Small-size bees reduce male fitness of the flowers of *Ononis masquillierii* (Fabaceae), a rare endemic plant in the northern Apennines

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Most plant species are visited by a diverse array of visitors that may contribute differently to reproductive success. In particular, the size of visitors, relative to flower size, may have severe implications for plant fitness, as it can affect the mechanics of the pollination process. *Ononis masquillierii*, a rare endemic legume in northern Italy, is visited by numerous bee species of different families and sizes. To evaluate the effect of bee guilds on seed set, we compared the relative efficiency of four size classes of bees visiting flowers in eight plant populations occurring in the Apennines. Size classes were assorted by body weight. Reproductive success was positively related to plant population size and affected by the visitation frequency of the bee size guilds: higher visitation rates by larger bees (medium–large Andrenidae and large Apidae) increased seed set, whereas high visitation rates by smaller bees (small Halictidae and small–medium Megachilidae) reduced seed set. We conclude that the influence of bee size on seed set is related to the pollen foraging techniques of the different bee guilds. Larger bees paying short visits to flowers remove one 'batch' of pollen and transfer it to another flower. In contrast, smaller bees enter the flower entirely and activate the pump-like pollen dispensing mechanism several times, resulting in a higher degree of selfing. These behaviours may result in differential seed sets as the flowers are self-incompatible, i.e. seed set may be reduced after visitation by smaller bees. Such variations in pollinator quality among populations coupled with the ephemeral nature of the habitat of *O. masquillierii* may represent a risk for its persistence. Our results show that such a risk can be confronted only through the conservation of large plant populations and of large bees capable of flying long-distances.   © 2011 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2011, **165**, 267–277.

ADDITIONAL KEYWORDS: foraging techniques – plant rarity – pollinator size – reproductive success – seed set – visitation frequency.

INTRODUCTION

Most entomophilous plant species are visited by a wide suite of pollinator species, the contribution of which to the pollination process may be highly variable. The relationship between pollinator morphology and floral traits is an important driver in the mechanics of pollination (Fukuda, Suzuki & Murata, 2001; Walker-Larsen & Harder, 2001; Dieringer & Cabrera, 2002) and variations in pollinator morphology may influence their effectiveness as pollinators, which again may play a critical role in determining plant reproductive success, through both male and female function. It has also been shown that behavioural

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patterns of pollinators on or in flowers may have important implications for pollination success (Potts, 2005; Young, Dunning & von Hasseln, 2007).

The size of pollinators in relation to flower size is an important attribute in pollination mechanics, as it can interfere with several aspects of the pollination process. Such aspects can be the amount and positioning of pollen adhered to the body of the pollinator (Tepedino, Sipes & Griswold, 1999), the ability of buzzing behaviour while foraging (Liu & Koptur, 2003) and the ability to trigger specialized pollination mechanisms (Stout, 2000; Pérez-Bañón *et al.*, 2003). Furthermore, foraging techniques by different pollinators tend to differ strongly in terms of flower handling time, which again can affect several components of the pollination process, such as the number of flowers visited per foraging bout, the distance travelled between flowers and the amount of pollen moved between flowers (Strickler, 1979; Konner, Davis & Rush, 1995; Hurlbert *et al.*, 1996; Gómez & Zamora, 1999; Ivey, Martinez & Wyatt, 2003). For example, pollinators that tend to fly short distances may potentially increase the number of within-plant movements as compared with long-distance fliers, and consequently reduce the quality component of pollination through increased selfing rates (Herrera, 1987).

The effectiveness of different pollinators can be estimated through quantification of the amount of pollen removed and/or deposited on floral stigmas (Herrera, 1987; Fishbein & Venable, 1996; Canto-Aguilar & Parra-Tabla, 2000), estimation of seed set after single (Dieringer, 1992; Olsen, 1997; Kandori, 2002) or multiple flower visits (Pérez-Bañón, Petanidou & Marcos-García, 2007) and analysis of external pollen load on pollinator individuals (Petanidou *et al.*, 1995, 1998; Tepedino *et al.*, 1999; Moeller, 2005; Pérez-Bañón *et al.*, 2007). The importance of each pollinator can then be calculated as the product of its visitation frequency and effectiveness. Quantifying pollinator effectiveness is, however, rarely feasible, as obtaining reliable assessment of pollinator efficiency is time-consuming. Therefore, the assessment of the contributions of different pollinators to the reproductive success of a plant species has to rely on easy-to-measure surrogates alone (e.g. visitation frequency). Recent studies have investigated the relative value of visitation frequency by a particular pollinator species to a particular plant species (Vázquez, Morris & Jordano, 2005) and considered the importance of pollinator visitation rate in determining pollinator importance (Sahli & Conner, 2006, 2007). These studies clearly show that visitation frequency is essential in determining the importance of a pollinator, whereas the contribution of pollinator effectiveness can be marginal. These results suggest that determining the most important pollinator(s) of a

plant species in many instances can be achieved by use of visitation frequency observations only. However, for plants with more specialized pollination mechanisms, this might not be the case and the importance of each pollinator must be evaluated through visitation frequency data coupled with observations of pollinator effectiveness during flower visits.

