 2-factor $R \times C$ contingency tables with the populations subordinate to the treatments. In the second sample lot (Sa2), the percentage of flowering plants was analyzed in the same way.

In the first sample lot (Sa1), means of biomass and height were compared using 2-way ANOVAs (considering treatment and population as main effects), whereas, in the second sample lot, means of height and all the flowering and reproductive parameters (except the percentage of flowering plants) were compared using 1-way ANOVA (considering treatment as principal factor). In both ANOVAs, the Brown–Forsythe correction was applied when variables were heteroscedastic. Mean of biomass in the second sample lot was compared between treatments using the nonparametric Mann–Whitney test because the data could not be normalized.

3. Results

3.1. Experimental pollinations

After the hand-pollination experiments in the 2 populations, none of the parameters analyzed (fruit- and seed-set, and seed weight) were significantly influenced by the population, by the treatment, or by its interaction (Table 1).

3.2. Vegetative traits

In Sa1, since none of the parameters were significantly influenced by the population (Table 2), the data of each treatment were pooled. Considering the populations together, there were no significant differences between treatments for any of the parameters (Table 2). In contrast, in Sa2, seed germination and seedling survival at the end of the first year (December 2006) were significantly higher in the outcrossed lineage (Table 2).

Table 1. Analyzed parameters (mean ± SD) in *Anagyris foetida* after hand-self-pollination (HSP) and hand-cross-pollination (HCP) and statistical results after applying generalized linear models (GZLM) or ANOVA. The number of individuals that produce at least one fruit in each treatment is indicated in the last column.

	Treatment	Fruit-set (%)	Seed-set	Seed weight	Individuals
Population 1	HCP	4.0 ± 5.6	2.6 ± 1.2	421 ± 32	5
	HSP	4.8 ± 7.1	3.3 ± 0.9	448 ± 33	7
Population 2	HCP	4.1 ± 3.2	3.0 ± 1.4	461 ± 41	8
	HSP	1.5 ± 2.2	2.4 ± 1.0	468 ± 55	5
	g.l.	Fruit-set (%)	Seed-set	Seed weight	
Population	1	Wald = 0.997 ^{ns}	Wald = 0.863 ^{ns}	F = 3.201 ^{ns}	
Treatment	1	Wald = 0.028 ^{ns}	Wald = 0.537 ^{ns}	F = 1.019 ^{ns}	
Population × Treatment	1	Wald = 2.620 ^{ns}	Wald = 1.384 ^{ns}	F = 0.322 ^{ns}	

Table 2. Seed germination, seedling emergence, and plant survival in both sample lots Sa1 and Sa2 of *Anagyris foetida*.

	Selfed (Sf)			Crossed (Cr)			Statistic Sf vs. Cr
	Pop1	Pop2	Statistic (Sf) Pop1 vs. Pop2	Pop1	Pop2	Statistic (Cr) Pop1 vs. Pop2	
Sa1							
N	119	90		105	61		
Germination (%)	93.3	96.3	$\chi^2 = 0.81^{ns}$	87.6	95.1	$\chi^2 = 2.47^{ns}$	$\chi^2 = 2.23^{ns}$
Emergence (%)	82.0	90.9	$\chi^2 = 2.95^{ns}$	91.3	91.4	$\chi^2 = 0.00^{ns}$	$\chi^2 = 2.59^{ns}$
Survival (%)	24.2	21.4	$\chi^2 = 0.27^{ns}$	26.2	24.5	$\chi^2 = 0.00^{ns}$	$\chi^2 = 0.22^{ns}$
Sa2	Selfed (Sf)			Crossed (Cr)			Statistic Sf vs. Cr
N	147			147			
Germination (%)	78.9			95.9			$\chi^2 = 19.32^{**}$
Emergence (%)	99.1			100.0			$\chi^2 = 1.45^{ns}$
Survival (%)	88.7			97.9			$\chi^2 = 9.10^*$

Survival refers to the final sampling campaign before the summer period of 2005 in Sa1, and to December 2006 in Sa2. Emergence percentage with respect to the total of germinated seeds is indicated, as also is the survival percentage with respect to the total of seedlings that emerged. Statistical results from comparing the 2 studied populations for each treatment are indicated in Sa1, whereas, in Sa2 results are given for the comparison between treatments. N, initial number of seeds used in each treatment and population. Pop1, population 1. Pop2, population 2. ns, not significant. *, P < 0.01. **, P < 0.001.

