I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text.

March 7, 2007
Daniele Vivarelli

Daniele Vivarelli

Alma Mater Studiorum – University of Bologna

Consequences of Plant Population Size for Pollinator Visitation and Plant Reproductive Success

Thesis submitted in partial fulfillment of the requirements for the degree of
Philosophiæ Doctor in the Faculty of Science, University of Bologna, Italy

May 2007

Ph.D. Program in Biodiversity and Evolution - XIX Cycle
BIO/02

Candidate: Daniele Vivarelli

Supervisor: Prof. Giovanni Cristofolini

Coordinator: Prof. Giovanni Cristofolini

to Claudia

Abstract

Habitat loss and fragmentation have a prominent role in determining the size of plant populations, and can affect plant-pollinator interactions. It is hypothesized that in small plant populations the ability to set seeds can be reduced due to limited pollination services, since individuals in small populations can receive less quantity or quality of visits. In this study, I investigated the effect of population size on plant reproductive success and insect visitation in 8 populations of two common species in the island of Lesvos, Greece (Mediterranean Sea), *Echium plantagineum* and *Ballota acetabulosa*, and of a rare perennial shrub endemic to north-central Italy, *Ononis masquillierii*.

All the three species depended on insect pollinators for sexual reproduction. For each species, pollen limitation was present in all or nearly all populations, but the relationship between pollen limitation and population size was only present in *Ononis masquillierii*. However, in *Echium plantagineum*, significant relationships between both open-pollinated and handcrossed-pollinated seed sets and population size were found, being small populations comparatively less productive than large ones. Additionally, for this species, livestock grazing intensity was greater for small populations and for sparse patches, and had a negative influence on productivity of the remnant plants. Both *Echium plantagineum* and *Ballota acetabulosa* attracted a great number of insects, representing a wide spectrum of pollinators, thereby can be considered as generalist species. For *Ballota acetabulosa*, the most important pollinators were megachilid female bees, and insect diversity didn’t decrease with decreasing plant population size. By contrast, *Ononis masquillierii* plants generally received few visits, with flowers specialized on small bees (*Lasioglossum* spp.), representing the most important insect guild. In *Echium plantagineum* and *Ballota acetabulosa,* plants in small and large populations received the same amount of visits per flower, and no differences in the number of intraplant visited flowers were detected. On the contrary, large *Ononis* populations supported higher amounts of pollinators than small ones. At patch level, high *Echium* flower density was associated with more and higher quality pollinators.

My results indicate that small populations were not subject to reduced pollination services than large ones in *Echium plantagineum* and *Ballota acetabulosa*, and suggest that grazing and resource limitation could have a major impact on population fitness in *Echium plantagineum*. The absence of any size effects in these two species can be explained in the light of their high local abundance, wide habitat specificity, and ability to compete with other co-flowering species for pollinators. By contrast, size represents a key characteristic for both pollination and reproduction in *Ononis masquillierii* populations, as an increase in size could mitigate the negative effects coming from the disadvantageous reproductive traits of the species. Finally, the widespread occurrence of pollen limitation in the three species may be the result of 1) an ongoing weakening or disruption of plant-pollinator interactions derived from ecological perturbations, 2) an adaptive equilibrium in response to stochastic processes, and 3) the presence of unfavourable reproductive traits (for *Ononis masquillierii*).

Acknowledgments

I was extremely fortunate to have Prof. Giovanni Cristofolini as my supervisor, without his wisdom, constant support and teaching, it would be impossible to complete this work. Thanks for giving me the opportunity to work in your lab, and for showing me that research is nothing but fun and a genuine attitude toward exploration.

I am still pleasantly astonished by Prof. Theodora Petanidou, a never-sleeping volcano of ideas and projects. Thanks for warmly welcoming me to your lab and for transmitting me your enthusiasm, and of course thanks for your insuperable greek *cuisine*.

Special thanks to Ellen Lamborn, I really benefited from our countless hours spent in constructive exchanging of ideas (including your english lessons!), thanks for your intellectual and logistic support.

I gratefully acknowledge Hjalmar Dahm (thanks for letting me drive your Lada Niva in Lesvos, and for your greek coffee breaks!), Michael Greenwell (ouzo?) and Milena Dawit for field assistance; thanks also to Marino Quaranta, Andrew Grace and the staff of the NHML for insect identification, and to Lucia Montagni and David Bianco for allowing me to have free access to the "Gessi Bolognesi e Calanchi dell'Abbadessa Regional Park". I also thank my friends and colleagues at the BES (Umberto, Annalisa, Marta, Licia, Claudia, Silvia, Lucia), the patience you had with me over these three years was infinite, I'm indebted to all of you.

This dissertion represents the final step of a 23 years-long journey, so thanks to my parents for all their efforts in guiding me and ensuring me a good education.

Finally, thanks to my *fiancée* Claudia, who has always been at my side, this work is dedicated to you.

Contents

1. Introduction

During the last few decades, an increasing emphasis have been placed on understanding the main drivers affecting plant-pollinator systems. The São Paulo Declaration on Pollinators (1998) raised international interest in pollinator conservation, and provided impetus for national and international research programmes aimed to evaluate the causes of pollinator declines and the consequences on agricultural and natural ecosystems.