Variation in visitation frequency and pollinator effectiveness can have serious consequences for plant reproduction, especially for rare plant species (Lau & Galloway, 2004) that depend on pollinators for seed set and have low and/or unpredictable pollinator availability (Pérez-Bañón *et al.*, 2003, 2007). Small and isolated plant populations may be prone to environmental, demographic and genetic hazards and to pollination limitation, increasing the likelihood of stochastic extinction (Menges, 1992; Young, Boyle & Brown, 1996; Holsinger, 2000; Oostermeijer *et al.*, 2000; Dauber *et al.*, 2010).

In this study we examined the pollinator-dependent reproductive vulnerability of *Ononis masquillierii* Bertol. (Fabaceae), a rare endemic from the northern Apennine range (Italy). In particular, we assessed the pollination success of *O. masquillierii* in populations with contrasting pollinator community composition. We grouped pollinators on the basis of their size (weight) and evaluated the contribution to seed set of each group separately, in terms of visitation frequency. The main aims of the study are to: (1) quantify the contribution to seed set in *O. masquillierii* of different pollinator size classes; and (2) illustrate the role of pollinator body size in the pollination process.

MATERIAL AND METHODS

STUDY AREA, PLANT SPECIES AND POPULATIONS

We conducted this study in June and July 2006 inside the 'Gessi Bolognesi e Calanchi dell' Abbadessa' Regional Park, Italy. The park (*c*. 5000 ha) is located in the hills south-east of the town of Bologna and is characterized by a series of gypsum outcrops and a deep karst system with large eroded landforms (gullies). Before the designation of the park in 1988, the area was strongly influenced by human-induced disturbances, mainly mining, grazing and crop cultivation. Today the park is heterogeneous, with sinkholes, woods, closed valleys, cliffs and gullies.

Ononis masquillierii is a perennial subshrub, endemic to the northern Apennines (Italy), from Emilia Romagna to the northern part of Marche and Tuscany. Preferred habitats are sunny slopes on dry, clay soils at elevations up to 600 m a.s.l. Such areas, locally known as 'calanchi', are characterized by parallel or fan-shaped furrows created by gully erosion and constitute fragile and unpredictable habitats for

Table 1. Characteristics of the eight study populations of *Ononis masquillierii* studied in 2006

Population	Altitude (m a.s.l.)	Location		Population size (no. of flowering racemes)		Population density (no. of flowering racemes/m²)	
		Latitude	Longitude	Round 1	Round 2	Round 1	Round 2
Abbadessa	215	44°24′29″N	11°26′42″E	605	681	0.72	0.81
Casola Canina	252	44°24′21″N	11°24′40″E	1151	2830	0.24	0.60
Castel de Britti	232	44°25′06″N	11°26′26″E	148	153	1.52	1.57
Croara	239	44°25′34″N	11°23′28″E	547	362	1.89	1.25
Eremo	290	44°24′47″N	11°24′02″E	1056	989	2.48	2.32
Flaminia	242	44°23′21″N	11°27′11″E	2550	4600	0.73	1.31
Monte Calvo	345	44°25′33″N	11°22′26″E	640	833	6.15	8.01
Monte Pieve	244	44°24′27″N	11°28′09″E	1057	606	1.12	0.64

a,s,l, above sea level.

plants. These habitats are highly fragmented as a result of the geological past and the history of human land use in the area (Petanidou & Lamborn, 2005).

Ononis masquillierii grows up to 40 cm high and reproduces both vegetatively and through seed set. Flowering takes place from late May to early July. Flowers are zygomorphic, with five petals arranged in a papilionoid structure. Flowers have a small, pink corolla (11–15 mm in depth) which exceeds the calyx. Flowers are usually arranged in dense racemes. The number of ovules per flower is invariably six (Galloni *et al.*, 2007). The zygomorphic outline of the flower suggests that it is pollinated predominantly by bees (Faegri & van der Pijl, 1979). Previous authors have described four main mechanisms of secondary pollen presentation for Fabaceae (brush, piston or pump, valvular and explosive; Galloni *et al.*, 2007 and references therein). Secondary pollen presentation implies the relocation of pollen from the anthers onto another structure of the flower, which acts as the presenting organ for pollination. For *O. masquillieri,* pollen presentation is mediated by a two-step 'pump' mechanism (Arroyo, 1981; López *et al.*, 1999; Galloni *et al.*, 2007). First, the pollen is released onto the keel petals and, second, as the pollinator enters the flower exerting pressure on the keel, a pump-like movement of the style and stamens extrudes a small amount of pollen through an opening at the keel tip. Pollen grains then adhere to the abdomen of the insect (sternotribic contact), and, because of the gradual release of pollen, the same flower can present pollen to a series of pollinators visiting the flower one after the other. The pump pollen release mechanism is present in other genera of Fabaceae and has already been found in other species of *Ononis* L. (Small, 1986; López *et al.*, 1999; Galloni *et al.*, 2007)