In both sample lots, the analysis of seedling height and biomass showed no differences between treatments at the end of the first year of the study (Table 3).

For Sa2, a major proportion of seedlings survived the first year (24% in Sa1 vs. 93% in Sa2), with some reaching maturity. In late May 2009 (the last date of sampling), the relative survival of plants with respect to the emerged seedlings was significantly greater in the outcrossed

lineage than in selfed (97.2% vs. 87.8%, Figure 1A) and the outcrossed plants were significantly taller (Figure 1B).

3.3. Reproductive fitness

In the first flowering season (2007–08), of the total of 238 live plants, 29 flowered (12 of 101 selfed plants and 17 of 137 outcrossed plants), while in the second flowering season (2008–09) 193 flowered (73 selfed plants and 120 outcrossed plants). Overall flowering plant percentage

Table 3. Height and biomass (mean ± SD) in both sample lots Sa1 and Sa2 of *Anagyris foetida* at the end of the first year of life of the plants. Statistical results are shown.

Sa1	Selfed		Crossed		Statistic		
	Pop1	Pop2	Pop1	Pop2	Treat	Pop	Treat*Pop
N	22	15	22	13			
Height (cm)	19.6 ± 3.5	18.9 ± 3.4	20.0 ± 3.2	20.5 ± 4.1	F = 1.33 ^{ns}	F = 0.03 ^{ns}	F = 0.53 ^{ns}
Biomass (mg)	775 ± 101	754 ± 109	788 ± 98	781 ± 120	F = 0.61 ^{ns}	F = 0.30 ^{ns}	F = 0.08 ^{ns}
Sa2	Selfed		Crossed		Statistic		
N	102		138				
Height (cm)	71.5 ± 14.7		75.1 ± 15.5		F = 1.05 ^{ns}		
Biomass (mg)	90.4 ± 72.8		115.3 ± 96.0		U = 6049 ^{ns}		

N, number of recorded individuals. Pop1, population 1. Pop2, population 2. Treat, treatment. ns, not significant. Statistic: Sa1, 2-way ANOVA ($F_{1,69}$, height and biomass); Sa2, 1-way ANOVA ($F_{1,238}$, height) and Mann-Whitney (U, biomass)

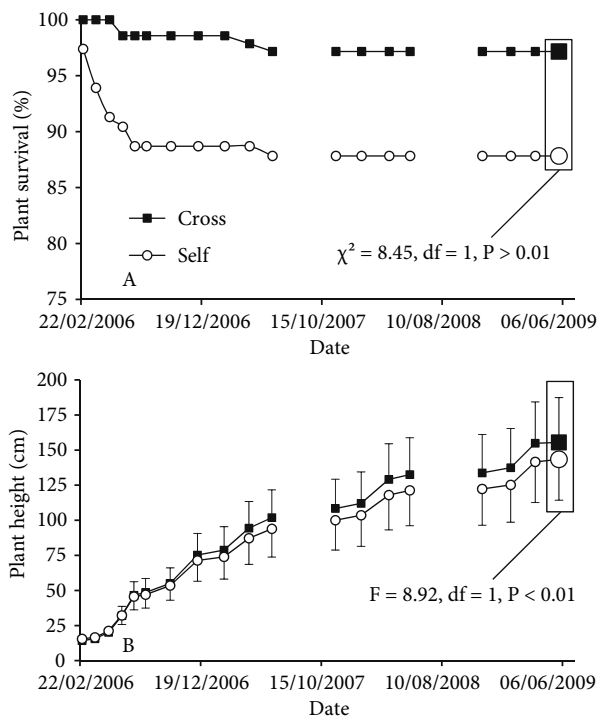


Figure 1. Plant survival with respect to the total emerged seedlings (A) and height (B) of selfed and outcrossed plants of sample lot Sa2 of *Anagyris foetida* from the time of transplantation to the experimental garden until the end of May 2009 (breaks in the curves correspond to the periods during which the plants are leafless). In B the vertical bars represent the SD. Statistical values in the last sampling date (rectangle) are indicated.

was significantly higher in 2008–09 than in 2007–08 (81% vs. 12%; $\chi^2 = 227.04, df = 1, P < 0.001$). In both seasons, the only parameters significantly different between both kinds of plants were the number of pollen grains per flower (greater in outcrossed plants) and the percentage of aborted pollen (greater in selfed plants) (Table 4). The rest of studied parameters did not show significant differences between treatments in the first flowering season. However, the percentage of flowering plants, number of flowers per plant, number of inflorescences per plant, nectar sugar content, and dry weight of the corolla were significantly higher in outcrossed plants in the second flowering season (Table 4).