It is in fact increasingly recognized that many ecosystems are facing a dramatic pollination crisis (Buchmann & Nabhan 1996, Kearns et al. 1998). A great number of studies have shown that plant-pollinator systems are under siege from natural and human-induced disturbances, such as fire (Petanidou & Ellis 1996, Potts et al. 2001, 2003), habitat fragmentation and isolation (Aizen & Feinsinger 1994a, Steffan-Dewenter & Tscharntke 1999, 2002), changes in land use (Kearns et al. 1998), pesticides (Batra 1982, O’Toole 1993), biological invasions (Brown & Mitchell 2001) and climate change (Price & Waser 1998, Warren et al. 2001). Additionally, surveys conducted on 258 species of angiosperms, indicate a high prevalence of pollen limitation (inadequate pollen receipt to fertilize all ovules), with 62% of the species pollen limited to some extent (Burd 1994, Knight et al. 2005a). Other authors, however, theoretically contest that pollen limitation is widespread within plant species (Burley & Willson 1983, Snow 1986, Walsh & Charlesworth 1992).

Many studies focused on whether pollen limitation could be governed by environmental factors, and several ecological perturbations have been proposed to have a major influence on it (Table 1). For example, the introduction of rewarding non-native plant species can increase pollination competition among plants, hence increasing pollen limitation in plants less attractive to pollinators, or wide-ranging shortfalls of pollinators, shifts in pollinator species composition, and accelerated rates of pollinator extinction may put into jeopardy pollination services to plants.

Habitat fragmentation is known to represent one of the most frequent and major threats to biodiversity (Oostermeijer 2003), and to plant-pollinator systems (Table 1). It has been defined as a process where “a large expanse of habitat is transformed into a number of smaller patches of smaller total area, isolated from each other by a matrix of habitats unlike the original” (Wilcove et al. 1986). Basically, the process of fragmentation is made up of three components, depending on the degree of habitat loss (Andrén 1994): 1) an extreme reduction (or complete loss) of habitat; 2) a reduction in size of habitat fragments; and 3) a lower connectivity of the remnant fragments. Therefore, fragmentation implies a subdivision of a continuous habitat into fragments, becoming progressively smaller and more isolated as the process increases, ultimately leading to population extinction when a critical threshold value of fragmentation is reached. From the plant's perspective, the lack of suitable habitat for expansion coming from habitat fragmentation may favour the occurrence of small arrays of plants, which in turn may cause pollination deficits (Widén 1993, Ågren 1996, Morgan 1999). However, pollinators may perceive habitat fragmentation in a different way than plants, as basic bee habitat requirements can be less dependent upon the distribution of flowering plants than expected (Cane 2001). For example, the availability of specific nesting attributes (soil texture, moisture, salinity) may be fundamental for the persistence of bee communities (Cane 1991).

Small and sparse natural plant populations can be more sensitive to habitat deterioration, due to the Allee effect, which suggests that low reproduction success (in terms of fertility and /or survival) can result from declined population size and density (Stephens et al. 1999). In fact, small and sparse populations may be more prone to pollen limitation than large ones (Table 1), and may show greater vulnerability to environmental, demographic and genetic hazards (Menges 1992, Young et al. 1996, Holsinger 2000), leading in the long or short term to a greater likelihood of stochastic extinction. There is in fact a large body of evidence that small populations are characterized by a lower genetic diversity than large ones (Ouborg et al. 1991, Weidema et al. 1996, Hensen & Oberprieler 2005), associated with higher levels of inbreeding depression and genetic drift (Ellstrand & Elam 1993, Fischer & Matthies 1998, Dudash & Fenster 2000, Lienert et al. 2002).

Previous studies have reported a positive relationship between plant reproductive success and population size (Ågren 1996, Hendrix & Kyhl 2000, Kéry et al. 2000, Oostermeijer et al. 2000, Mavraganis & Eckert 2001, Tomimatsu & Ohara 2002, Brys et al. 2004, Kolb 2005, Ward & Johnson 2005, Hensen & Wesche 2006) or plant density (Kunin 1993, 1997; Bosh & Waser 1999, Kirchner et al. 2005) and size and density often correlate (Ågren 1996). Two possible explanations for these observed patterns are that the quantity and/or quality of pollen receipt are insufficient in such populations (Table 1). First, small and scattered populations generally result less attractive to pollinators (Jennersten 1988, Kunin 1997), having lower visitation rates and pollen deposition on stigmas. Second, in such populations, pollinators are more likely to favour shorter flight distances, thus increasing within-plant movements and pollen transfer. This can directly affect the quality of the pollination service by increasing the frequency of geitonogamy, ultimately leading to inbreeding depression in self-compatible plant species (De Jong et al. 1993, Karron et al. 1995), or resulting in a high fraction of incompatible conspecific pollen deposited on stigmas in self-incompatible species, that may physically or chemically prevent outcross pollen from contacting the stigma and from performing pollen tube germination and fertilization success (stigma clogging) (Shore & Barrett 1984, Scribailo & Barrett 1994, Murphy 2000).