We selected eight study populations of *O. masquillierii*, six inside the park and two just outside the park limits, at elevations between 215 and 345 m a.s.l. (Table 1). All the study populations were separated by > 1 km or/and by natural barriers too difficult to be crossed by bees (e.g. dense woods). Within each population we selected four patches of flowering racemes, each at a distance of at least 2 m from the other patches. Patches were selected to be representative of each study population and were used to observe pollinator visitation rates to the flowers. Number of flowering racemes per patch ranged between 19 and 1743 (average = 268 ± 41 SE, $N = 64$). Voucher specimens from all study populations are preserved at the Herbarium of the University of Bologna (BOLO).

ANTHESIS

In order to determine the flower lifespan, we followed 20 flowers from bud to withering in the largest of the eight populations (Flaminia; Table 1). We selected the buds on 20 different racemes, separated by ≥ 1 m. We also ensured that the buds were located in different positions within the racemes. We marked the buds and followed them every 4 h during daytime, i.e. from 08:00 to 20:00 h. Anthesis was considered to start when the flag (posterior petal) was fully erect, i.e. when pollinator visits were possible.

We assessed stigma receptivity and pollen viability on 15 flowers of different anthesis stages (i.e. five buds, five freshly open and five senescent flowers) all randomly selected in the same population (Flaminia). Assessment of stigma receptivity was carried out by using the Peroxtesmo KO test (Macherey-Nagel 90609). We tested pollen viability using the 3,3′-diaminobenzidine (DAB) test (Sigma D-4168, according to Dafni, Pacini & Nepi, 2005).

Although there are no nectariferous species of *Ononis* (Muller, 1883), we carried out some measure-

ments in order to make sure that flowers of *O. masquillierii* were exclusively pollen-rewarding. Nectar was measured as standing crop on 15 flowers, in the second large population (Casola Canina), by inserting Drummond microcaps into the staminal column.

POLLINATION TESTS

Information on the plant breeding system was collected through pollination treatments applied to 20 racemes selected at random in the vicinity of the four patches (five plants per patch) in the Flaminia population. When the stigma was receptive, one flower was hand pollinated with pollen from two flowers within the same raceme (self-pollination to test the self-compatibility level); a second flower was hand pollinated with pollen from two flowers from two racemes found at a distance of > 10 m from the recipient (cross-pollination to test the maximum seed set potential); and a third flower was untreated to test for spontaneous self-pollination. All three flowers in each raceme were covered by a hydrophilic plastic bag (Osmolux) to prevent insect visits after pollination treatment. In order to test for unintentional effects of the bag on flower, fruit and seed development, we compared the seed set resulting from the above hand-crossing treatments with hand-crossing treatments performed in the same population (Flaminia) but without using plastic bags.

To measure natural seed set after open pollination, we chose 40 racemes in each study population at random (ten in the vicinity of each of the four patches). On each raceme, we randomly selected and marked one freshly open flower and left it uncovered to be freely visited by insects. To assess the contribution of insect activity to seed set, we performed supplementary hand-pollination tests on additional flowers selected in each of the 40 racemes. On each raceme, we randomly selected, marked and cross-pollinated by hand one freshly open, receptive flower. As above, cross-pollination by hand was carried out using pollen collected from two distant plants or racemes (> 10 m from the recipient). After manual crossing we left the flowers unbagged until fruit harvest.

Each hand pollination was performed as a single application of an abundant quantity of pollen to the recipient flower. In all flowers used as pollen donors and recipients, we ensured maximum pollen viability and stigma receptivity based on the results of the viability/receptivity tests described above. We harvested fruits approximately 30 days after all treatments. Recovery of the marked flowers was between 75 and 100% for the different treatments (Table 3). We determined reproductive success resulting from the different treatments for each population (pooled over all patches) and we expressed it as the number of viable seeds per flower treated.