In the first season of flowering, fruit-set was very small (only 1 outcrossed and 2 selfed individuals produced fruits). It was thus impossible to analyze any of the fruiting parameters, although the selfed plants presented slightly higher values (Table 4). In the second flowering season, 97 outcrossed plants (81% of the flowering plants) and 58 selfed plants (77% of the flowering plants) produced fruit, and all the fruiting parameters presented significant differences between both kind of plants, with fruit-set and number of seeds per fruit being greater in the selfed plants and seed weight greater in the outcrossed plants (Table 4).

3.4. Inbreeding depression

None of the parameters studied during the first year of the life cycle of *A. foetida* (seed germination, seedling emergence, seedling survival, seedling height, and seedling biomass) were significantly influenced by the treatment

Table 4. Flowering percentage and reproductive parameters in the sample lot Sa2 of *Anagyris foetida* in the flowering periods 2007–08 and 2008–09 (mean ± SD).

Parameters	2007–08		2008–09		
	Selfed	Crossed	Selfed	Crossed	
<i>Flowering</i>					
N	12	17	75	120	
FP (%)	11.88	12.41	74.3	87.6	$\chi^2 = 6.98^{**}$
Fl/Pl	33.8 ± 42.3	15.0 ± 17.4	310.9 ± 380.5	467.6 ± 505.9	$F_{1,193} = 5.32^*$
Infl/Pl	5.75 ± 6.41	2.94 ± 2.41	52.6 ± 67.5	78.8 ± 86.7	$F_{1,193} = 4.97^*$
Fl/Infl	5.07 ± 3.99	4.96 ± 2.89	6.26 ± 2.46	6.14 ± 1.95	
NV (µL)	10.42 ± 5.77	10.83 ± 4.39	5.91 ± 4.99	6.96 ± 5.38	
NSA (mg)	0.90 ± 0.35	0.92 ± 0.24	0.46 ± 0.36	0.58 ± 0.40	$F_{1,193} = 3.91^*$
Calyx weight	11.24 ± 1.79	11.96 ± 1.39	10.28 ± 1.89	10.08 ± 1.70	
Corolla weight	17.38 ± 3.54	18.16 ± 1.25	17.49 ± 2.71	18.22 ± 1.76	$F_{1,193} = 5.12^*$
PG (×10 ⁴)	11.54 ± 4.74	15.47 ± 3.61	21.20 ± 7.18	23.79 ± 5.45	$F_{1,27} = 6.46^*$ $F_{1,193} = 7.88^{**}$
APG (%)	8.70 ± 4.35	2.52 ± 1.95	13.70 ± 11.36	5.75 ± 4.91	$F_{1,27} = 21.16^{***}$ $F_{1,193} = 43.23^{***}$
Ovules/ovary	8.71 ± 1.21	8.76 ± 0.94	8.77 ± 0.75	8.85 ± 0.82	
<i>Fruiting</i>					
N	12/2	17/1	75/58	120/97	
Fruit-set	1.75 ± 3.01	0.96 ± 2.35	7.53 ± 9.48	5.13 ± 5.32	$F_{1,153} = 5.00^*$
Seeds/fruit	3.70 ± 1.63	3.20 ± 1.64	2.69 ± 0.60	2.33 ± 0.74	$F_{1,153} = 6.67^*$
SW (mg)	421 ± 51	403 ± 21	314 ± 43	364 ± 44	$F_{1,153} = 46.96^{***}$

APG, aborted pollen grains. Fl/Infl, number of flowers per inflorescence. Fl/Pl, number of flowers per plant. FP, flowering plants. Infl/Pl, number of inflorescences per plant. N, number of recorded individuals, in *Fruiting* section the first number corresponds to the recorded individuals used to calculate fruit-set, whereas the second number is used to calculate seeds per fruit and seed weight. NSA, nectar sugar amount. NV, nectar volume. PG, total pollen grains. SW, seed weight. Statistical information is only shown when there are significant differences between treatments. *, P < 0.05. **, P < 0.01. ***, P < 0.001.

