Among-individual variation in pollen limitation and inbreeding depression in a mixed-mating shrub

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Running-head: *Individual variation in pollen limitation and inbreeding depression*

Background and Aims. Variation in inbreeding depression (δ) among individual plants is considered to play a central role on mating system evolution and population genetics. Moreover, such variation could be linked with individual susceptibility to pollen limitation (PL) because those individuals strongly affected by δ for seed production will require more outcross pollen for setting a given number of fruits or seeds. However, there is a lack of studies testing explicitly for associations between PL and δ at the individual plant level. This study assess the extent of the among-individual variation in PL and δ, the consistency of δ across life stages and the relationships between individual PL and δ in the mixed-mating shrub *Myrtus communis*.

Methods. Controlled hand-pollinations were performed in a natural *M. communis* population. Marked flowers were monitored until fruit production and a greenhouse experiment was conducted with the seeds produced.

Key results. Compared to selfing, outcross-pollination enhanced the seed number per fruit, germination rate and seedling growth, but did not enhance fruit set. Only seed number per fruit was pollen limited and, thus, cumulative pollen limitation depended more on pollen quality (outcross pollen) than on quantity. The effects of δ varied considerably across life stages and individual plants. Cumulative δ was high across individuals (mean $\delta = 0.65$), although there was not positive correlations between δ values at different life stages. Interestingly, maternal plants showing stronger δ for seed production were more pollen limited, but they were also less affected by δ for seedling growth because of a seed size/number trade-off.

Conclusions. Results show a general inconsistency in δ across life stages and individuals, suggesting that different deleterious loci are acting at different stages. The

association between δ and PL at the individual-level corroborates the idea that pollen limitation may be 'genotype-dependent' regardless of other factors.

Key words: Inbreeding depression, family-level, mixed-mating systems, *Myrtus communis*, pollen limitation, progeny performance, seed number/seed size trade-off, seed production

INTRODUCTION

Seed production in plants may be compromised by pollen limitation, defined as insufficient pollen receipt in terms of quantity and/or quality (e.g. self- or cross-pollination; Burd, 1994; Ashman *et al*., 2004; Knight *et al*., 2005; Aizen and Harder, 2007). Pollen limitation can be influenced by several ecological factors such as plant size (Dudash, 1993), flowering phenology (Santrandreu and Lloret, 1999), floral neighbourhood (Jakobsson *et al*., 2009) and pollinator visits in the case of animal-pollinated species (González-Varo *et al*., 2009*b*).

In hermaphroditic plants with mixed mating strategies, sexual reproduction occurs by either self-fertilization (selfing) or mating with other individuals (outcrossing; see Goodwillie *et al*., 2005). Selfing offers the opportunity to ensure reproduction in environments where outcross-pollen availability is unpredictable (Knight *et al*., 2005; Morgan and Wilson, 2005); however, it entails the problem of inbreeding depression, i.e. the reduction in fitness of selfed progeny relative to outcrossed progeny (Schemske 1983; Dudash, 1990; Holsinger, 1991; Byers and Waller, 1999). A higher frequency of deleterious recessive alleles occurring in homozygosis in the selfed progeny has been outlined as the cause of inbreeding depression (Dudash and Carr, 1998; Charlesworth and Charlesworth, 1999; Fox *et al*., 2008). Because of this conflict, inbreeding depression plays a central role in the evolution of self-fertilization in plants, and it is considered as a main selective force in the maintenance of mixed-mating systems (Goodwillie *et al*., 2005; Harder *et al*., 2008).

Inbreeding depression seems to vary between populations (Goodwillie and Knight, 2006), life-history stages (Husband and Schemske, 1996) and maternal families (Sakai *et al*., 1989; Holtsford, 1996; Dudash *et al*., 1997; Mutikainen and Delph, 1998),

i.e. between seeds/seedlings belonging to the same mother plants. Nevertheless, variation at the family-level (also called individual-level) has captured just a modest attention in studies about inbreeding depression in plants (Byers and Waller, 1999). Extant theoretical and empirical studies (most based on data from herbs) have suggested that values of inbreeding depression at the individual-level may be as relevant as population values to mating system evolution and population genetics, and have called for further studies on this issue (Holtsford, 1996; Mutikainen and Delph, 1998; Fishman, 2001; Picó *et al*., 2004; Oakley and Winn, 2008; Waller *et al*., 2008). Differences among plants in their genetic load (i.e. number of deleterious alleles) would explain among-individual variation in inbreeding depression (Dudash *et al*., 1997; Fishman, 2001; Picó *et al*., 2004). Moreover, possible variation in inbreeding depression among plants (genotype-dependent) is expected to be linked with individual differences in pollen limitation because those individuals showing high levels of inbreeding depression for seed production should be more prone to pollen limitation in terms of quality; i.e. they will require more outcross pollen for setting a given number of fruits or seeds (see Aizen and Harder, 2007; Harder and Aizen, 2010). However, there is a lack of studies testing explicitly for associations between pollen limitation and inbreeding depression at the individual plant level, which is the most relevant level from an evolutionary perspective.

Inbreeding depression is also of major concern in conservation biology since small populations are becoming increasingly common due to the pervasive effects of the loss and fragmentation of natural habitats (Ellstrand and Elam, 1993; Dudash and Fenster, 2000, Ouborg *et al*., 2006). The among-individual variability in inbreeding depression could safeguard population persistence from such threats if some individuals

are able to cope with inbreeding depression (Picó *et al*., 2004). Thus, evaluating the extent of the among-individual variation in inbreeding depression across different life stages is also an essential tool for understanding the demographic and genetic factors that may threaten the persistence of small populations (see Ouborg *et al*., 2006).