Ecological perturbation	**Predicted consequence for pollen limitation**	**Explanation**	**Empirical support**
Presence of other plant species	$PL_{\text{coflowering species}} > PL_{\text{single flowering species}}$	Co-flowering results in pollinator competition, increased heterospecific pollen delivery, and/or stigma clogging by heterospecific pollen[a]	Campbell 1985, Gross 1996, Gross & Werner 1983
	$PL_{\text{coflowering species}} < PL_{\text{single flowering species}}$	Co-flowering results in increased pollinator attraction[b]	Moeller 2004
Plant population size/density	$PL_{\text{in small populations}} > PL_{\text{in large populations}}$	Small populations have reduced pollinator visitation, pollen deposition[c], ratio of conspecific to heterospecific pollen delivered[d] and more intraplant pollinator visits[e]	Ågren 1996, Davis et al. 2004, Forsyth 2003, Knight 2003, Kunin 1997, Moeller 2004, Sih & Baltus 1987, Waites & Ågren 2004, Ward & Johnson 2005
Pollinator loss	$PL_{\text{with fewer pollinators}} > PL_{\text{with more pollinators}}$	Pollinator visitation rate increases with pollinator abundance and diversity[f]	Liu & Koptur 2003
Resource additions	$PL_{\text{in resource rich habitat}} > PL_{\text{in resource poor habitat}}$	Seed production depends solely on pollen receipt when resources are unlimited	Galen et al. 1985
Habitat size and isolation	$PL_{\text{in fragmented habitat}} > PL_{\text{in continuous habitat}}$	Habitat fragmentation reduces the abundance of plants and/or pollinators, alters pollinator composition[g]	Cunningham 2000, Groom 2001, Johnson et al. 2004, Moody-Weis & Heywood 2001, Steffan-Dewenter & Tscharntke 1999, Wolf & Harrison 2001
Plant enemies (herbivores, pathogens)	$PL_{\text{with high enemy abundance}} > PL_{\text{with low enemy abundance}}$	Enemies decrease pollinator attraction and pollinator visitation[h]	None
	$PL_{\text{with high enemy abundance}} < PL_{\text{with low enemy abundance}}$	Enemies decrease plant resource status; plants become more limited by resources than pollen	Parker 1987
Plant mutualists (mycorrhizal fungi)	$PL_{\text{with high mutualist abundance}} < PL_{\text{with low mutualist abundance}}$	The presence of soil mutualists facilitates plant resource acquisition, pollinator visitation and seed set[i]	None

(continued)

Ecological perturbation	Predicted consequence for pollen limitation	Explanation	Empirical support
Pollinator predators	$PL_{\text{with pollinator predators}} > PL_{\text{without predators}}$	Predators reduce pollinator abundance and visitation rate[j]	Knight et al. 2005b
Non-native plant species	$PL_{\text{non-native plants}} > PL_{\text{native plants}}$	Non-native plants lack effective pollinators[k]	None
	$PL_{\text{non-native plants}} < PL_{\text{native plants}}$	Non-native plants have a higher frequency of autogamy[l]	None
Non-native pollinators	$PL_{\text{with non-native pollinator}} > PL_{\text{without non-native pollinator}}$	Non-native pollinators compete with native pollinators, and are less efficient pollinators of crops and wild plants[m]	None

[a] Campbell & Motten 1985; Caruso 1999, 2001; Galen & Gregory 1989; Waser 1983

[b] Moeller 2005, Rathcke 1983

[c] Fausto et al. 2001, Feinsinger et al. 1991, Regal 1982, Whitehead 1983

[d] Caruso 2002, Kunin 1993

[e] Franceschinelli & Bawa 2000, Iwaizumi & Sakai 2004, Kinkhamer & De Jong 1990, Mustajärvi et al. 2001

[f] Buchmann & Nabhan 1996, Kearns et al. 1998, Thomson 2001

[g] Jennersten 1988, Linhart & Feinsinger 1980

[h] Mothershead & Marquis 2000; Steets & Ashman 2004; Strauss et al. 1996; Irwin, Brody & Waser 2001

[i] Wolfe et al. 2005

[j] Dukas 2001, Dukas & Morse 2003, Muñoz & Arroyo 2004, Suttle 2003

[k] Parker 1997

[l] Rambuda & Johnson 2004

[m] O'Toole 1993, Paini 2004, Paton 1993, Sugden & Pyke 1991

Table 1 (From Knight et al. 2005a)

Even if there is a large body of evidence that extrinsic factors may influence the seed output, such as plant population size or density, intrinsic factors (relative to the plant reproductive traits) may play an important role as well. Plant species may in fact not be equally sensitive to pollen limitation, and many plant traits are supposed to influence to some extent its likelihood and its magnitude (Larson & Barrett 2000, Knight et al. 2005a).

Among these, a major role can be attributed to all the traits responsible for flower attractiveness (flower size and display, nectar secretion, flower shape) and the traits that prevent the plants to be strictly dependent on pollinators (capacity of autogamy, clonal propagation).

For example, autogamous species (or allogamous species capable of vegetative propagation) are expected to be less dependent on external pollen vectors than obligate outcrossers; these species will therefore be less exposed than others to the detrimental effects of insect paucity or inconstancy, and will persist even at low plant population densities (Morgan et al. 2005). According to Burd's (1994) hypothesis, the additional source of pollen represented by the self-pollen in the selfing species would account for the lower pollen limitation compared with the outcrossing species.

Another factor that can have a strong influence on pollen limitation involves the degree of plant specialization. Given the profound differences in terms of body size, behavior and feeding requirement among pollinator taxa, plant specialization often means modification in the morphology of the flowers (Fægri & van der Pijl 1979), even if species-specific pollination is quite rare (Waser et al. 1996). For example, large-flowered and nectariferous species are expected to result more attractive to pollinators, and will likely less suffer from low insect visitation rate than their counterparts (Bell 1985, Stanton & Preston 1988, Klinkhamer & De Jong 1990, Eckhart 1991, Vaughton & Ramsey 1998, Valido et al. 2002, Momose 2004).