(selfed vs. outcrossed) in either lot (Sa1 or Sa2). These parameters were not strongly affected by ID in the early stages of the life cycle of this species (Figure 2). All the parameters studied had values of ID greater than 0, i.e. the selfed seedlings had lower values than the outcrossed ones, except germination in Sa1 (Figure 2). In Sa1, the total ID was very low ($\delta = 0.13$); in contrast, in Sa2 it was moderate ($\delta = 0.45$) (Figure 2).

In the 2007–08 season, the outcrossed plants only had values clearly greater than the selfed ones in male fitness (pollen grains per flower and percentage of viable pollen). For this season, regardless of the estimate of female fitness, the ID at the reproductive level was absent in the first year of flowering (Figure 3); it needs to be interpreted with caution due to the small proportion of

plants that flowered and then produced mature fruit. In the 2008–09 season, ID_{rep_potential} was moderate and ID_{rep_real} was low in value (0.54 and 0.33, respectively; Figure 3). If the 2 flowering seasons are considered together, the values of ID are the same as those obtained in the second flowering season.

Overall the effect of ID could only be quantified in Sa2, as this was the only sample lot that reached maturity (flowering). At the end of the third season of the study (May 2009), its ID at the vegetative level was 0.32. Considering both vegetative and reproductive parameters, the ID was 0.69 considering the ID_{rep_potential} and 0.54 considering the ID_{rep_real}. These results show the species to have medium/high values of ID, affecting both its vegetative and reproductive characters.

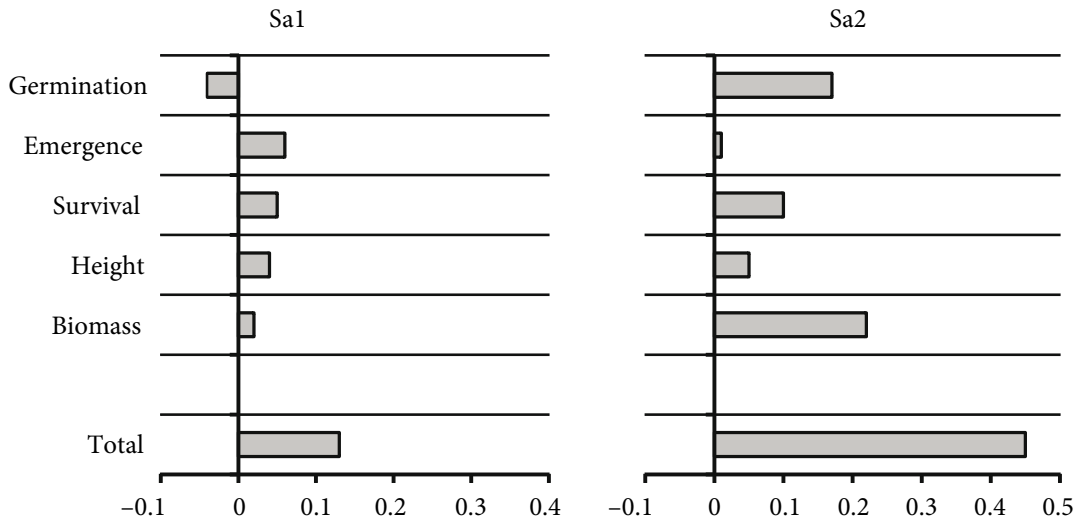


Figure 2. Inbreeding depression coefficients (δ) for different vegetative parameters and total in the first year of life of both sample lots Sa1 and Sa2.