In this paper we evaluate the extent of the among-individual variation in pollen limitation and inbreeding depression, the consistency of individual inbreeding depression across life stages, and the relationships between pollen limitation and inbreeding depression at the individual-level in the Mediterranean shrub *Myrtus communis* (commonly named myrtle). For this purpose, we performed controlled self- and cross-pollinations and conducted a greenhouse experiment with the seeds produced. Myrtle is insect-pollinated and self-compatible, although the number of seeds per fruit strongly depends upon outcross pollen (González-Varo *et al*., 2009*b*). Mating system analyses using allozyme markers have revealed a mixed-mating system across populations (González-Varo *et al*., 2010), showing that seed crops in myrtle are generated by a mixture of self- and cross-fertilization events (mean outcrossing rate [t_m] = 41% in five populations; González-Varo *et al*., 2010).

We expect to find consistency in inbreeding depression effects at the individual-level across life stages if the deleterious alleles responsible for inbreeding depression are roughly the same. Moreover, if seed production is strongly pollen limited in terms of quality (i.e. outcross-pollen limited), then we expect that the level of pollen limitation of individual plants should be related to their susceptibility to inbreeding depression for seed production.

MATERIALS AND METHODS

The plant species

The Mediterranean myrtle (*Myrtus communis*) is a common sclerophyllous shrub and the sole representative of the Myrtaceae in the Mediterranean Basin. It grows up to 4 m in height, and occurs as main component of late successional woodlands in fertile soils and warm habitats. It blooms massively during July in the study area. Myrtle flowers are hermaphrodite, with a white open dish corolla up to 3 cm in diameter and a life span of 1–3 days, they have one style, many stamens (>50) and contain ca. 70 ovules (González-Varo *et al*., 2009*b*). A single individual can produce over 30,000 flowers although only ~15% are open simultaneously (Sofia Nora, unpubl. data). Insects, mostly hymenopterans and dipterans, visit myrtle flowers in search of pollen (González-Varo *et al*., 2009*b*). The fruits are dark blue berries 7.9 ± 0.6 mm (mean ± SD) in diameter when ripen in November, that contain 7.7 ± 3.8 seeds weighing 6.6 ± 2.1 mg each in the study population ($n_{\text{plants}} = 12$, $n_{\text{fruits}} = 233$, $n_{\text{seeds}} = 223$). Endozoochorous seed dispersal is carried out by frugivorous passerine birds and carnivorous mammals (Traveset *et al*., 2001; González-Varo, 2010).

Study population

We studied a myrtle population located in Binifaldó (39º50′ N, 2º54′ E, Mallorca, Balearic Islands), at 530 m a.s.l. in the Sierra de la Tramuntana. The climate of the region is Mediterranean, with a mean annual temperature of 17 ºC and rainfall (1000–1200 mm) concentrated between autumn and spring. The landscape is mostly covered by mixed forest of Aleppo pines (*Pinus halepensis*) and helm oaks (*Quercus ilex*) with the understory dominated by the shrubs of *Erica multiflora* and *Myrtus communis*. The myrtle population is large (> 1,000 individuals) and shrubs grow densely in the moistest

soils. In this site, the main myrtle pollinator is the honeybee (*Apis mellifera*), accounting for 56% of insect visits, while dipterans (32% of visits) and other bee species (12% of visits; mainly the bumblebee *Bombus terrestris*) account for the remaining (42 censuses, each of 5-min observation period; J.P. González-Varo and A. Traveset, unpubl. data).

Controlled pollinations

We conducted controlled hand-pollinations in July 2008 over 10 flowering individuals of similar size (ca. 2 m height) and flower crop. On each plant, we applied three pollination treatments: (1) 'Self-pollination': flowers were bagged and hand pollinated with pollen from other flowers of the same plant; (2) 'Cross-pollination': open flowers were hand pollinated with outcross pollen (without bagging); and (3) 'Control': flowers were left to be naturally pollinated by insects. Each treatment was replicated on 20−22 flowers per plant, over 2–4 branches per treatment. Branches were covered with gauze bags (20 × 40 cm, < 0.1 mm mesh light) for the bagging treatment (Self-pollination). Flowers were pollinated by gently touching the stigma with anthers from other flowers of the same plant or from two distant (≥ 20 m) myrtles in the population, depending on the treatment (Self-pollination and Cross-pollination, respectively). Bags were removed one week after hand pollinations when stigmas were dry. The flowers used for Cross-pollination were recently opened (identified by the light colour of the anthers) and hand pollinated in the morning. Flowers used as Controls were floral buds chosen the day before opening, aiming to avoid flower alteration, e.g., fallen petals, when marking them with plastic rings. Overall, 622 flowers were marked (207−208 flowers per treatment) with plastic rings in the flower pedicel and regularly monitored until fruit production.

Fruit and seed production

In September 2008, all developed unripe marked fruits were noted to obtain the fruit set for each plant and treatment. In early November, ripe fruits were collected and dissected in the laboratory, and the number of seeds per fruit was counted. At this date, fruit loss due to fruit consumption by frugivores was negligible (2.4% of marked fruits). Once dissected, we weighed all the seeds of each marked fruit (n = 1–23 seeds per fruit) to the nearest 0.1 mg. Overall, 540 marked fruits were noted, 527 of which were collected and dissected, and 3,528 seeds were weighed (mean individual seed mass within each fruit was calculated for data analyses).

Progeny performance

Progeny performance, measured as seed germination and seedling growth, was evaluated in a greenhouse experiment by sowing seeds from controlled pollinations. In total, we sowed 300 seeds: 10 individuals × 3 pollination treatments × 10 seeds per treatment and individual. We used only one (randomly selected) seed per fruit (n_{fruits} = 300). In late November 2008, seeds were individually and randomly planted in trays of 60 pots (5 × 5 cm). Each pot was filled with horticultural mixture in which the seeds were planted at a depth of ~ 0.5 cm. Trays were watered twice a week to ensure permanent humidity, and their position in the greenhouse was randomized fortnightly. Seed germination and survival were monitored every 3–5 days until the day 102, when monitoring started to be weekly until the end of the experiment in June 2009 (day 231 after sowing). We considered that seed germination had taken place when cotyledons were fully emerged. At the end of the experiment, all seedlings (except 30 that were left

alive) were harvested and dried at 60ºC during 72 h to obtain seedling dry biomass. We calculated a biomass 'growth rate' (g day^{-1}) for each seedling by dividing its dry biomass by its growth time ($t_{growth} = t_{total} - t_{emergence}$; being t_{total} the total number of days of the experiment and $t_{emergence}$ the number of days until seedling emergence). Although absolute seedling biomass and biomass growth rate were highly correlated ($r^2 = 0.97$, $p < 0.0001$), we used the latter to control for the among-seedling differences in emergence date.