Again, in constant pollinator regimes there is a clear selective advantage on specialization, as restricted floral access can improve the quality of the pollen receipt via better pollen placement on pollinators (Kunin & Shmida 1997), promoting plant reproductive isolation and speciation rate (Sargent 2004). Therefore, for a rare plant's perspective, specialization can counterbalance the lack of showy floral displays and copious rewards. Nevertheless, many studies highlighted how pollination systems are mostly governed by extreme and unpredictable fluctuations in pollinator abundance and composition at both temporal and spatial scale (Herrera 1988, Petanidou & Ellis 1993, Tepedino et al. 1999, Petanidou & Potts 2006). In such conditions, specialist plant species, that can rely on a narrow spectrum of suitable pollinators, are expected to exhibit higher pollen limitation than generalist species (but see Larson & Barrett 2000), as a consequence of a higher likelihood to lose (temporarily or definitely) the few appropriate pollinators which the plant has specialized on.

When insufficient pollen delivery comes from both extrinsic (relative to ecological perturbations) and intrinsic (relative to the reproductive characteristics of the species) factors, the species may enter in a vicious circle of events, in which the decrease of population size is not buffered by a proper ability of assuring the reproductive success, potentially leading to an ever-decreasing population size and fitness process, and ultimately ending with extinction.

The Mediterranean region is in unison considered one of the richest bee and plant community worldwide (Petanidou & Lamborn 2005 and references therein), and this diversity is maintained despite both ecological constraints (e.g. summer drought) and heavy anthropogenic perturbations (e.g. fire, grazing, land use) that have created a spatially heterogeneous and fragmented landscape. This translates in a strong dependance of Mediterranean plants on pollination mediated by insects, and, in the meanwhile, in a high exposure for Mediterranean plant-pollinator communities to the risk of weakening or disruption of the interactions.

This study examines the pollination ecology of three different plant species (*Echium plantagineum* (Boraginaceae), *Ballota acetabulosa* (Labiatae), *Ononis masquillierii* (Fabaceae)), occurring in the Mediterranean Basin, with particular emphasis on the effect of plant population size on reproductive success and pollination. The main question is whether small populations are at reproductive disadvantage over large ones, and to what extent this is pollination-related.

2. Materials and Methods

2.1. The species

***Echium plantagineum* L.**

Echium plantagineum L. (Boraginaceae) is an annual or biennial plant, 20 to 60 cm high and with a rosette morphology. It is mainly found on roadsides, fields, degraded pastures, and sandy areas near to the sea, at altitudes of 0-1300 m. Even if native to the western Mediterranean Basin, it has been introduced to many other countries (mainly Australia and USA), where it has quickly become a noxious weed of temperate pastures (Piggin & Sheppard 1995). In the Mediterranean, it flowers from early April to early-middle May.

Inflorescences are 5-15 cm high usually with 2-8 ascending branches. Flowers are zigomorphic, with a shallowly five-lobed infundibuliform corolla (18-30 mm), blue becoming pink through purple, usually with two exerted stamens (Fig.1). The calyx is 7-10 mm long at anthesis, but up to 15 mm in fruit. The fruits are four rough, wrinkled to fine-tubercled nutlets.

It produces large amounts of nectar (Corbet & Delfosse 1984, Corbet et al. 1991), and it's mainly pollinated by bees.

Fig.1. Flower of *Echium plantagineum* L. (Photo: http://perso.orange.fr/scanice/botanique_flore.htm).

Ballota acetabulosa **(L.) Bentham**

Ballota acetabulosa (L.) Bentham (Labiatae) is an evergreen perennial shrub, woody at base, growing up to 80 cm high. It cannot grow in the shade, it requires dry or moist soils (on rough grounds) and it's drought tolerant. Its distributional range is represented by the Aegean area, including Turkey, Greece and Crete. It flowers from early June through July.

The small zigomorphic flowers have a purple and white corolla (15-18 mm), and are arranged in verticillasters along the ascending stems. The calyx is grey-green, hairy and apically expanded (12-15 mm), and holds the corolla tube; it persists after flower's dehiscence (Fig.2). Flowers have a nectary placed at the base of the superior ovary, producing nectar (Petanidou et al. 2000).

The flat, felted thick leaves are well adapted to xerophytic conditions, having a high specific dry weight and a reduced inner air volume; at maturity, the percentage of internal exposed surface per leaf area increases as an adaptation to water paucity (Psaras & Rhizopoulou 1995).

The fruits are four nutlets, held in the same receptacle at the base of the calyx.

Like most of the Labiatae of the Mediterranean, *B. acetabulosa* is mostly pollinated by bees, which can find in its flowers a large source of nectar and water, necessary to contrast the severe summer drought (Petanidou & Voukou 1993).

Fig.2. Flower of *Ballota acetabulosa* (L.) Bentham visited by *Eucera* sp. (Photo: Hjalmar Dahm).

Ononis masquillierii Bertol.

Ononis masquillierii Bertol. (Fabaceae) is a perennial, leguminous shrub, endemic to the northern Appennines (Italy), and found from Emilia Romagna to the northern part of Marche and Tuscany. The species grows up to 40 cm high on dry and clay soils, at altitudes of 0-600 m, where it prefers sunny slopes. It flowers from late May to early July.