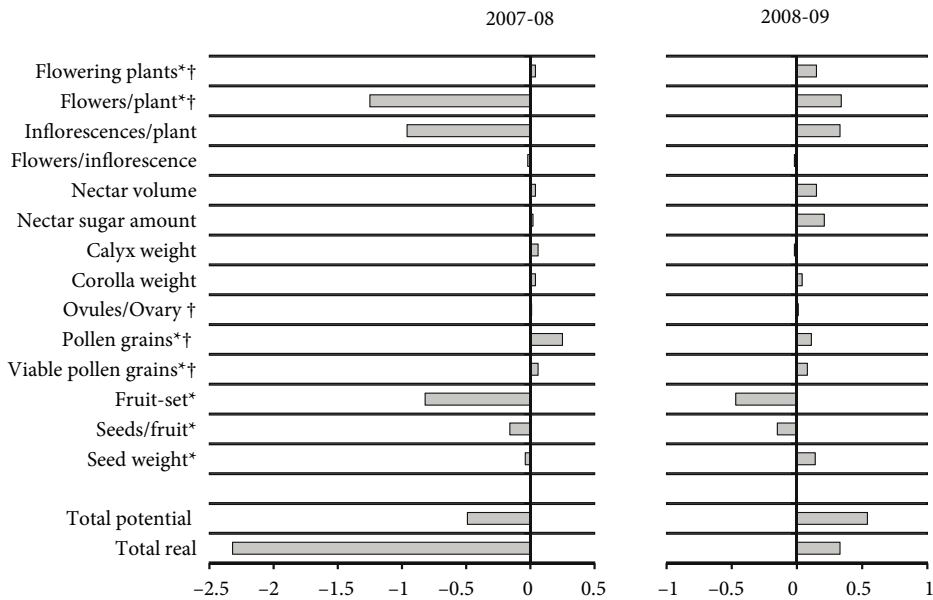


Figure 3. Inbreeding depression coefficients (δ) for reproductive parameters, $ID_{rep_potential}$ (Total potential) and ID_{rep_real} (Total real) in 2007/08 and 2008/09 flowering seasons of the sample lot Sa2. Parameters used to calculate $ID_{rep_potential}$ are marked with * and to calculate ID_{rep_real} with †.

4. Discussion

Our results show the existence of moderate ID in the 2 populations studied of *Anagyris foetida*, a Mediterranean woody plant, as shown by both vegetative and reproductive parameters. The values of ID during the first year of life were not very high (0.13 to 0.45), consistent with those of species that reproduce predominantly by selfing. This multiyear study shows that the intensity of ID may be underestimated in studies that only consider the early

stages of the life cycle. The severest values of ID at the reproductive level coincide with reports in other works that they occur in later stages of the life cycle of long-lived species. Furthermore, this ID was expressed mainly in the male rather than the female function. All these aspects are discussed below.

4.1. Experimental pollinations

After hand self- and cross-pollinations, the fruit-set was highly variable among individuals. Rather than to the

genotype of the individuals, this variability could be due to the strong resource constraints that *Anagyris foetida* adults are subjected to (Valtueña et al., 2012). Indeed, only a very small proportion of flowers (less than 5%: Valtueña et al., 2008a) produce fruit, while the proportion initiating fruit is much higher (greater than 60%: Valtueña et al., 2010). For this reason, we take the germination of the seed to be the starting point for the study of ID. In particular, for its calculation in the early stages of the life cycle we do not take into account the values of fruit-set, seed-set, or seed weight obtained after the hand pollinations. Indeed, although the absence of maternal lineages to study the magnitude of ID in the present study is an important handicap, our results could represent the real situation in the populations better than a study by using the same initial number of seeds from each individual and treatment; this is due to the fact that in natural conditions not all the individuals produce similar numbers of fruits and seeds, and probably they have different selfing rates.

4.2. Effect of ID in the first stages of the life cycle of *Anagyris foetida*

All the parameters studied during the first year of life in both sample lots, except for seed germination in Sa1, presented greater values in the outcrossed plants, although the differences were only significant in seed germination and seedling survival in Sa2. The greater values in the outcrossed plants caused a mild ID. This could be because in populations of long-lived plants there is no purging via selection of the genetic load (recessive deleterious mutations) resulting from a high rate of selfing (see revision in Husband and Schemske, 1996; Ishida, 2006). The significant reduction in the germination percentage of selfed seeds in Sa2 relative to Sa1 (both groups of seeds came from the 2003–04 crop) and the absence of any such reduction in the outcrossed seeds indicates that the effect of ID on the germination of selfed seeds in woody species could be enhanced by increasing seed age. This species forms seed banks in the soil (Valtueña et al., 2008b) and so it is to be expected that those that are selfed would be less likely to germinate with increasing age in the soil. Moreover, germination may be influenced by differences in the individual mother plants, which may be decisive in the performance of their progeny (Shi et al., 2005).

