

RESEARCH PAPER

Female reproductive output in a Mediterranean shrub: effects from inflorescence to population

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ABSTRACT

Inflorescence effects have been poorly studied, in spite of the functional relevance of the inflorescence in fruit and seed ecology. The present study focused on the effects of inflorescence size and phenology, and flower position within the inflorescence, in relation to fruit and seed production of the Mediterranean shrub *Ononis fruticosa*. Variability in fruit and seed production, seed weight and germination were estimated and modelled. Results confirmed that the most important predictors in seed production were inflorescence flowering time and flower position within the inflorescence. Thus, the number of mature seeds per fruit was higher in earlier inflorescences and in basal positions. On the other hand, predation was higher in fruits in basal positions. In fact, seed predation seemed to be the most important factor controlling final seed production. Models at the plant level suggested a negative incidence of geitonogamous pollination and resource limitation, which were also observed at the fruit level. This study confirmed the relevance of inflorescence effects on the reproductive output of *O. fruticosa*. Although the underlying processes could not be identified, our results provide several hypotheses for future experimental studies.

INTRODUCTION

Seed production is the outcome of a complex process involving a diverse arrangement of non-excluding factors. Among these, studies have highlighted pollinator activity (Wilcock & Neiland 2002; Ashman *et al.* 2004; Knight *et al.* 2005), seed predation (Krupnick *et al.* 1999; Mothershead & Marquis 2000), resource availability (Medrano *et al.* 2000; Holland *et al.* 2004) and architectural/phylogenetic constraints (Diggle 1995, 1997; Herrera 1999). The relative importance of these factors and their interactions vary at different spatial and temporal scales.

Inflorescences are complex functional units in which most of the factors affecting fruit and seed production occur (Herrera 1997; Harder *et al.* 2004). Flower position is the most relevant observed source of variation in seed production within inflorescences (Nicholls 1987; Herrera 1991; Moravcová *et al.* 2005). This variability may depend on plant resources, which influence both the initial number of ovules per flower and, later, the probability of each ovule becoming a mature seed (Guitián & Navarro 1996).

However, in some cases, experimental inflorescence manipulation has shown that position effects are not related to resource availability. Architectural constraints may be responsible for these findings (Ashman & Hitchens 2000; Medrano *et al.* 2000; Gómez & Zamora 2003). Attention must also be paid to the possible relation among position-related variability and pollinator and seed predator activity. Pollination processes may influence seed production through the non-random fertilisation of flowers in the same inflorescence (Tremblay 2006). In addition, seed predation (Ehrlén 1996; Brody & Mitchell 1997; Kudo & Molau 1999; Kudo *et al.* 2001) may be affected by fruit and seed position within the plant, which can then cause position dependence in final seed production.

Other possible sources of variation remain poorly explored. For instance, seed production may vary with inflorescence phenology, as a result of progressive resource depletion during ontogeny and/or phenological variation in floral display intensity, which may also be related to inflorescence size. In any case, the simultaneous availability of flowers may also increase the attraction to

pollinators and the incidence of seed predators. In addition, a higher number of open flowers may enhance pollinator visits (Kudo & Harder 2005), but may also produce a relatively lower proportion of probed flowers and a higher frequency of geitonogamous self-pollination (Mitchell *et al.* 2004; Miyake & Sakai 2005). The balance between pollination probability and predation risk may determine final fruit production (Krupnick *et al.* 1999; Mothershead & Marquis 2000; Herrera 2000) and, thus, may have shaped flowering and fruiting phenology in the course of evolution (Augsburger 1981; Gómez 1993; Brody 1997).

In this context, we conducted a field survey on seed production of *Ononis fruticosa*, a Mediterranean leguminous shrub. A complete set of reproductive variables were estimated to test the hypothesis that variability in seed production is related to: (i) inflorescence phenology; (ii) fruit position along the inflorescence axis; and (iii) inflorescence size. Since the effect of the factors involved may appear hierarchically structured, we built our models by means of hierarchical mixed generalised models, that is, with fruits nested in inflorescences, inflorescences nested in plants, and plants nested in populations. The final output of this complex balance may depend on the analysis scale and, thus, variables have been independently modelled at the plant and fruit scale when possible (Albert *et al.* 2001; Aragón *et al.* 2007).