POLLINATOR VISITATION

In each plant population we monitored pollinator activity twice during peak flowering. Observations were made during optimal weather conditions, i.e. with a temperature > 12 °C and avoiding cloudy, rainy or windy days. Each observation round lasted 1 day and consisted of four 15-min observations carried out in the morning and another four in the afternoon (two 15-min observations per patch, one in the morning and one in the afternoon). We randomized the sequence in which the eight plant populations were observed and the order in which the patches were observed within each population. We carried out the pollinator observations in a portion of each patch (hereafter called 'observation unit'). Observation units varied in shape and size, but contained between ten and 100 flowers, depending on the size and the number of plants in the patch. During each observation period, we recorded each insect that entered the observation unit and visited at least one flower. For each insect we recorded its identity (see below for the taxonomic resolution applied), flower handling time (the amount of time spent per flower) and behaviour on flowers (positioning and movements on and in flowers). We considered only bees as pollinators and we assigned them, on the wing, roughly to genus (*Lasioglossum*, *Megachile*, *Bombus,* etc.). We also observed a limited number of butterflies and wasps visiting the flowers. However, as they did not contact any floral reproductive organs (wasps did not land on flowers and butterflies were too light to activate the pollen dispenser mechanism), we did not consider them as pollinators and excluded them from the analyses. Following the four observation periods, we spent another 30 min collecting bees visiting *O. masquillierii* flowers within the plant population. We captured bees that had just visited a flower using a net, killed them with ethyl acetate and put them in separate vials for further analysis in the laboratory, including taxonomic identification and examination of pollen carried on the body.

We identified all collected bees to genus and individually weighed them in the laboratory. Based on their weight, we assigned the bees to the following size classes (hereafter referred to as 'size guilds'): small bees (including *Lasioglossum* spp.), small–medium bees (including *Megachile*, *Osmia* and *Anthidium* spp.), medium–large bees (including *Andrena* and *Eucera* spp.), and large bees (including *Anthophora* and *Bombus* spp.) (Table 2). The four size groups significantly differed in body weight (Kruskal–Wallis test $H_{(3,112)} = 91.717$, $P < 0.001$; Mann–Whitney

Table 2. Flower visiting bees collected in the eight study populations of *Ononis masquillierii*

Taxon	Body dry weight (g, mean ± SE)	Bee size guild	N
Halictidae			
Lasioglossum spp.	0.0046 ± 0.0005	Small bees	26
Andrenidae			
Andrena spp.	0.039 ± 0.002	Medium–large bees	12
Apidae			
Eucera spp.	0.052 ± 0.003	Medium–large bees	24
Anthophora spp.	0.084 ± 0.001	Large bees	2
Bombus spp.	0.070 ± 0.005	Large bees	13
Megachilidae			
Anthidium s.l. spp.	0.024 ± 0.004	Small–medium bees	6
Osmia s.l. spp.	0.022 ± 0.002	Small–medium bees	22
Megachile s.l. spp.	0.018 ± 0.002	Small–medium bees	7

N refers to the number of individuals caught from all eight populations.
Total time spent in each population was 2 h spread over two observation rounds.
SE, standard error.

U-test: $0 < U < 1198$, $P < 0.001$, with Bonferroni corrections applied). At the end, the pollinator observations were matched with the identifications of the bees collected in the field, so that each bee observed visiting a flower was assigned to a particular size guild.

For each plant population, we calculated visitation rate for the different pollinator size guilds as the mean number of visits to *O. masquillierii* per flower per hour, based on all observations conducted in the population. We pooled pollinator observation data from all four patches in each population in all analyses of pollinator activity. Our analyses are therefore based on variations among populations and do not concern variation observed at the patch level. All insect specimens collected are held at the Department of Evolutionary Experimental Biology, University of Bologna.

INSECT FIDELITY

We examined insect fidelity based on the identity of the pollen adhered to the bodies of pollinators that we collected just after they had visited a flower of *O. masquillierii*. We removed pollen samples from the insect bodies using standard size round pieces (5 mm in diameter) of fuchsin glycerine-gel using the protocol of Beattie (1971) and Petanidou *et al.* (1995). We pressed once and firmly a piece of gel on the part of the body that was responsible for the active collection of pollen by bees (corbiculae for bumblebees, ventral scopae for Megachilidae and hind legs for *Andrena*, *Anthophora*, *Eucera* and *Lasioglossum*) and then fixed the pieces on slides. We counted pollen grains under a light microscope (all pollen grains per slide for Megachilidae and Halictidae; ten transects per slide for the remaining pollinator groups). We expressed fidelity as the proportion of *O. masquillierii* pollen grains out of the total number of pollen grains counted (*Ononis masquillierii* + foreign). Pollen of different species of the genus *Ononis* could not be clearly differentiated under the microscope, but we assumed that *O. masquillierii* pollen was dominant in our samples as it was the only *Ononis* sp. in the study areas and the pollinators were collected after they had visited a flower of *O. masquillierii*.