During the first year of life, there were no differences between the survival of selfed and outcrossed plants in either Sa1 and Sa2, the value of ID for this parameter being very low in both these sample lots ($\delta = 0.06$ and 0.08 , respectively). The absence of any difference between selfed and crossed plants could be interpreted as reflecting a lack of ID at the level of this parameter. The ID of plants grown under controlled conditions is generally considered to be lower than that under natural conditions (Dudash, 1990) because the stress in natural conditions is presumably

greater than that in greenhouses or experimental gardens. However, in their meta-analysis Angeloni et al. (2011) found no significant differences in the magnitude of ID between field studies and greenhouse or common garden studies. Our results support the findings of these authors, although in our case they could reflect the similarity in the main environmental factors (substrate, humidity, light, and temperature) between the experimental garden and the natural populations studied.

4.3. Effect of ID in the mature stage of the life cycle of *Anagyris foetida*

At the end of the last year of the study (2008–09) in Sa2—the only sample lot to reach maturity—the value of the ID considering only vegetative parameters was 0.32, slightly lower than that obtained at the end of the first year of life (0.45). However, this difference did not really reflect an improvement in the vegetative parameters of the selfed plants after the first year, but instead was due to it being impossible to determine the plants' biomass after that year, and this parameter was therefore not included in the calculation of the ID. Indeed, when this parameter is also excluded from the calculation of the ID at the end of the first year of the study, the value becomes 0.30, almost the same as that at the end of the third year. This shows that, at the vegetative level, ID is expressed in the early stages of the life cycle (germination, and seedling emergence and establishment), which are critical to the plant's survival, as evidenced by the null or low survival to the first year of life of both kinds of plants under natural conditions.

In the first flowering season (2007–08), several flowering and fruiting parameters had higher values in selfed plants than in outcrossed plants. This could imply the existence of outbreeding depression, but in the second flowering season almost all of the results were reversed, with higher values in the outcrossed plants. Because of this, and together with the low percentage of flowering plants in the first flowering season, the results obtained in this season should be treated with caution. Additionally, this highlights the need to study more than one flowering season in long-lived species.

The parameter that showed the greatest variation between selfed and outcrossed plants in the 2 seasons of flowering was the production of flowers per individual. Indeed, this was the main parameter contributing to the negative values of ID during the first season and positive values in the second. Previous studies found that flower production was one of the reproductive traits that show higher values of ID, although these studies were developed on herbaceous plants, either annual (*Mimulus guttatus* DC.) (Willis, 1999) or perennial (*Silene virginica* L.) (Dudash and Fenster, 2001).

The floral parameters studied that were not directly included in the fitness did not differ significantly between

the selfed and outcrossed plants, so that the effect of ID in them was null or nearly null. In general, parameters not involved in fitness seem to be less affected by ID (DeRose and Roff, 1999). Thiele et al. (2010) proposed the lower effect of ID in floral traits than in fitness traits could be because selection acts more strongly on flowers than on fitness or because selection acting on floral traits may be stabilizing, in contrast to selection on fitness traits that likely is directional. Indeed, previous studies showed ID has hardly any effect on flower size or on the floral pieces such as sepals or petals (Ellmer and Andersson, 2004; Thiele et al., 2010).

It has been shown that selfing is followed by a decline in male reproductive capacity, affecting both the amount of pollen produced (Carr and Dudash, 1997; Goodwillie and Knight, 2006) and its viability (Willis, 1993; Carr and Dudash, 1997; Ellmer and Andersson, 2004), and this was confirmed in the present study. This negative effect has important implications for the maintenance of populations and for the species' colonization capacity. Indeed, this favored outcrossing over selfing, especially in individuals originating from several generations of selfing or populations with a high rate of selfing. In our 2 populations, we had observed previously a possible effect of ID on pollen viability, with very large interindividual variations, and even individuals whose male flowers could be considered sterile (Valtueña et al., 2008a).

With respect to female reproductive capacity, the number of ovules per ovary was similar between the selfed and outcrossed plants. This could reflect strong selective pressure on this parameter in order to maximize the reproductive capacity of the flowers in terms of ovary size. This is supported by the low variation in the number of ovules per ovary between individuals and populations (Valtueña et al., 2008a).