MATERIALS AND METHODS

Study species

Ononis fruticosa L. (Fabaceae) is a shrub which occurs in the western half of the Mediterranean basin. In the Iberian Peninsula, it is mainly found in the eastern half over a wide altitudinal range (400–1500 m) on calcicole and gypsum outcrops. Knowledge of the biology of this species is scarce and mainly deals with developmental physiology (Palacio & Monserrat-Martí 2006; Palacio *et al.* 2006) and factors controlling seed germination after fire (De Luis *et al.* 2006).

Flowers are grouped (1–3) in axillary units that form a paniculated lax inflorescence. Fruits are 16–30 mm pods containing 3–4 seeds. Pollination seems to be completely dependent on pollinator activity, as bagged inflorescences did not produce any fruits (Sánchez, unpublished data).

Study site and data collection

This study was conducted in 2004 near Morata de Tajuña, 40 km southeast of Madrid, central Spain (40°12'N, 3°25'W, 660 m a.s.l.). The climate is dry Mesomediterranean with a mean annual temperature of 14.2 °C and an average annual rainfall of 438 mm (data from Arganda del Rey thermopluviometric station, $n = 29$ years). 2004 was a relatively wet year (540 mm). The area is covered by a mosaic of gypsum and calcicole vegetation and traditional olive groves.

Two *Ononis fruticosa* populations were selected in the study area. The populations were located on a northwest-facing slope with a distance between them of around 1 km. Thirty reproductive plants per population were randomly selected, tagged and measured (height and two maximum crown diameters) before the onset of flowering. Plant volume was estimated as an ellipsoid ($V = 4/3\pi abc$). Distance from each individual to the three nearest *O. fruticosa* neighbours was also recorded.

Plants were visited every 10 days during the blooming period (from April to July). At each census, we counted the number of inflorescences in bloom per plant and estimated inflorescence size through the number of flowers and flower pedicels (sign of previous presence of flowers). Up to 20 inflorescences were marked with coloured plastic strips to identify inflorescence flowering date. Mature seed pods from marked inflorescences were later harvested and stored in paper bags. Inflorescence phenology and seed pod position on the inflorescence axis was noted before harvesting. Each fruit was assigned to one of three phenology periods: 1 May–20 May, 21 May–10 June, 11 June–30 June 2004; and to one of three position categories, defined according to inflorescence size: basal (1–3 most basal position pods), apical (1–3 most distal position pods) and intermediate (pods not included in the two previous categories).

In order to estimate fruit set per plant we calculated the total number of flowers and fruits per plant. Total number of flowers per plant was estimated as the product of three variables: (i) number of inflorescences per plant (sum of inflorescences in bloom observed throughout all visits); (ii) number of pedicels per inflorescence (counted in 20 inflorescences and 30 plants); and (iii) number of flowers per pedicel (counted in up to 130 pedicels belonging to 11 different plants). Total number of fruits per plant was recorded when flowering was finished. Persistent calyx presence was considered an indicator of previously dispersed pods.

All collected pods were dissected in the laboratory and signs of herbivory were noted. Number of non-fertilised ovules, mature seeds and aborted seeds were recorded in those pods without signs of predation. Mature seeds were weighed to the nearest 0.0001 g. Seeds were mechanically scarified and placed on moistened filter paper in Petri dishes, noting their mother plant and inflorescence flowering date. All Petri dishes were placed in a germination chamber at 20 °C and 16 h/8 h photoperiod. Germination was monitored every 2–3 days over 50 days.

Total number of ovules was used as an indicator of initial reproductive investment. Since the observed rate of passive autogamy was null (Sánchez, unpublished data), pollination success was estimated by recording the number of fecundated ovules (mature seeds + aborted seeds) per fruit, and ovule set as the percentage of fecundated ovules per fruit (Augsburger 1981). Number of mature seeds and seed set (percentage of mature seeds per fruit) were used as a measure of reproductive success after predispersal seed predation. The number of aborted seeds

was recorded and abortion rate was calculated as the percentage of aborted seeds per fruit.

In order to estimate some phenological predictors, we considered flowering peak as the moment when the maximum number of inflorescences was in bloom. Flowering synchrony (S_i ; flowering overlap with any other plant in the studied population), was calculated as follows (Augspurger 1983):

$$S_i = \frac{1}{n-1} \sum_{j \neq i} \frac{a_{ij}}{b_i}$$

where n is the total number of plants in the studied population, b_i is the number of days plant i is in flower, and a_{ij} is the number of days plant i and j overlap in their flowering periods. This index ranges between 0 and 1 (0 = no overlap; 1 = highest synchrony).