Flowers are borne singly at each node, usually in dense racemes, and are zygomorphic. They are made up of five petals, arranged in a papilionoid structure: the posterior petal (flag or banner), two lateral petals (wings) and two petals partially fused together to form a boat-shaped keel at the base of the flower, that encloses the stamens and the stigma. Flowers have a small, pink corolla (11-15 mm) which exceeds the calyx (Fig.3) and the fruits or legumes are 3-6 mm long carrying 1-2 dark brown seeds.

Ononis masquillierii propagates both vegetatively and by seed. Pollination is predominantly mediated by bees. Secondary pollen presentation is mediated by a “pump” mechanism (Arroyo 1981, Westerkamp 1997, López et al. 1999). First, the pollen is released onto the keel petals, then insect’s pressure on the keel forces a pump-like action of stigma and style which extrudes a small amount of pollen through an opening at keel tip. Pollen grains adhere to the insect’s abdomen (sternotribic mechanism), and, due to the gradual pollen emptying, more than one visit per flower is possible.

Fig.3. Flowers of *Ononis masquillierii* Bertol. (Photo: Daniele Vivarelli).

Voucher specimens of both *Echium plantagineum* and *Ballota acetabulosa* are preserved at the Laboratory of Biogeography and Cultural Ecology, Department of Geography, University of the Aegean, while the ones relative to *Ononis masquillierii* at the Herbarium of the University of Bologna (BOLO).

2.2. Population characteristics

The study on *Echium plantagineum* and *Ballota acetabulosa* was carried out during spring and summer 2005 in the island of Lesvos, belonging to the East Aegean Greek islands (Fig.4). The island (about 70 km long by 45 km wide at its maximum point) is characterized by a rugged topography, and, from a geological and vegetational point of view, divided into two different parts by the Gulf of Kalloni. The eastern part is in fact mainly formed of crystalline rocks, igneous peridotites and ophites, and is dominated by olive groves and, at higher altitudes, by pine forests; the western part is formed of igneous tertiary rocks, and phrygana is prevalent (Fig.5)

The study relative to *Ononis masquillierii* was conducted during June and July 2006 in the “Gessi Bolognesi e Calanchi dell’Abbadessa Regional Park,” near Bologna, Italy (Fig.6). The Park (5.000 ha) is located on the hills surrounding Bologna, and is characterized by a series of gypsum outcrops, a deep karst system and large erosional landforms (gullies). In the past, the park was strongly subject to human-induced disturbances (mainly mining, grazing, agricultural practices), but today the Park represents a very rich heterogeneous area, with sinkholes, woods, closed valleys, cliffs and gullies.

For each species, eight populations were selected. Each population was separated by at least 1 km from any other population, or natural barriers occurred between them (Fig.4, Fig.6). For *Ononis masquillierii*, all populations (except two) were located inside the “Gessi Bolognesi e Calanchi dell’Abbadessa Regional Park”. For *Echium plantagineum* and *Ballota acetabulosa*, two qualitative size categories were defined (small and large), based on approximate numbers of individuals (Table 2). For both species, small populations were about half the size of large ones. Population size was estimated by roughly counting all the flowering plants in the area delimited by the limits of four 100 meters transects in the four cardinal directions, starting from the core area of the population. For *Ononis masquillierii*, it was impossible to distinguish and count all the individuals due to the vegetative propagation nature of the species, thus I counted all the flowering racemes in the population instead of the flowering plants. In this case, the variable “population size” was continuous, as the exact number of flowering racemes was recorded; for this species, location details can be found in Table 3. The eight *Ononis masquillierii* populations in this study covered a 24-fold range in size. In addition, only for *Ononis masquillierii*, population density was calculated as the number of *Ononis masquillierii* flowering racemes per population area. For each population of this species, both population size and density estimates were done twice (one per round, see “Pollinator visitation”), and I considered the means as final values.

Within each population, four patches of flowering plants (or racemes) were randomly selected, separated by 2-10 m. For each species, the size (area encompassed by the outer limits of the patch, m^2) and density (number of flowering individuals (or racemes) per unit area) relative to each patch were determined. Patch density was calculated by counting the total number of flowering plants (racemes) within the patch (therefore calculating plants (racemes) $/m^2$) for *Ballota acetabulosa* and *Ononis masquiliierii*, whereas for *Echium plantagineum* patch density was sampled by using a quadrat (0.25 m^2) haphazardly placed within the patch (N = 10 for large patches, N= 5 for small patches, repeated for each round), then by counting the number of individuals within the quadrat. Density resulted from the mean of the measures. In addition, for each patch of *Ballota acetabulosa*, I determined the mean plant size (area occupied, m^2), by haphazardly selecting 11 plants (10 from pollination test focal plants, and 1 from insect observation focal plants (per round), see below).

In each patch, one observation unit was chosen for insect observations (see below). Observation units varied in size and shape, but contained approximatively from 10 to 100 flowers. For *Echium plantagineum*, five “focal” plants within the observation area were chosen, and, for each focal plant, the distance to the four nearest neighbouring flowering plants in four quadrants was measured (spatial distribution, cm). The average values were used at patch level. Moreover, only for *Echium planatgineum*, all the co-flowering species occurring in the observation units were identified (at species or genus level).

The values of each variable used in the analyses (patch size, density, plant size and spatial distribution) came from the mean of the three (two in *Ononis masquillierii*) rounds.