Data analyses

Variation in fruit/seed production and progeny performance. The effects of pollination treatment (T), individual (I) and their interaction (T × I) on seed production (fruit set, seed number per fruit and seed mass) and progeny performance measurements (germination rate and biomass growth rate) were tested with Generalized Linear Models (hereafter, GLMs; GLZ procedures of STATISTICA 6.0, StatSoft, 2005). Because seedling mortality was very low (4.5%, $n = 176$ germinated seedlings) and only occurred in five out of the ten families studied, we excluded the life stage 'seedling survival' from these analyses and also from the subsequent inbreeding depression analyses. Binomial distribution and logit-link function were used for modelling fruit set and germination rate, Poisson distribution and log-link function for seed number per fruit, and normal distribution and identity-link function for seed mass and biomass growth rate. To control for the effect of seed size on progeny performance, we included seed mass in the models of germination rate and seedling growth rate as a continuous covariate. The importance of the pollination treatment and individual plant on seed production and progeny performance was evaluated using the deviance quotients

(percentage of relative variance) provided by GLMs (e.g. García *et al*., 2005). All sample sizes are given in Table S1 (Supplementary material).

Pollen limitation. For each individual plant, fruit set and mean number of seeds per fruit obtained in Control and Cross-pollination treatments were used to calculate a pollen limitation index (PL) expressed as: $PL = 1 - C/X$, where C and X represent the fruit set or seeds per fruit of treatments Control and Cross-pollination, respectively (following Tamura and Kudo, 2000). This standard index has a straightforward biological interpretation (e.g., Lázaro and Traveset, 2006; González-Varo *et al*., 2009*b*), ranging from zero (when both treatments produce the same fruit set and/or seeds per fruit, i.e. no pollen limitation) to one (when natural pollination does not produce seeds and hand-pollination does it, i.e. maximum pollen limitation); a negative value means that naturally pollinated flowers received more and/or better pollen than hand-pollinated ones. We calculated the pollen limitation of the population by averaging the individual PL values. At the individual level, a PL value was considered significant if fruit set and/or seed number per fruit differed significantly among C and X treatments at $p < 0.05$ (using GLMs, *t*-tests or Mann-Whitney *U*-test, depending upon sample size and the distribution of the response variable; see above). At the population level, significant pollen limitation was tested by means of a *t*-test against zero. To estimate the multiplicative pollen limitation throughout fruit set and seeds per fruit, we calculated the cumulative pollen limitation index in each individual as $PL_{cumulative} = 1 - (C_{fruit\ set}\ C_{seeds\ per\ fruit})/(X_{fruit\ set}\ X_{seeds\ per\ fruit})$ (see González-Varo *et al*., 2009*b*).

Inbreeding depression. Inbreeding depression coefficient (δ) was calculated for each life stage and individual mother plant as $\delta = 1 - (w_s/w_x)$, where w_s and w_x are the fitness of selfed and outcrossed progeny, respectively (according to Ågren and Schemske, 1993). Positive δ values indicate that outcrossed progeny are more fit than the selfed progeny, whereas negative δ values mean the opposite. We estimated the inbreeding effects for: (1) fruit set, (2) seed number per fruit, (3) seed germination and (4) biomass growth rate. For each life stage, we calculated the inbreeding depression of the population by averaging individual δ values. At the individual level, a δ value for a given life stage was considered significant if w_s and w_x were significantly different at $p < 0.05$ (GLMs, *t*-tests or Mann-Whitney *U*-test). At the population level, significant δ for each stage was tested by means of a *t*-test against zero. We finally calculated the predispersal δ (across fruit set and seed number per fruit), the postdispersal δ (across germination and seedling growth) and the cumulative δ (across all life stages studied) of each maternal plant by multiplying fitness values (w_s and w_x) across life stages and then applying the formula above (following Ågren and Schemske, 1993; see also Harder *et al*., 2008). Predispersal and postdispersal inbreeding depression differ biologically between them in the degree of dependency on maternal resources, being complete in the first and partial (indirect through seed size) in the later (Harder *et al*., 2008).

We used Spearman's correlations (using individual means as replicates) to examine correlation between pollen limitation and inbreeding depression values at different life stages.

RESULTS

Fruit and seed production

Fruit set was very high across pollination treatments (85–89%) and did not differ significantly between them (see Fig. 1, Table 1). However, fruit set differed among individuals (68% of variance) and between pollination treatments within them (T × I). Seed number per fruit differed significantly between pollination treatments and individuals, and there was also a significant interaction T × I. This was higher for Cross-pollination (mean = 7.8 seeds per fruit), intermediate for Control (mean = 6.1) and lower for Self-pollination (mean = 5.5; see Fig. 1). Seed mass was also significantly different between pollination treatments and individual plants, being different the effect of treatments across the different individuals (T × I). Conversely to seed number per fruit, seed mass was higher for Self-pollination (mean = 7.1 mg), intermediate for Control (mean = 6.6 mg) and lower for Cross-pollination (mean = 6.0 mg; see Fig. 1). These results revealed the existence of a trade-off between seed number and average seed mass within myrtle fruits (log regression: $r = -0.66$, $p < 0.001$, $n = 527$ fruits). Total seed mass per fruit was, however, higher for Cross-pollination (mean = 41.8 mg) than for Self-pollination and Control, which showed similar mean values (35.0 and 34.5 mg, respectively). Most variance in seeds per fruit and seed mass was accounted by individuals (61–75%), while the interaction (16–22%) and pollination treatments (9–17%) accounted for a lower variance (see Table 1).