Data analysis

Collected data were used to analyse female reproductive output at two different scales: fruit and plant. Data had an unbalanced, hierarchical structure at both scales with fruits nested within inflorescences, inflorescences nested within plants and plants nested within populations. In order to overcome the correlation among data points due to our data structure, we used a multilevel approach considering inflorescences, plants and populations as random factors and applying mixed modelling (Verbeke & Molenberghs 1997). As the response variables clearly departed from the standard normality assumption, we used generalised linear mixed models (GLMM; McCullagh & Nelder 1989). Error distribution of the response variables was

checked to choose the appropriate response and link function. We used the restricted maximum-likelihood (REML) method and, because our data were unbalanced, we used Satterthwaite's method to determine the approximate denominator degrees of freedom for tests (Verbeke & Molenberghs 1997).

Dependent variables at the fruit scale were: initial reproductive investment, number of fecundated ovules, ovule set, mature seeds, seed set, number of aborted seeds, abortion rate and predation occurrence. GLMMs performed to explain variability in these variables considered fruits within an inflorescence, inflorescences within plants, plants within populations and population as random factors. Some fixed factors, which *a priori* may be relevant for reproduction at the fruit scale, were also included: inflorescence flowering time, inflorescence size, pod position on the inflorescence axis, plant size, neighbour distance, plant flowering peak and plant flowering synchrony (Table 1).

Total number of flowers and fruits, and fruit set were dependent variables used to analyse female reproductive output at the plant scale. Predictors included population, as a random factor, and plant size, neighbour distance, flowering peak and synchrony and total flower production as fixed factors (Table 1).

Seed weight and germination percentage were also modelled with GLMM. A model was performed with individual seed weight data, with plants within population and population as random factors. Fixed factors for this model were all the variables in Table 1. Another model was fitted to explain variability in mean seed weight per plant and per inflorescence flowering time. In this last

Table 1. Description of the independent variables used in GLMM analyses to explain variability in female reproductive output at the fruit and plant scale and variability in seed weight.

level	variable	description
plant	Plant size	Ellipsoid volume ($V = 4/3\pi abc$)
	Neighbour distance	Mean distance to three nearest neighbour
	Flowering synchrony	Mean number of days that the flowering of a plant overlaps with the flowering of every other plant in the population
	Flowering peak	Moment with a maximum number of inflorescences in bloom
	Flower production	Total number of flowers estimated at the end of the season
	Fruit production	Total number of fruits estimated at the end of the season
	Fruit set	Percentage of flowers becoming fruits, estimated at the end of the season
inflorescence	Flowering time	Inflorescence blooming moment
	Inflorescence size	Number of pedicels
fruit	Pod position	Position on the inflorescence axis

case, population was included as a random factor and all those explicative at the plant level as fixed factors (Table 1).

Germination percentage was modelled as percentage of germinated seeds per Petri dish after 50 days of incubation, with population and plant as random factors, and inflorescence flowering time as a fixed factor.

Due to the complexity of the models and the relatively large number of predictors, we only considered main effects. Effects of random factors were tested using Wald Z-statistic tests in those models with large sample size: all models in Table 4 ($n > 300$) and the model on individual seed weight ($n = 428$). For those models at the plant scale ($n = 60$, see Table 5) and on mean seed weight per plant ($n = 42$) and germination percentage ($n = 53$), a random factor effect was tested with a log-likelihood ratio test, as recommended for relatively small sample sizes (Little *et al.* 2006). Fixed factors were tested with F-tests because a quasilielihood algorithm was used to overcome some difficulties in the nature of the data (Little *et al.* 2006).

Seed weight was also modelled with GLMM. A model was prepared with individual seed weight data (dependent variable, $n = 428$), with plants within a population and population as random factors. Fixed factors for this model were all the variables in Table 1. Another model was fitted to explain variability in mean seed weight per plant ($n = 42$) and per inflorescence flowering time. In this last case, population was included as a random factor and all those explainable at the plant level as fixed factors (Table 1).

Germination percentage was analysed with GLMM, with percentage of germinated seeds per Petri dish ($n = 53$) after 50 days of incubation as a dependent variable, population and plant as random factors, and inflorescence flowering time as a fixed factor.

All GLMM computations were performed using SAS Macro program GLIMMIX, which iteratively calls SAS Procedure Mixed until convergence (GLIMMIX ver. 8 for SAS/STAT; available on <http://www.sas.com.techsup/download/stat/>).