The values of fruit-set and seed number per fruit were higher in the selfed plants in the 2 years overall. In the second year, a possible explanation is the resource limitation that the species is subject to, so that, having produced fewer inflorescences and flowers, the selfed plants would have more resources to channel to fruit and seed formation (Valtueña et al., 2012). It is known that greater resource availability leads to increases in reproductive parameters after selfing, reducing the effect of ID (Shi et al., 2005). Furthermore, this increase comes at the cost of progeny of poorer quality, as is reflected in lower seed weight of the selfed plants (Shi et al., 2005). This would mean a loss of survival capacity during the early stages of the plant's life since greater seed weight is known to provide a greater capacity for survival in the early stages of a plant's development (Stanton, 1984; Seiwa, 2000; Naito et al., 2005). The results of the first year are difficult to interpret in this sense because selfed plants produced

more flowers. The difference in fruit-set between selfed and outcrossed plants in the second flowering season ($\delta = -0.47$) was lower than that in the first flowering season ($\delta = -0.82$), whereas the negative values of ID were the same in seed number per fruit ($\delta = -0.15$). The uniform value obtained in this second parameter could indicate that it is subject to higher genetic control and the clear existence of outbreeding depression because of the rupture of some coadapted gene complexes or underdominance at some loci; therefore, heterozygosity in the responsible genes is a disadvantage (Lynch, 1991). In contrast, the results in fruit-set could be because this parameter may depend more on external factors (as pollination, environmental parameters, or resource availability) and so the variations between years should be higher.

The absence of ID in the individuals' female reproductive capacity has major ecological implications because selfed seeds could give rise to new individuals capable of reproduction by outcrossing due to the low viability of their own pollen. The probability of pollen flow from distant individuals is possible in this species because of the large radius of action of their pollinators (passerine birds), and this would allow new populations to become established. In contrast, if female reproductive capacity was reduced, the establishment of new populations would be limited by the dispersal of outcrossed seeds. There is support for this theory in that *Myrtus communis* L. (González-Varo et al., 2010) shows a higher rate of outcrossing in individuals of small populations connected to individuals of large populations. This implies that individuals established in a new area could originate a new population with pollen flow from the original large population or other close populations.

At the level of reproduction, the value of ID depended on the parameters used in the calculation ($\delta_{\text{rep_real}} = 0.33$ and $\delta_{\text{rep_potential}} = 0.54$), compared to a value of 0.32 at the vegetative level. Since the value of ID during the first year of life was 0.29 considering the same parameters used to calculate the vegetative ID at the mature stage, this simply shows that the magnitude of ID has a greater effect in later stages of the life cycle, mainly affecting reproductive parameters (in particular, flower production and male reproductive capacity). This phenomenon is characteristic of long-lived species that reproduce mainly by selfing (Husband and Schemske, 1996), in which early ID is caused by recessive lethal mutations and late by recessive and only mildly deleterious mutations, which are very difficult to purge even in highly inbred populations.

In the same way as before, the overall (vegetative + reproductive) value of ID in the populations studied varied according to which reproductive parameters were analyzed ($\delta_{\text{rep_real}} = 0.54$ and $\delta_{\text{rep_potential}} = 0.69$). This is considerably higher than that reported for selfed angiosperms ($\delta =$

0.22) by Husband and Schemske (1996). Some theoretical studies predict that, at values of δ above 0.5, xenogamy would be evolutionarily stable, while full autogamy should evolve towards lower values of that parameter (Lande and Schemske, 1985). According to this prediction, the values close to 0.5 found for *Anagyris foetida* would not be high enough to maintain the characters that facilitate xenogamy based on the trade-off between the cost (ID) and benefit (increased gene transfer) of autogamy.

In conclusion, ID in the populations of *Anagyris foetida* studied was manifest throughout the plant's life cycle. It was weak in the early stages, was similar under controlled and natural conditions, and none of the studied parameters was predominant in the ID value. In the mature stage, it was manifest mainly in the individuals' reproductive capacity, affecting male fitness in both pollen production and viability, without female fitness being affected. Considering several years of its life cycle, we found values of the ID that were medium to high resulting from its effect on both vegetative and reproductive parameters. This proves beyond any room for doubt that ID would be underestimated if one were only to study the initial stages of the life cycle or only one type of parameter (vegetative or reproductive). The present

results have major implications for the conservation of the populations studied, since a high rate of selfing in them would lead to a progressive increase in individuals that are sterile (due to malformations in their flowers) or that have reduced male function, and thus jeopardize the long-term viability of the populations. Indeed, the low probability to reach the mature stage and the mortality of adult individuals because of human activities draw a future framework of the populations with a small number of individual that could be very close genetically. This would imply high rates of selfing with the subsequent problem for long-term species survival. However, it is necessary to conduct more studies to determine the selfing rate in the studied populations and the effect of the ID in posterior generations to confirm this hypothesis.

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