Progeny performance

Percent germination was significantly higher for seeds from Cross-pollination (79%) than for seeds from Self-pollination and Control, which showed very similar percentages (52% and 45%, respectively; see Table 1, Fig. 1). Moreover, germination rates differed significantly among families (i.e. seeds belonging to the same individual;

41% of variance), being the effect of treatments different across families (T × I; 30% of variance) (Table 1, Fig. 1). Seed mass had a negative, but small (5% of variance) effect on germination. Pollination treatments also produced significant differences in seedling growth rate (21% of variance), which was higher for Cross-pollination (0.0129 g day^{-1}), intermediate for Control (0.0115 g day^{-1}) and lower for Self-pollination (0.0101 g day^{-1}) (see Fig. 1); mean ± SE seedling dry biomass was 2.52 ± 0.10 g for Cross-pollination, 2.28 ± 0.16 g for Control and 1.98 ± 0.09 g for Self-pollination seedlings. Seedling growth rate also varied significantly among families (27% of the variance) and between treatments across the different families (T × I; 36% of the variance) (Table 1, Fig. 1). In this case, seed mass showed positive effects on growth rate, although it accounted for only 6% of the variance.

We acknowledge that we might have somewhat underestimated germination in the Control treatment (see Fig. 1) because some fruits within this treatment were not fully ripe when collected, but bluish green. This was probably due to a lapse of time (of a few days) in fertilization date among treatments since, while self- and cross-pollination flowers were hand-pollinated the same day, flowers marked as Control (buds) depended on pollinator visits (1−5 days after). As expected, seedling growth was clearly higher in Control treatment than in Self-pollination (Fig. 1).

Pollen limitation

Fruit set did not appear to be pollen limited, either at the population or individual level (Fig 2). At the population level, mean $PL_{fruit\ set}$ = 0.01, and values ranged from –0.21 to 0.15 among individuals (Fig. 2). By contrast, seed number per fruit was pollen limited at both levels, with 60% of plants (6 out of 10) showing significant pollen limitation. At

the population level mean $PL_{seeds\ per\ fruit} = 0.19$, and values ranged from –0.42 to 0.50 among individuals (Fig. 2). Finally, cumulative pollen limitation was not significant at the population level ($PL_{cumulative} = 0.20$) and values ranged from –0.68 to 0.57 among plants (Fig. 4a).

Pollen limitation for fruit set ($PL_{fruit\ set}$) and seeds per fruit ($PL_{seeds\ per\ fruit}$) were not significantly correlated (Spearman's $r_s = 0.382$, $p > 0.28$, $n = 10$). However, the cumulative pollen limitation ($PL_{cumulative}$) was correlated with both $PL_{fruit\ set}$ ($r_s = 0.648$, $p = 0.043$) and $PL_{seeds\ per\ fruit}$ ($r_s = 0.879$, $p < 0.001$), but more strongly with the latter.

Inbreeding depression

At the population level, inbreeding depression (δ) for fruit set was not significant, and only one individual showed a significant $\delta_{fruit\ set}$ value (Fig. 3). The average across individuals was $\delta_{fruit\ set} = 0.04$, ranging from –0.15 to 0.28 (Fig. 3). Seed number per fruit, percentage of seed germination and seedling growth rate were affected by inbreeding depression (δ) both at the population- and individual-level. Six out of ten plants showed significant $\delta_{seeds\ per\ fruit}$ values. At the population level, mean $\delta_{seeds\ per\ fruit} = 0.22$, ranging from –0.35 to 0.55 across individuals (Fig. 3). Three out of ten plants showed significant $\delta_{germination}$ values. At the population level, mean $\delta_{germination} = 0.34$, ranging from –0.14 to 0.75 across individuals (Fig. 3). Finally, three out of nine plants showed significant $\delta_{seedling\ growth}$ values, all of them showing positive values (see Fig. 3). At the population level, mean $\delta_{seedling\ growth} = 0.26$, and values ranged from 0.13 to 0.45 across individuals (Fig. 3).

Besides the strength of inbreeding depression effects, life stages also differed in the heterogeneity of such effects across the different individuals. As shown by the

coefficient of variation (CV = SD×100/mean), both seeds per fruit (CV_{δ} = 138%) and germination rate (CV_{δ} = 99%) showed much more variability in inbreeding depression effects across mother plants than seedling growth (CV_{δ} = 45%; see also Fig. 3). Also, it is worth noting that seeds per fruit and germination showed negative inbreeding depression values (yet non-significant), while all $\delta_{seedling\ growth}$ values were positive.

The magnitude of predispersal inbreeding depression ($\delta_{predispersal}$ = 0.20, range = −0.73 to 0.61; Fig. 4b) was non-significant at the population level and mean value was 2.4-fold lower than the highly significant mean value of postdispersal inbreeding depression ($\delta_{postdispersal}$ = 0.47, range =−0.02 to 0.80; Fig. 4c). Moreover, the effects of inbreeding depression were much more heterogeneous at predispersal (CV_{δ} = 229%) than at postdispersal stages (CV_{δ} = 61%; see Fig. 4b, 4c). Regarding the cumulative inbreeding depression, it was highly significant at the population level ($\delta_{cumulative}$ = 0.65), with all plants showing high positive values (range = 0.44–0.89; Fig. 4d). Thus, the cumulative effects of inbreeding depression showed a relatively low variability among mother plants (CV_{δ} = 23%).

Relationships in inbreeding depression between life stages

In general, inbreeding depression was not consistent among individuals and life stages, as shown by the multiple non-significant correlations found (see Table 2). Interestingly, there was a rather strong and negative correlation between $\delta_{seeds\ per\ fruit}$ and $\delta_{seedling\ growth}$ (Spearman's r_s = –0.817, p = 0.007), indicating that those plants that suffered a higher inbreeding depression for setting seeds had a lower inbreeding depression for seedling growth. These correlations also showed that $\delta_{cumulative}$ was mainly determined by $\delta_{germination}$ (r_s = 0.689, p = 0.04).