RESULTS

Flowering phenology at the two studied populations lasted from mid-April to early July, with the flowering peak in mid-June and an average synchrony of 0.77 ± 0.12 among plants (Table 2). Plant size ranged from less than 0.1 to 2.9 m^3 . Mean distance to the three nearest neighbours was around 1 m. However, this distance increased to over 2 m for more isolated plants (Table 2). All selected plants flowered, and only two plants produced no fruits. Total number of flowers per plant varied greatly, ranging from 33 to 5143 flowers. Total number of fruits per plant ranged from 0 to 1124 pods and mean fruit production was 187.93 ± 243.10 . Mean fruit set was 17.82 ± 13.76 (Table 2). A total of 974 fruits were examined and 67.36% were parasitised. Inspection of non-parasitised fruits showed that mean

Table 2. Mean values (\pm SD) of explanatory variables at the plant scale.

	plant size (m^3)	neighbour distance (cm)	flowering synchrony	flowering peak	flower production	fruit production	fruit set	seed weight (mg)	germination %
population 1	0.71 ± 0.60	100.11 ± 69.01	0.75 ± 0.12	59.90 ± 14.97	927.94 ± 1026.91	180.81 ± 289.49	12.94 ± 10.60	9.4 ± 1.7	46.00 ± 26.25
population 2	0.30 ± 0.31	104.44 ± 61.94	0.80 ± 0.12	62.93 ± 9.86	1031.78 ± 922.85	194.33 ± 197.21	22.71 ± 14.94	9.4 ± 1.6	67.12 ± 28.63

Table 3. Mean values (\pm SD) for different stages of ovule development within fruits of *Ononis fruticosa*.

	examined fruits	parasitised fruits %	ovules	aborted seeds	mature seeds
population 1	443	67.04	15.18 \pm 2.71	1.97 \pm 2.91	2.36 \pm 1.79
population 2	531	67.42	16.28 \pm 2.66	2.35 \pm 1.99	2.06 \pm 2.32
total	974	67.36	15.78 \pm 2.73	2.20 \pm 2.10	2.17 \pm 2.46

Table 4. Generalised linear mixed models used at the fruit scale.

level	variable	models				
		ovules	fecundated ovules	aborted seeds	mature seeds	predation incidence
population	Residual population variance component	*	n.s.	n.s.	n.s.	n.s.
plant	Plant size	n.s.	n.s.	n.s.	n.s.	n.s.
	Neighbour distance	+, **	-, *	n.s.	n.s.	n.s.
inflorescence	Flowering peak	n.s.	n.s.	n.s.	n.s.	n.s.
	Flowering synchrony	n.s.	n.s.	-, *	n.s.	n.s.
	Residual plant (pop.) variance component	*	*	**	*	*
	Flowering time	-, *	-, **	-, *	-, *	n.s.
	Inflorescence size	n.s.	n.s.	+, **	n.s.	n.s.
fruit	Residual inflor. (plant) variance component	-	***	***	**	*
	Pod position	-, ***	-, *	n.s.	n.s.	-, *
	Residual fruit (inflor.) variance component	***	***	***	***	***

Signs (+, -) show the sign of each estimated coefficient and asterisks show significance for the Wald test (random effects) and Type III F-tests (fixed effects): * \leq 0.05; ** \leq 0.01; *** \leq 0.001; n.s. = non-significant.

seed set was 13.75%, and average abortion rate reached 13.94% for the two studied populations (Table 3).

Female reproductive output at the fruit scale

As 18 plants produced no fruits from marked flowers, they were excluded from the fruit-scale analysis. Thus, 42 plants and 974 mature fruits were considered. Signs of predation were found in 656 fruits, which were excluded from all fruit-scale models except for predation occurrence. None of the predictors were significantly related to ovule set, seed set and percentage of aborted seeds per fruit. The other models revealed that all dependent variables showed significant residual variability among fruits, inflorescences and plants. However, only number of ovules varied between the two studied populations (Table 4). Estimates suggested a decreasing amount of variability from the fruit to the population level; that is, there was more variability among fruits within inflorescences than among inflorescences within plants or among plants within populations.

Number of ovules per fruit was higher for more isolated plants, earlier inflorescences (Fig. 1a) and flowers in basal positions in the inflorescence (Fig. 2). However, number of fecundated ovules per fruit was lower for more isolated plants, but higher in earlier inflorescences (Fig. 1b) and at basal positions in the inflorescence. Number of aborted ovules was higher in less synchronous plants and in earlier and larger inflorescences. Finally,

earlier inflorescences produced a higher number of mature seeds per fruit (Fig. 1c) and predation occurrence was higher in fruits in basal positions (Table 4).