DATA ANALYSES

To assess how pollinators (grouped into size guilds) might affect the seed set of *O. masquillierii*, we ran two separate multiple regression analyses. In both analyses, the response variable was the 'pollination limitation', which was taken as the difference between the average number of seeds per flower after cross-pollination by hand minus the average number of seeds after open pollination. In the first analysis, predictor variables were related to the quantity of pollinator services, i.e. the visitation frequencies by each of the four size guilds. In the second analysis, predictor variables were related to the quality of pollinator services measured as insect fidelity, i.e. the ratio of *O. masquillierii* pollen to the total number of pollen grains carried on the bodies of different bee size guilds.

A Shapiro test revealed that, for several variables, our data violated the assumption of normal distribution and equality of variance required for the use of

parametric statistics. In cases when these assumptions were not met, we used the non-parametric Kruskal–Wallis ANOVA or Mann–Whitney *U*-tests. Where necessary, we applied a Bonferroni correction. Where normality assumptions applied, we used simple or multiple linear regressions. All statistical analyses were performed using R version 2.6.0. (R Development Core Team, 2008).

RESULTS

ANTHESIS AND FLOWERING CHARACTERISTICS

In all study sites, the flowering period of *O. masquillierii* lasted from late May to early July, i.e. *c.* 40 days. Flower lifespan was short (1.31 days ± 0.04 SE, $N = 20$ flowers).

Tests performed showed that stigmas were not receptive during the bud stage, but became receptive in freshly open flowers and remained receptive until the flowers withered. Pollen was viable from bud through all anthesis stages. Nectar was not present in any flower.

BREEDING SYSTEM AND PLANT REPRODUCTIVE SUCCESS

The fruits (capsules), 3–6 mm long, usually carried zero to two dark brown seeds. Seed set after hand crossing did not vary between bagged and non-bagged flowers (Mann–Whitney *U*-tests: $304 < U < 377$, $P = 0.42$), implying no significant influence of bags on seed set.

Seed set varied significantly among pollination treatments. Spontaneous selfing produced no seeds, whereas hand selfing resulted in a seed set lower than after hand crossing (Mann–Whitney *U*-tests: $50 < U < 350$, $P < 0.001$; Fig. 1). Seed set after open pollination was lower than after hand-cross treatments in all eight study populations (Mann–Whitney *U*-tests: $137 < U < 910$, $P < 0.008$; Table 3).

POLLINATORS

We collected a total of 112 pollinators belonging to eight different bee genera on the flowers of *O. masquillierii* during the 16 h of flower visitor collections (all eight plant populations included) (Table 2). All pollinators collected were female bees foraging for pollen.

Small bees (Halictidae) had the highest visitation rate in five of the study plant populations. Large bees were absent in three populations and showed the lowest visitation rate among the represented bee size guilds in two populations (Table 4). A linear
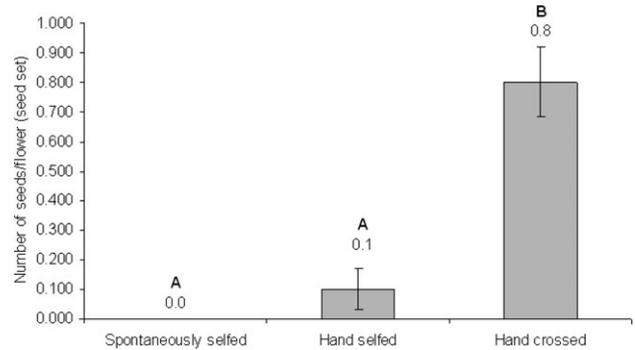

Figure 1. The reproductive success of *Ononis masquillierii* after different pollination treatments of the flowers (spontaneously selfed, hand selfed and hand crossed). Values over the columns are averages over all flowers treated. Error bars represent standard error (SE). Significant differences are indicated by the different letters ($P < 0.001$). Note that the maximum number of seeds is six (see text).

regression showed a negative relationship between the visitation rate by the different size guilds and their body weight ($R = -0.97$, $P = 0.03$, $N = 4$; Fig. 2). The four size guilds also varied in their handling time per flower (Kruskal–Wallis $H_{(3,40)} = 26.215$, $P < 0.001$). This variation was related to the small bees having a significantly longer handling time per flower than the other guilds (Mann–Whitney *U*-tests: $1 < U < 166$, $P < 0.001$, $N = 40$, Bonferroni corrections applied; Fig. 3). Smaller bees spent more time as they were moving freely inside the flowers compared with larger bees that never entered the flowers; they just landed briefly at the exterior and collected a batch of pollen. The analysis of pollen loads on the scopae and corbiculae of the four pollinator size guilds showed a lower fidelity for small–medium bees (Megachilidae) compared with the other three bee size guilds (Kruskal–Wallis $H_{(3, 112)} = 22.985$, $P < 0.001$; Mann–Whitney *U*-test: $120 < U < 724$, $P < 0.002$, Bonferroni corrections applied).