Relationships between inbreeding depression and pollen limitation

Cumulative pollen limitation ($PL_{cumulative}$) of mother plants was positively and highly significantly correlated with their degree of inbreeding depression for seed production ($\delta_{predispersal}$: $r_s = 0.794$, $p = 0.006$; Fig. 5). Moreover, individual pollen limitation for seed number per fruit ($PL_{seeds\ per\ fruit}$) was near-significantly correlated with individual inbreeding depression at this stage ($\delta_{seeds\ per\ fruit}$: $r_s = 0.612$, $p = 0.060$). These results indicated that those plants more sensitive to inbreeding depression for setting seeds exhibited higher levels of pollen limitation.

DISCUSSION

The finding that open pollinated myrtle flowers produced intermediate results between the Self-pollination and Cross-pollination treatments across life stages indicated that these flowers received a mixture of self and outcross pollen, as occurs in other populations of this species (González-Varo *et al*., 2010). Compared to selfed progeny, outcross-pollination enhanced seed number per fruit, germination rate and seedling growth rate. Interestingly, we detected a trade-off between seed number and seed size that depended on pollination type: outcrossed flowers set more seeds per fruit than selfed flowers, but the former were ~20% lighter. The significant effects of the interaction between pollination treatment and individual across life stages showed that mother plants responded differently to pollination treatments. Moreover, the fact that 61–75% of the variation in seed production measurements (i.e. fruit set, seed number per fruit and seed mass) was accounted for by the mother plant suggested that maternal

effects are playing an important role in generating fitness differences among families in response to pollination (Roach and Wulff, 1987).

Among-individual variation in pollen limitation and inbreeding depression across life stages

While fruit set in *M. communis* is mostly limited by pollen quantity, seed number per fruit is constrained by pollen quality because it strongly depends on outcross pollen (González-Varo *et al*., 2009*b*; this study). Our results indicated that the study population and most target individuals were much more pollen limited in quality (i.e. outcross-pollen limited) than in quantity (Aizen and Harder, 2007). Besides the likely differences among plants in outcross pollen receipt, differences among individuals in such 'quality-pollen limitation' seem to depend on their susceptibility to inbreeding depression for setting seeds. This fact could even explain the finding of negative pollen limitation values for seeds per fruit (see next sections).

With the exception of fruit set, all life stages studied showed significant inbreeding depression values both at the population and individual level. Germination rate, in particular, was critically affected by inbreeding depression, with half plants showing values of $\delta_{germination} > 0.5$. The small number of plants showing significant $\delta_{germination}$ and $\delta_{seedling\ growth}$ despite their high and positive values possibly reflected the smaller sample size for these life stages (seeds or seedlings per individual and treatment). Seed number per fruit, germination rate and seedling growth not only varied in the strength of inbreeding depression effects (δ-values) but also in the heterogeneity of such effects across the different individuals. Among-individual variation in inbreeding depression at different life stages could reflect differences in either the

number or type of deleterious mutations. Two main types of deleterious mutations are usually considered responsible for inbreeding depression: (1) deleterious mutations of large effect (lethal), typically found in a few loci, and (2) mildly deleterious mutations in many loci across the genome (Husband & Schemske 1996; Charlesworth & Charlesworth 1999; Fox *et al*., 2008). While deleterious alleles of strong effect might be responsible for the non-viability of selfed embryos and thus prevent seed production (seeds per fruit) and/or germination, the additive effect of multiple mildly deleterious alleles might deteriorate the development (metabolism and physiology) of selfed progeny, thus lowering seedling growth (Husband and Schemske, 1996; Charlesworth and Charlesworth, 1999). The fact that some individuals had such deleterious alleles of large effect and others did not could explain the higher among-family variation found for $\delta_{\text{seeds per fruit}}$ and $\delta_{\text{germination}}$ (Koelewijn, 1998; Fox *et al*., 2008). This mechanism could also explain the negative inbreeding depression values observed if any of the two pollen donors chosen for the Cross-pollination treatment shared these lethal/harmful alleles with any of the target plants (i.e. biparental inbreeding; see Dudash, 1990; Waser and Price, 1994). Conversely, mildly deleterious alleles in many different loci should be more homogeneously distributed across plants, as they might be more difficult to purge due to their smaller effects (Charlesworth and Charlesworth, 1999; see also below the effect of the trade-off seed number/size on $\delta_{\text{seedling growth}}$). Hence, this type of alleles might explain the general positive $\delta_{\text{seedling growth}}$ values across plants as well as the lower among-individual variability in $\delta_{\text{seedling growth}}$.

The cumulative inbreeding depression in myrtle was very high both at population- and individual-level (mean = 0.65), with all mother plants showing positive values and most of them showing values of $\delta_{\text{cumulative}} > 0.5$ (see Fig. 4b). These results

are striking because our study species shows high selfing rates (an average of 65% in two large populations; González-Varo *et al*., 2009*a*) and theoretical models predict that when inbreeding depression values are larger than 0.5, selection favours complete outcrossing (see Lande and Schemske, 1985; Husband and Schemske, 1996; but see Goodwillie *et al*., 2005; Morgan and Wilson, 2005). Likewise, when considering only postdispersal stages ($\delta_{postdispersal}$), inbreeding depression was ~0.5, and a recent model developed by Harder *et al*. (2008) predicts that selection favours for complete outcrossing when $\delta_{postdispersal} \geq 0.5$. Since it is difficult to detect strong inbreeding depression effects under non-stressful conditions, such as those in the greenhouse, we would expect stronger inbreeding depression effects under field conditions (Dudash, 1990; Ramsey and Vaughton, 1998; Koelewijn, 1998; Hayes *et al*., 2005), where selection for higher outcrossing rates should be even stronger. Finally, the distribution of cumulative inbreeding depression among individuals contrasts with other studies reporting either positive or negative cumulative values (e.g. Mutikainen and Delph, 1998; Picó *et al*., 2004), suggesting that selection will tend to favour outcrossed progenies across life stages.