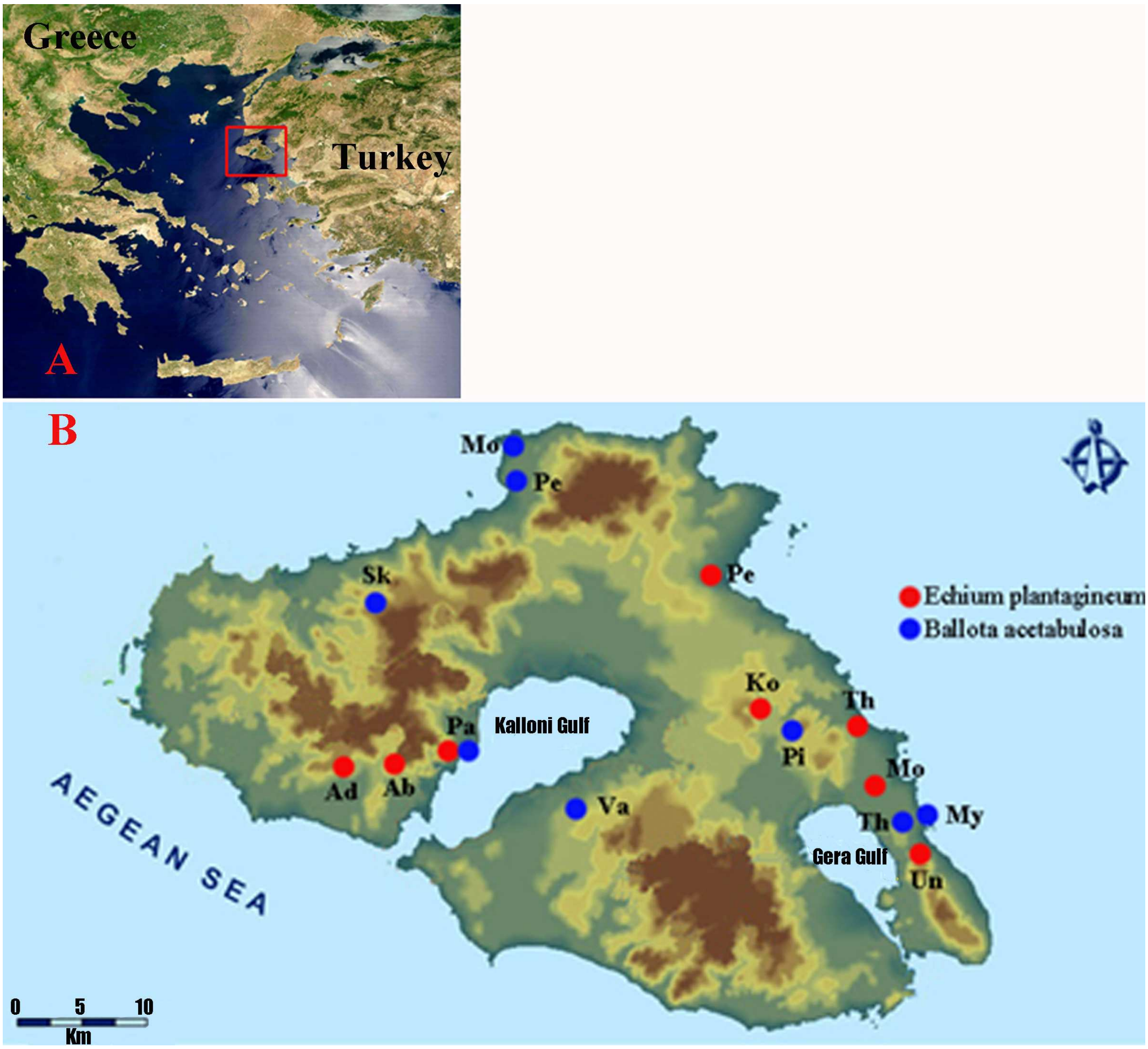


Fig.4. Study area of *Echium plantagineum* and *Ballota acetabulosa*. (A) location of Lesvos in the Aegean Sea; (B) studied populations (red circles: *Echium plantagineum*, blue circles: *Ballota acetabulosa*). Population names are displayed next to each circle (see Table 1 for details).