DEPENDENCE OF SEED SET ON PLANT POPULATION SIZE

Population size had a significant positive effect on seed set after free pollination. This resulted from the regression of seed set (data from Table 3) used as dependent variable vs. population size taken as average sizes of rounds 1 and 2 (Table 1): $R^2 = 0.58$, $P = 0.03$

Plant population size had a significant negative effect on pollination limitation. This resulted from the regression of pollination limitation (data from

Table 3. Reproductive success of *Ononis masquillierii* in the eight study populations after cross-pollination by hand and open pollination (average number of seeds per flower ± SE)

Population	No. of fruits recovered at harvest	Seed set after cross-pollination by hand (no. of seeds ± SE)	Seed set after open pollination (no. of seeds ± SE)	Pollination limitation (mean no. of seeds ± SE)	*P*-value
Abbadessa	33	0.85 ± 0.08	0.30 ± 0.08	0.55 ± 0.12	> 0.01
Casola Canina	33	0.82 ± 0.08	0.24 ± 0.08	0.58 ± 0.09	> 0.01
Castel de Britti	32	0.97 ± 0.05	0.09 ± 0.05	0.88 ± 0.13	> 0.01
Croara	34	0.82 ± 0.07	0.18 ± 0.07	0.64 ± 0.14	> 0.01
Eremo	31	0.94 ± 0.08	0.29 ± 0.08	0.65 ± 0.15	> 0.01
Flaminia	31	0.84 ± 0.09	0.39 ± 0.09	0.45 ± 0.15	> 0.05
Monte Calvo	30	0.90 ± 0.08	0.23 ± 0.08	0.67 ± 0.15	> 0.01
Monte Pieve	32	0.91 ± 0.07	0.19 ± 0.07	0.72 ± 0.13	> 0.01

Pollination limitation is the difference between these two values.
P-values are from Mann–Whitney *U*-test on pairwise differences in seed set between cross-pollination by hand and open pollination.
SE, standard error.

Table 4. Visitation rate (visits/flower/hour) of the different pollinator size guilds measured in the eight *Ononis masquillierii* populations

Population	Visitation rate (visits/flower/h)				
	All visitors	Small bees	Small–medium bees	Medium–large bees	Large bees
Abbadessa	0.0189	0.0103	0.0039	0.0029	0.0019
Casola canina	0.0157	0.0125	0.0006	0.0013	0.0013
Castel de Britti	0.0394	0.0223	0.0141	0.0030	0
Croara	0.0100	0.0021	0.0080	0	0
Eremo	0.0324	0.0087	0.0182	0.0007	0.0048
Flaminia	0.0130	0.0056	0	0.0068	0.0006
Monte Calvo	0.0099	0.0084	0.0006	0	0.0009
Monte Pieve	0.0177	0.0082	0.0077	0.0019	0
Overall Populations	0.0196	0.0098	0.0066	0.0021	0.0012

Data are expressed as means and are calculated over all observations and rounds in each population.

Table 3) used as dependent variable vs. population size (numbers as above): $R^2 = 0.57$, $P = 0.03$.

DEPENDENCE OF SEED SET ON INSECT VISITATION RATES AND ATTRIBUTES

Overall insect visitation rates had no significant effect on seed set. This resulted from the regression of seed set after free pollination (data from Table 3) used as dependent variable vs. overall visitation (pooled data from rounds 1 and 2; Table 4): $R^2 = 0.14$, $P > 0.05$. Similarly, regression of pollination limitation (Table 3) over overall visitation showed no significant impact ($P > 0.05$).
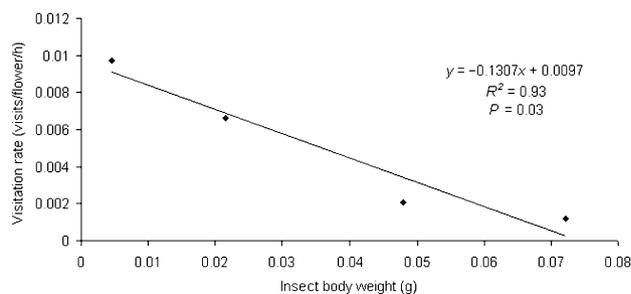

$$y = -0.1307x + 0.0097$$
$$R^2 = 0.93$$
$$P = 0.03$$

Figure 2. Mean visitation rate (visits/flower and hour) vs. mean body weight (g) of the four bee groups visiting *Ononis masquillierii* flowers in all eight populations studied. Bee body weights are given in Table 2.