Correlation between inbreeding depression values: the effect of the trade-off seed number/seed size

We found a general lack of consistency among individuals in inbreeding depression across life stages. The single exception was the negative correlation between $\delta_{seeds\ per\ fruit}$ and $\delta_{seedling\ growth}$. However, it is worth mentioning that this relationship was an indirect effect because $\delta_{seedling\ growth}$ was highly negatively correlated with the difference in seed mass (seed number-dependent) between selfed (heavier) and outcrossed (lighter) seeds:

the higher the difference, the lower the $\delta_{\text{seedling growth}}$ (Spearman's $r_s = -0.90$, $p < 0.001$; see Fig. S1 in Supplementary material). Thus, by compensating selfed seeds with a larger size, the trade-off between seed number and seed size seems to counteract a component of predispersal inbreeding depression ($\delta_{\text{seeds per fruit}}$) at a postdispersal stage ($\delta_{\text{seedling growth}}$). These results disagree with the theoretical model developed by Sakai and Ishii (1999), which predicted an opposite trade-off between seed number and seed size, i.e. few large outcrossed seeds *vs*. many small selfed seeds.

We found that cumulative inbreeding depression ($\delta_{\text{cumulative}}$) was mainly determined by $\delta_{\text{germination}}$, the life stage with the highest δ values. Hence, germination resulted to be the most critical stage in terms of susceptibility to inbreeding depression. This finding is consistent with the results of a recent study reporting that percent germination was the life stage most correlated to the outcrossing rates in fragmented *M. communis* populations (53% and 73% in the most selfed and outcrossed populations, respectively; González-Varo *et al*., 2010).

Linking pollen limitation to inbreeding depression

To our knowledge, this is the first study explicitly testing for correlations between pollen limitation and inbreeding depression at the individual plant level. As expected, we found that pollen limitation ($PL_{\text{cumulative}}$) of individual plants was positively associated with their susceptibility to inbreeding depression ($\delta_{\text{predispersal}}$ or $\delta_{\text{seeds per fruit}}$). We acknowledge that ten mother plants seem to be a modest sample size to achieve robust conclusions. However, and in spite of a lower statistical power, results show a clear and significant relationship between the magnitude of pollen limitation of individual plants and their genetic load (inbreeding depression; see Fig. 5). Pollen

limitation is a phenomenon that depends on several ecological factors, either intrinsic or extrinsic to the plants (Ashman *et al*., 2004; Knight *et al*., 2005). Our results show that pollen limitation may be 'genotype-dependent' regardless of other intrinsic individual plant characteristics such as plant size (Dudash, 1993), flower size (Totland *et al*., 1998) or flowering phenology (Santrandreu and Lloret, 1999), which may depend on age or resources (see Campbell and Halama, 1993). Although our study focuses on a self-compatible species, it is not difficult to guess that biparental inbreeding could contribute in a same way (i.e. qualitatively) to pollen limitation in self-incompatible species (Dudash, 1990; Waser and Price, 1994).

Concluding remarks

Our study shows that the effects of inbreeding depression in *M. communis* vary considerably across life stages and individuals, some life stages showing a greater among-individual variation in inbreeding depression (seed number per fruit and germination) than others (seedling growth). Although cumulative inbreeding depression was high across plants, we found a general inconsistency in inbreeding depression across life stages, suggesting that different deleterious loci are acting at the different stages. Interestingly, a trade-off seed number/size in *M. communis* was found to buffer the effects of inbreeding depression on emerged seedlings ($\delta_{\text{seedling growth}}$) in those plants in which selfed seeds are much larger than outcrossed ones (i.e. with high $\delta_{\text{seeds per fruit}}$). Finally, our study also shows that variation in pollen limitation among plants may be partly explained by differences among plants in inbreeding depression (genetic load). Hence, maternal plants suffering from strong inbreeding depression for seed production

were more pollen limited but less affected by inbreeding depression at postdispersal stages ($\delta_{\text{seedling growth}}$).

From an ecological and evolutionary viewpoint, our study empirically contributes to the understanding of what factors and mechanisms maintain high selfing rates given that selfed offspring should be so infrequently recruited into the adult population. Spatiotemporal variation in selective pressures imposed by either abiotic conditions (e.g. rainfall) or biotic interactions (e.g. herbivory) may differentially filter the selfed progenies of different individuals depending on the life stage mostly affected by a given selective pressure.

SUPPLEMENTARY MATERIAL

Supplementary material consists of the following table and figure: Table S1: sample sizes (*n*) for the different life stages studied (per pollination treatment and individual mother plant). Fig. S1: Relationship between the magnitude of inbreeding depression (δ) for seedling growth and the difference in mean seed mass among selfed and outcrossed seeds in the mother plants studied.

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TABLE 1. *Results of generalized linear models (GLMs) testing for the effects of pollination treatment, individual plant and their interaction (T × I) on seed production and progeny performance measurements. Seed mass was included as covariate in models of progeny performance measurements. In each Chi-square, subscripts denote degrees of freedom. The percentage of relative variance (RV) derived from GLMs is also shown.* n = *flowers, fruits, seeds or seedlings.*

		Treatment			Individual plant			T × I			Seed mass		
LIFE STAGE	n	χ^2_2	*p*	RV	χ^2_9	*p*	RV	$\chi^2_{15\text{-}18}$	*p*	RV	χ^2_1	*p*	RV
Seed production													
Fruit set	622	1.8	ns	1.7	73.9	***	68.1	32.8	**	30.2	–	–	–
Seeds per fruit	527	84.2	***	16.9	307.1	***	61.4	108.7	***	21.7	–	–	–
Seed mass†	527	45.9	***	8.5	406.5	***	75.2	88.2	***	16.3	–	–	–
Progeny performance													
Germination	300	33.7	***	31.9	35.5	***	33.6	31.4	*	29.7	5.0	*	4.8
Growth rate	134	21.2	***	31.2	18.5	*	27.2	24.2	*	35.6	4.1	*	6.0

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, no significant effects.

† Mean individual seed mass of each fruit was calculated for data analyses (total = 3,528 seeds weighed).