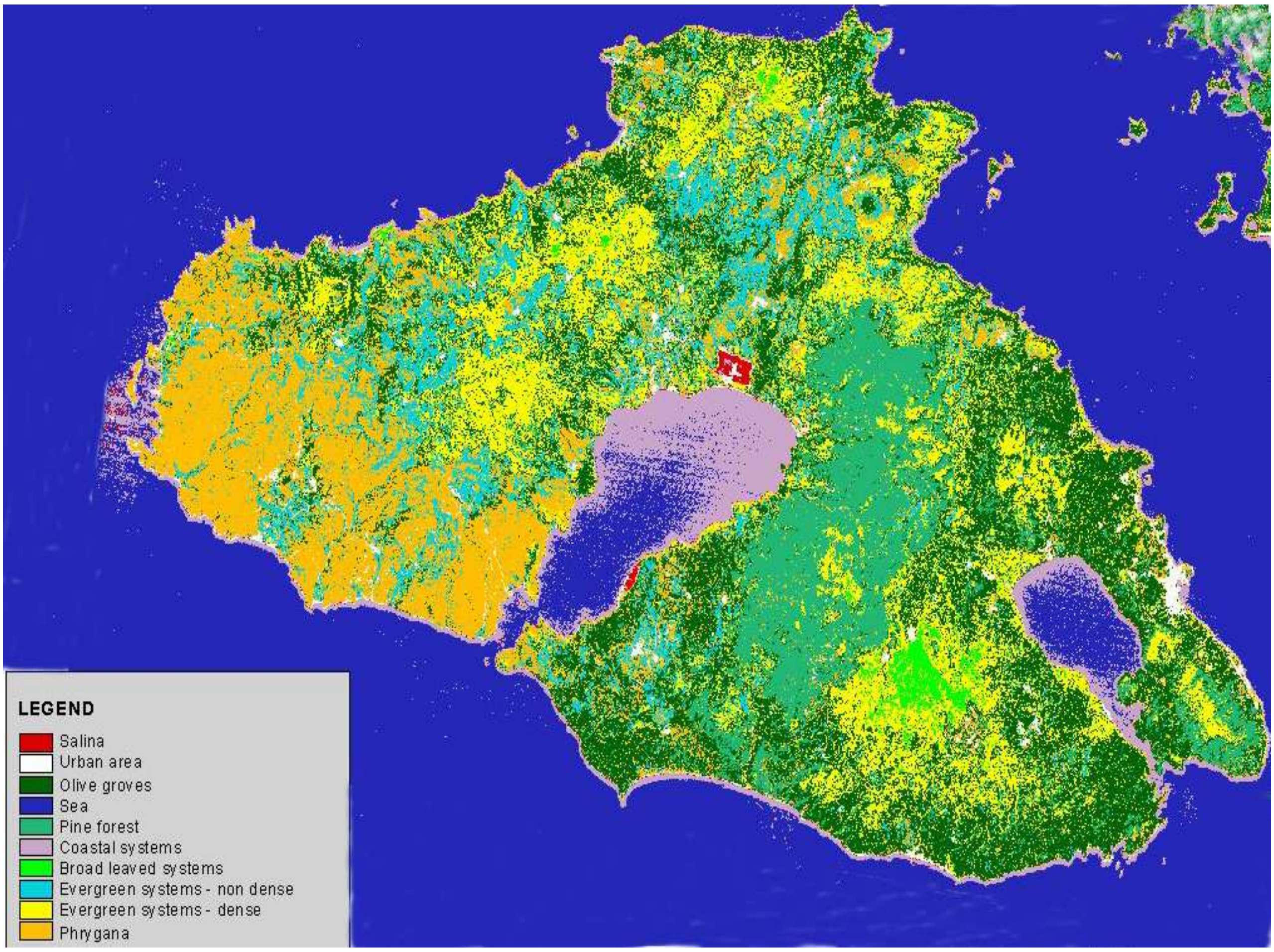


Fig.5. Map of Lesvos showing the main vegetational habitats. Source: University of the Aegean.

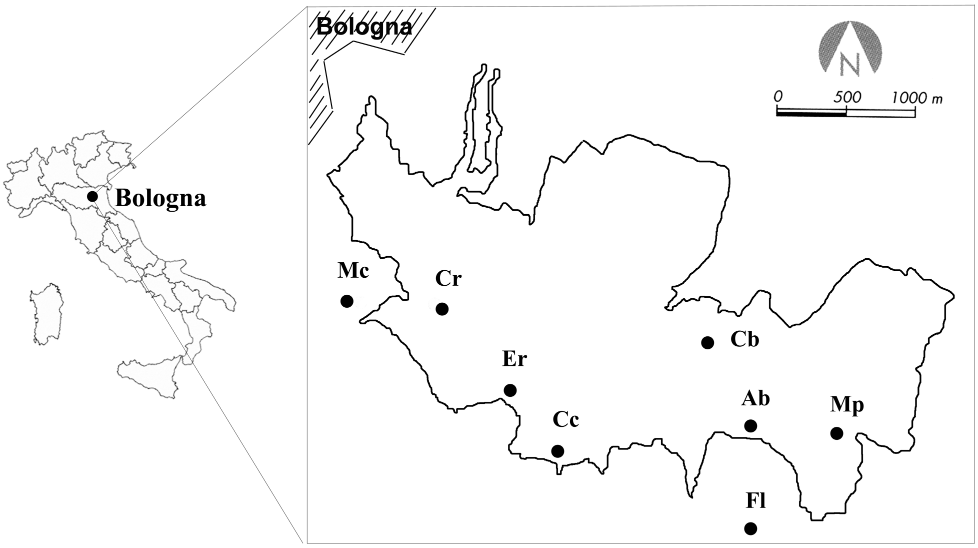


Fig.6. Study area of *Ononis masquillierii*, located in the surrounding of Bologna, Itlay. The box shows the enlarged area, corresponding to the "Gessi Bolognesi e Calanchi dell'Abbadessa Regional Park". Sampled populations are indicated by solid circles and site abbreviations.

Species					
	Echium plantagineum			*Ballota acetabulosa*	
Population	Abbreviation	Size	Population	Abbreviation	Size
University	Un	large	Theatre	Th	large
Agra bridge	Ab	large	Vasilika	Va	large
Pedi	Pe	large	Molyvos	Mo	large
Agra donkey	Ad	large	Skalachori	Sk	large
Parakoila	Pa	small	Parakoila	Pa	small
Moria	Mo	small	Mytilini castle	My	small
Komi	Ko	small	Petra	Pe	small
Thermi	Th	small	Pigi	Pi	small

Table 2. Name and size of the 8 studied populations of *Echium plantagineum* and *Ballota acetabulosa*.