The variation in pollination limitation among the studied populations was explained by the visitation frequencies shared out by the four size guilds (model A, Table 5). Higher visitation frequencies by small and small–medium bees increased pollination limitation (negative effect on seed set), whereas increased visitation frequencies by medium–large and large bees decreased pollination limitation (positive effect on seed set).

Because of insufficient data (in some populations, bees of certain families or size guilds were not present) we carried out the analysis of pollinator fidelity by pooling bee data into two size classes: smaller bees comprising small and small–medium bees; and larger bees comprising medium–large and large bees. The results indicate that pollination limitation was unaffected by bee fidelity (model B,
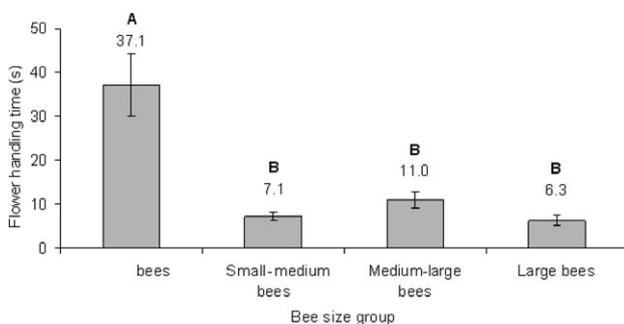

Figure 3. Average flower handling times for the different size guilds of bees visiting *Ononis masquillierii* (all eight populations pooled). Numbers over the columns also show average handling time. The different letters stand for significant differences between them ($P < 0.001$). Error bars represent standard error (SE).

Table 5), which is not surprising as the pollinator fidelity was high for all size guilds.

DISCUSSION

In this study we have shown that seed set in *O. masquillierii* is positively affected by plant population size and body size of the bees visiting the flowers. The first is a phenomenon known from many plant species (Petanidou, den Nijs & Ellis-Adam, 1991; Oostermeijer *et al.*, 2000; Dauber *et al.*, 2010 and references therein). The second result highlights the importance of pollinator body size in reproductive success: higher visitation frequencies by larger bees increased seed set in *O. masquillierii*, whereas higher visitation rates by smaller bees reduced seed set in the same populations.

To understand better the contrasting effects of visitation frequencies by different bee size guilds on seed set in *O. masquillierii*, it is necessary to explore the exact nature of the pollination mechanism of this plant. *Ononis masquillierii* has complex zygomorphic flowers, the pollination of which totally depends on bees, as is the case with many other Mediterranean legumes (Free, 1993; Pérez-Bañón *et al.*, 2003 and references therein). The reproductive output of the eight study populations was not equally high and this was related to the differential frequency of the bee size guilds visiting the flowers of the eight populations, with smaller bees reducing and larger bees increasing seed set. This appears, at a first glance, to be a mismatch between pollinator size and flower morphology, as the small corolla of only 11–15 mm appears to suit smaller bees. A more careful inspection of the data and the behaviour of the bee–flower relationships could give explanations for these results.

Table 5. Results from two multiple linear regression models describing the dependence of pollination limitation of *Ononis masquillierii* populations (i.e. the difference in seed set between hand-cross control and open pollination) on: (model A) bee visitation rates by the four size guilds (small, small–medium, medium–large and large bees), and (model B) insect fidelity values for the above bees grouped into two size classes, i.e. smaller bees (small and small–medium) and larger bees (medium–large and large)

	Regression coefficient	t	P-value
Model A ($R^2 = 0.947$; P-value $= 0.03$, $N = 8$)			
Visitation rate (visits/flower and h) of small bees	9.609	3.06	0.055
Visitation rate (visits/flower and h) of small–medium bees	11.262	3.63	0.036
Visitation rate (visits/flower and h) of medium–large bees	−22.824	−2.83	0.066
Visitation rate (visits/flower and h) of large bees	−43.298	−3.63	0.036
Model B ($R^2 = 0.445$; P-value $= 0.31$, $N = 8$)			
Insect fidelity of small bees and small–medium bees	−0.0087	−1.31	0.261
Insect fidelity of medium–large bees and large bees	−0.0006	−0.17	0.874

$N = 8$ equals the number of *Ononis masquillierii* populations.

Overall, smaller bees were more frequent visitors to *O. masquillierii* flowers (Table 4), implying a more dominant role of smaller bees in the pollination process. In addition to their apparent importance, smaller bees spent significantly longer periods in handling the flowers. During the flower handling period, smaller bees entered the flowers with the entire body and collected pollen while moving freely inside the flowers. Being able to activate the pump-like pollen dispensing mechanism of the flower a number of times, these bees could release several 'batches' of pollen during a single visit, resulting in high pollen expenditure coupled with limited movements to other flowers, leading to reduced male fitness. However, such a behaviour as this could increase the probability of passing its own pollen to stigmas within the same flower, resulting in limited seed set as a result of self-incompatibility and possible stigma clogging preventing further application of conspecific, non-self pollen (Shore & Barrett, 1984; Scribailo & Barrett, 1994; Murphy, 2000).