TABLE 2. *Spearman's correlation coefficients between inbreeding depression (δ) values at different life stages in* Myrtus communis *families, and also between the values at each stage and the cumulative inbreeding depression across all the stages. For clarity, significant correlation coefficients are in bold.*

LIFE STAGE	$n_{families}$	Fruit Set	Seeds per fruit	Percentage germination	Biomass growth rate
Fruit set	10	–			
Seeds per fruit	10	0.345 ^{ns}	–		
Percentage germination	10	–0.476 ^{ns}	–0.146 ^{ns}	–	
Biomass growth rate	9	–0.467 ^{ns}	**–0.817** **	0.227 ^{ns}	–
CUMULATIVE	9	–0.200 ^{ns}	0.500 ^{ns}	**0.689** *	–0.267 ^{ns}

*, $p < 0.05$; **, $p < 0.01$; ns, no significant correlation.

FIG. 1. Mean fruit set, number of seeds per fruit, seed mass, percentage of seed germination and seedling growth rate in the different pollination treatments both at the population level and for each individual plant (family). Vertical bars denote standard errors.

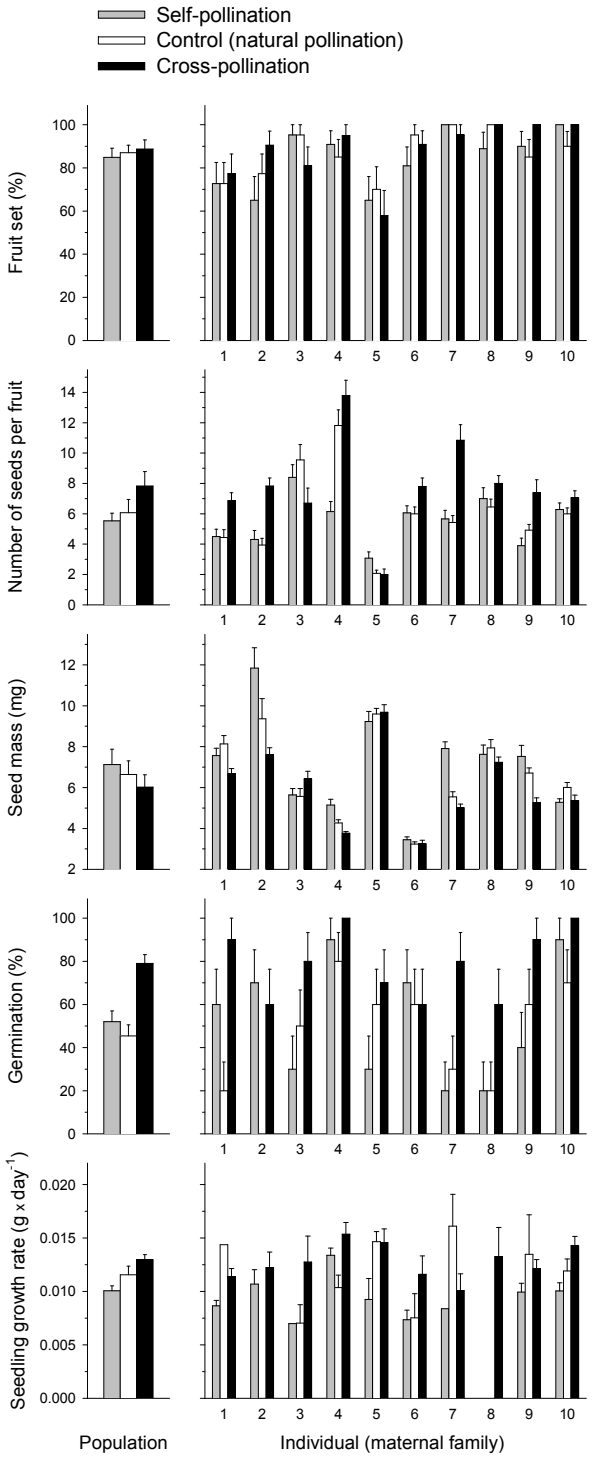

FIG. 2. Pollen limitation (PL) values for fruit set and seed number per fruit at the population level (mean ± 95% CI) and for each individual studied. Positive values indicate that hand-pollinated (i.e. 'Cross-pollination') flowers produced more fruits/seeds than those naturally pollinated (i.e. 'Control'), whereas negative values indicate the opposite. At the population level, *p*-values testing significant differences from zero (*t*-tests) are shown. At the individual level, black bars indicate that 'Control' and 'Cross-pollination' treatments significantly differed from one another ($p < 0.05$).

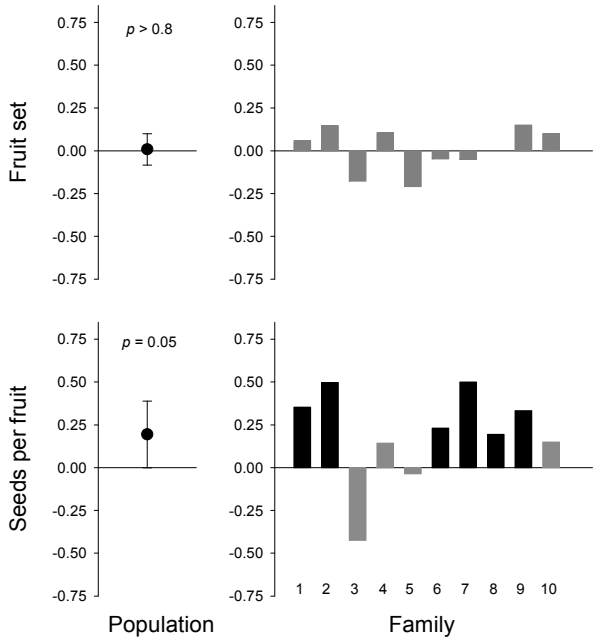

FIG. 3. Inbreeding depression coefficients (δ) for different life stages at the population level (mean ± 95% CI) and for each individual studied. Positive values indicate that outcrossed progeny outperformed selfed progeny whereas negative values indicate the opposite. At the population level, *p*-values testing significant differences from zero (*t*-tests) are shown. At the individual level, black bars indicate that selfed and outcrossed progeny significantly differed from one another ($p < 0.05$). na: data not available due to the lack of selfed seedlings.