Female reproductive output at the plant scale

The residual population variance component was not significant in any of the three models examined. Observed number of flowers was higher in plants with a later flowering peak. Total number of fruits was higher for plants with a greater distance to their neighbours, and plant fruit set was positively related to neighbour distance (Table 5).

Seed weight and germination

Models based on individual seed weight values ($n = 428$) revealed that the residual plant variance component significantly differed from zero ($Z = 2.52$, $P = 0.058$) and that earlier inflorescences produced heavier seeds ($F = 29.25$, $P < 0.0001$) (Fig. 1d). Models based on mean weight values per plant and per inflorescence flowering time showed that plants with an earlier flowering peak ($F = 6.75$, $P = 0.014$) and earlier inflorescences ($F = 5.49$, $P = 0.025$) produced heavier seeds. Mean germination percentage after 50 days of incubation was 59.56 ± 29.65 , and germination percentage was higher for those seeds from later inflorescences ($F = 9.14$, $P = 0.006$).

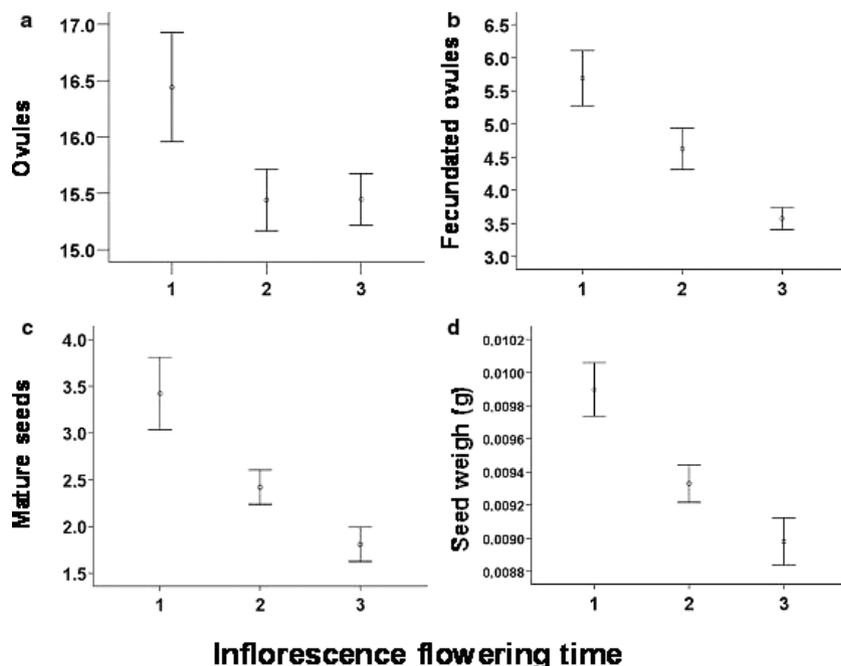


Fig. 1. Effect of inflorescence flowering phenology on number of ovules per fruit (a), fecundated ovules per fruit (b), mature seeds per fruit (c), and seed weight (d), for the 60 *Ononis fruticosa* plants studied (mean ± SE) (1 = 1 May–20 May; 2 = 21 May–10 June; 3 = 11 June–30 June 2004).

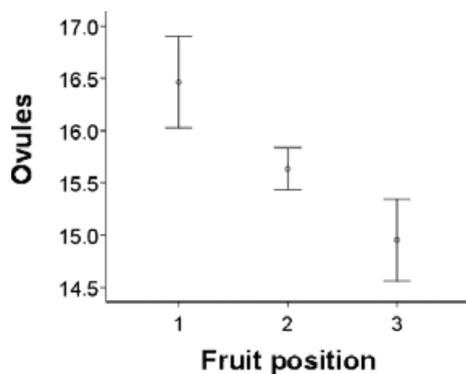


Fig. 2. Effect of fruit position on the inflorescence on number of ovules per fruit for the 60 *Ononis fruticosa* plants studied (mean ± SE) (1 = basal; 2 = intermediate; 3 = apical).

Table 5. Generalised linear mixed models used at the plant scale.

model	fixed factors			
	size	neighbour distance	flowering peak	synchrony
flowers	n.s.	n.s.	+, ***	–
fruits	n.s.	+, **	n.s.	n.s.
fruit set	n.s.	+, *	n.s.	n.s.