Larger bees, in contrast, were restricted to the external part of the flower, but still able to collect pollen. Unlike smaller bees, larger bees only activated the pollen dispenser mechanism once, i.e. when leaving the flower and thus released only one 'batch' of pollen per visit, which was carried to other flowers. Moreover, larger bees were able to carry the pollen longer distances than smaller bees, thus overcoming the geitonogamy barriers that might restrict seed set in this self-incompatible and highly vegetatively reproducing plant.

In no case do we expect interspecific pollen transfer to play a role, as all pollinator size guilds showed high fidelity to *O. masquillierii* flowers. Thus, we conclude that the major factor affecting the quality of pollination of *O. masquillierii* is the behaviour of smaller bees foraging on the flowers.

The low reproductive success of *O. masquillierii* seems to be the result of low pollinator visitation rate (quantity component) and the predominance of inefficient pollinators (quality component). On average, a flower received less than one pollinator visit and, out of six ovules, less than one developed into seed. Approximately 83% of visits were made by smaller bees, with a flower handling time almost three times greater than that of larger bees. Even if the negative fitness effects of low-efficiency small-sized pollinators could have been masked by the low pollinator visitation rate, the relationship between seed set and the visitation rate by the different pollinator size guilds, coupled with the observations of their foraging behaviour, suggest that small-sized pollinators can decrease the male component of plant fitness by reducing the pollen available to more efficient pollinators.

It is also possible that the sequence of different pollinator size guild visits to flowers of *O. masquillierii* affected the resulting seed set. For instance, a first visit by a smaller bee could prevent the correct and functional mechanism of pollination of a further visit by a larger bee on a stigma already covered by conspecific self pollen.

The rarity and limited distribution of *O. masquillierii* seem to be mainly a result of abiotic environmental constraints (Burylo, Rey & Delcros, 2007). It is an early succession pioneer species requiring suitable 'calanchi' habitats, limiting its expansion. In addition, the species patchy distribution makes its persistence dependent on the ability of pollinators to move between more or less isolated populations (Amarasekare, 2004). This, combined with the fact that larger bees can forage at greater distances than smaller bees, leads us suggest that larger bees are the most important pollinator of *O. masquillierii*, especially in isolated populations.

The absence or scarcity of larger bees on flowers of *O. masquillierii* in all plant populations does not imply a general absence of this pollinator guild in the study areas. For example, other co-flowering species could have attracted larger bees. *Ononis masquillierii* coexisted with other bee-pollinated species well adapted to the selective substrate of the gullies, primarily other Fabaceae (*Hedysarum coronarium* L., *Dorycnium hirsutum* Ser. and species of *Trifolium* L.) or it was surrounded by arid-meadow Lamiaceae (e.g. *Ajuga genevensis* L and *Stachys officinalis* Franch.). Unlike the majority of its neighbouring species, *O. masquillierii* is nectarless and foraging choices of pollinators could be influenced by floral rewards of all the plant species of the community. The small, nectarless, short-living flowers could make *O. masquillierii* a poor competitor. The present study documented that larger bees were important pollinators for *O. masquillierii*, but we did not address the problem of evaluating the principal causes of their general scarcity as *Ononis* pollinators, or whether they were more abundant on other plant species. Whether there was a wide-ranging shortfall of large-sized bees or not, their preservation could potentially improve the pollination services to *O. masquillierii*.

Further to what was found in earlier studies, i.e. that visitation frequency is important for the reproductive success of plants (e.g. Dauber *et al.*, 2010), here we demonstrated that the quality of pollinator visits is of great importance to the reproductive success of *O. masquillierii*. Size of plant populations still plays a major role in this respect, maybe more effective in attracting large size bees while these are moving between populations, even if there is no evidence of that in the present study. This interplay between large plant populations with large bees is

important in view of conserving this rare endemic species in the Apennines.

CONCLUSIONS AND IMPLICATIONS FOR CONSERVATION

Although it was beyond the scope of this study to test: (1) the links between seed production and recruitment; (2) whether the widespread inadequate pollination would have any consequences on population establishment and conservation; or (3) whether the low seed set would be sufficient for population growth and persistence, recruitment through seedling establishment is likely to be mediated by larger bees. Then, from the perspective of *O. masquillierii*, the preservation of its most effective pollinators coupled with the conservation of its preferred habitat could ensure sexual reproduction and space for it to grow, two conditions for the long-term survival.

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