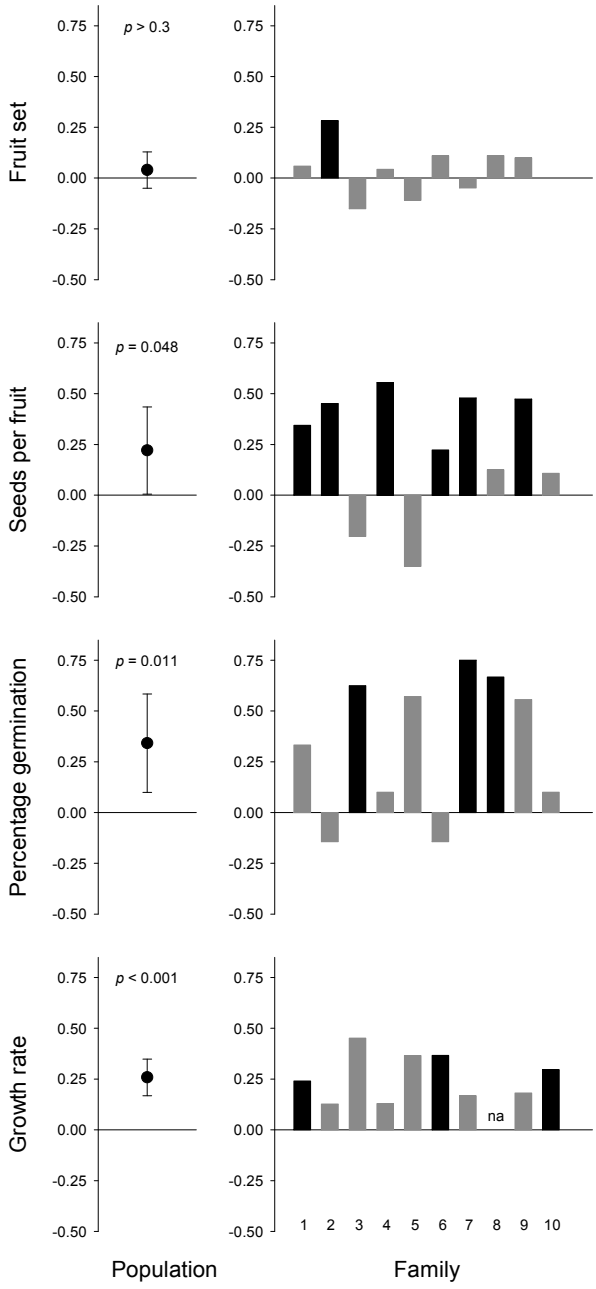

FIG. 4. Population (mean ± 95% CI) and family values for (a) cumulative pollen limitation (cumulative PL across fruit set and seed number per fruit); (b) predispersal inbreeding depression (cumulative δ across fruit set and seed number per fruit); (c) postdispersal inbreeding depression (cumulative δ across germination and seedling growth); and (d) cumulative inbreeding depression (cumulative δ all life stages studied; see text for details). At the population level, *p*-values testing significant differences from zero (*t*-tests) are shown. na: data not available due to the lack of selfed seedlings.

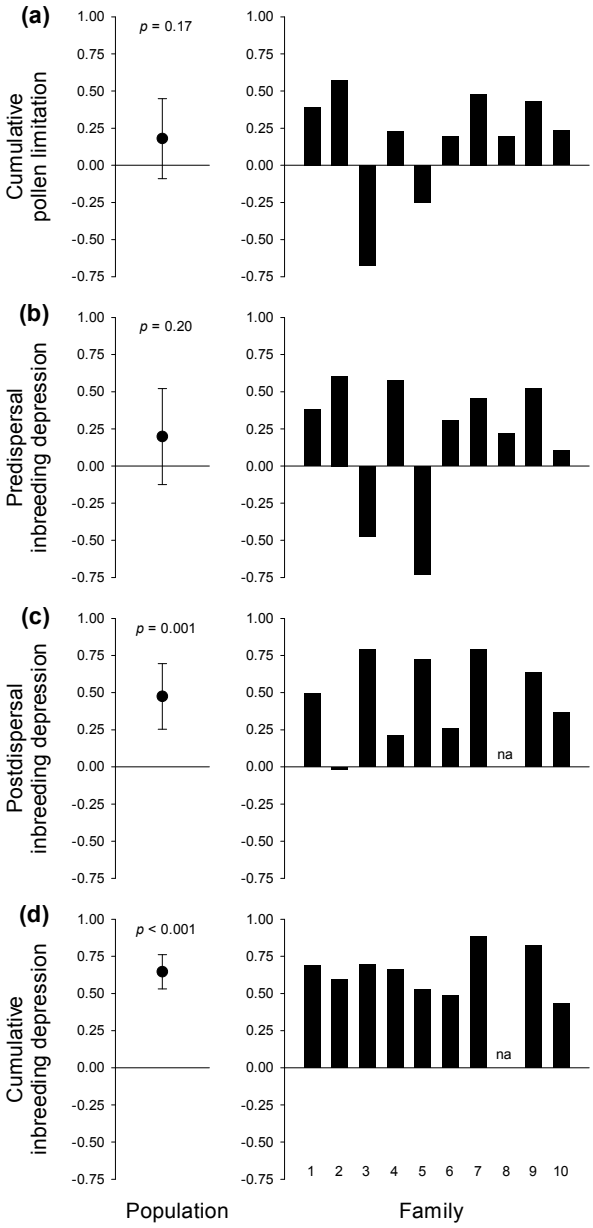

FIG. 5. Relationship between the magnitude of inbreeding depression coefficient for seed production ($\delta_{predispersal}$) and the cumulative pollen limitation index ($PL_{cumulative}$) in the ten *Myrtus communis* mother plants studied. Both parameters were calculated as the product between values (δ and PL, respectively) of fruit set and seed number per fruit.