Signs (+, –) show the sign of each estimated coefficient and asterisks show significance for Type III F-tests (fixed effects): *≤0.05; **≤0.01; ***≤0.001; n.s. = non-significant.

DISCUSSION

Inflorescence effects were found in *Ononis fruticosa* at both the flower and plant scale. The most important effect in seed production was inflorescence flowering time (Fig. 1). Earlier inflorescences presented a higher number of ovules, fecundated ovules, mature seeds and aborted seeds (Table 4). Our results suggest that the widely known correlation between flowering time and female reproductive output (Kelly & Levin 2000; Galloway 2002) may be mediated in certain cases by inflorescence phenology. Among the mechanisms that have been proposed (Rathcke & Lacey 1985), resource limitation seems the most likely process in this shrub. As the reproductive season progresses, maternal resources are divided among an increasing number of structures, producing a decrease in ovules per fruit, seeds per fruit and seed weight (Medrano *et al.* 2000; Holland *et al.* 2004). It is widely known that weather affects reproduction in Mediterranean plants (García *et al.* 2002) due to water shortage (Gómez 1993; Rabasa *et al.* 2007). *Ononis fruticosa* suffers an extended Mediterranean summer drought, and low soil water content probably limits nutrient uptake early in the season (Aragón *et al.* 2007).

The preliminary analysis of variation in the number of fecundated ovules per fruit suggests that pollination limitation may also have occurred. However, ovule set (percentage of swollen ovules per fruit) did not vary significantly over the study period, showing similar pollination success independent of inflorescence flowering

time. Thus, variability in fecundated ovules seems to be due to variation in the number of ovules per flower over the flowering period rather than to variation in pollinator availability. Another outstanding inflorescence effect was that larger inflorescences presented a higher number of aborted seeds per fruit. The most plausible explanation is the higher probability of geitonogamous crosses promoted by successive insect probing on flowers of the same inflorescence in relation to a higher floral display (Mitchell *et al.* 2004; Miyake & Sakai 2005). Alternatively, this higher abortion rate could be due to resource limitation, as larger inflorescences had to distribute their resources among a higher number of fruits (Holland *et al.* 2004). All these findings seem to be translated in terms of seed weight, since seeds coming from earlier inflorescences were heavier. However, seed weight variation was not correlated with an increase in seed germination percentage under controlled conditions.

Both inflorescence phenology and pod position on the inflorescence axis were relevant for final seed production per fruit. Basal pods had a higher number of ovules (Fig. 2) and fecundated ovules, but suffered a higher risk of predation. Experimental studies should be carried out in order to dissect the potential effect of plant architecture and resource limitation on ovule number (Ashman & Hitchens 2000; Medrano *et al.* 2000; Wolfe & Denton 2001). In any case, predation risk seems to be more related to the behaviour of herbivores which may actively select pods with higher seed content. Seed predation seems to be the main factor controlling final seed production in *O. fruticosa*, since more than 65% of the inspected fruits showed some sign of predation. Moreover, predation affected plants and inflorescences of all sizes throughout the reproductive period (Table 4). High rates of pre-dispersal seed predation have been found in other *Fabaceae* shrubs (Bossard 1991; Rabasa *et al.* 2007) and in other Mediterranean shrubs (Herrera 1993).

Among plant level predictors, distance to nearest neighbours was the most relevant for female reproductive output at both the fruit and plant scale. Pods of more isolated plants had a higher number of ovules per flower, and at the plant scale, they had higher total fruit production and fruit set. This may be due to intra-specific competition. Several studies in arid and semiarid environments have shown that little distance variation among plants can be critical in determining the level of soil interference among plants (Fowler 1986). However, ovule set (just marginally significant, $F = 3.35$, $P = 0.074$) and number of fecundated ovules (Table 4) decreased as distance increased, probably due to the effect of plant density on pollinator activity (Grindeland *et al.* 2005) and the need for xenogamous pollen.

Our modelling approach suggests that variation decreases from fruit to population. In addition, inflorescence predictors best explained the observed variability among fruits, and thus were more relevant for female reproductive output. More specifically, our results revealed, as hypothesised, that the most explanatory factor

is inflorescence phenology, and secondarily flower position on the inflorescence axis. Both sources of variation suggest a certain level of resource limitation as the main underlying process controlling final female reproductive output. However, an experimental approach is needed to test the importance of resource limitation and other alternative hypotheses. These findings suggest that inflorescence scale should be taken into account in future studies of the processes involved in fertility, as some of these processes cannot be detected at the plant scale.

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