Population	Abbreviation	Elevation (m)	Location		Population size	Population density
			Latitude	Longitude		
Abbadessa	Ab	215	44°24’29’’ N	11°26’42’’ E	643	0.77
Casola Canina	Cc	252	44°24’21’’ N	11°24’40’’ E	1991	0.42
Castel de Britti	Cb	232	44°25’06’’ N	11°26’26’’ E	151	1.54
Croara	Cr	239	44°25’34’’ N	11°23’28’’ E	455	1.57
Eremo	Er	290	44°24’47’’ N	11°24’02’’ E	1023	2.40
Flaminia	Fl	242	44°23’21’’ N	11°27’11’’ E	3575	1.02
Monte Calvo	Mc	345	44°25’33’’ N	11°22’26’’ E	737	7.08
Monte Pieve	Mp	244	44°24’27’’ N	11°28’09’’ E	832	0.88

Table 3. Size (mean number of flowering racemes), density (mean number of flowering racemes/m²) and location of the 8 studied populations of *Ononis masquillierii*.

2.3. Flowering characteristics

In order to determine the phenology of the flower, for each species 20 flowers were studied daily, every 4 hours, from bud until they withered. For each species one large population was selected for this study (“Un” for *Echium plantagineum*, “Th” for *Ballota acetabulosa*, “Fl” for *Ononis masquillierii*). Each initial bud was selected at random on 20 different plants (or racemes, separated by at least 1m from any other in *Ononis masquillierii*), and on different positions within the plant, and marked with coloured plastic clips or with waterproof paint. Flower anthesis was considered to start when the flower opened or, in *Ononis masquillierii*, when the flag was fully erect, thereby enabling insect visits.

In *Echium plantagineum* and *Ballota acetabulosa*, stigma receptivity and pollen viability were determined by observing morphological changes in stigmas and anthers of 15 flowers of different ages, collected at “Un” and “Th” populations respectively. In *Ononis masquillierii*, for stigma receptivity and pollen viability, three stages of flowering were identified as: 1) bud, 2) freshly open flower and 3) senescent flower. Stigma receptivity and pollen viability were assessed on five different flowers per stage at “Fl” population by using the Perotexsmo KO test and the DAB test respectively (Dafni et al. 2005). In addition, on the same flowers the anther dehiscence was recorded.

In *Ononis masquillierii*, nectar standing crop was also measured on five flowers per stage, this time in another large population (“Cc”), by inserting “drummond” microcapillary tubes along the staminal column, and measuring the length of nectar secretion in mm using a ruler.

2.4. Pollination tests

In order to evaluate the occurrence of pollen limitation, supplementary hand vs free pollination tests were performed in each of the eight populations of each plant species, during peak flowering. Forty focal plants were chosen in each population, ten in each patch, by selecting the closest plant to five random points chosen on each of two crossed transects through the centre of the patch. On each plant, two freshly open and receptive flowers were selected, marked and randomly assigned to two different treatment groups: hand cross pollination (overall N=320) or free pollination (overall N=320). Manual outcrossing was performed with pollen from three other individuals within the same population. The remaining flowers were left as an open pollinated control.

In order to gain information about the breeding system of each plant species, tests of self-compatibility and spontaneous self-pollination were carried out on 20 plants randomly chosen at “Pe”, “Th” and “Cc” populations (for *Echium plantagineum*, *Ballota acetabulosa* and *Ononis masquillierii* respectively), five plants per patch. Three comparable unopened buds were marked on each plant, and assigned to the following pollination treatments: hand-selfing (overall N=20), spontaneous-selfing (overall N=20) and hand-cross control (overall N=20). After that, the whole inflorescence was covered with a hydrophilic plastic bag to prevent insect visits. When the flowers opened, one flower was hand pollinated with self pollen, by taking two flowers of the same plant as pollen donors, the second flower was left unmanipulated and the third flower was pollinated with outcrossed pollen from two nearby individuals. This hand-crossing treatment was to test for a bag effect on flower, fruit and seed development, and the results of this final treatment were compared with the productivity after hand crossing in natural conditions in the same population.

All hand-pollinations consisted of a single application of an abundant quantity of pollen. Particular attention was paid to the stage of flowers used as pollen donors and receptors; in all cases pollen at the maximum viability was used as donor, and fully receptive stigma were used as receptors. Outcrossing pollen donors were selected at a distance of at least 10 metres from the focal plant, to avoid the possibility of selfing from vegetative propagules. In *Ononis masquillierii*, an estimate of the initial number of ovules was assessed from 10 freshly open flowers at “Cc” population, while for both *Echium plantagineum* and *Ballota acetabulosa* the number of ovules per flower was invariably four.

At the end of the flowering season, fruits were collected from all the marked flowers, approximatively one month after pollination treatments, and viable seeds were counted. Reproductive success was determined as the number of filled viable seeds over the initial number of ovules (seed set). The small “Th” population of *Echium plantagineum* was completely lost because of the mechanical cutting of the vegetation.

As a great percentage of *Echium* flowers was subject to livestock grazing, I evaluated the intensity of grazing by means of the same procedure used for determining patch density (see above), and by counting the number of grazed and ungrazed *Echium* plants within the quadrats at the end of the flowering season. Grazing was determined as the ratio of grazed Vs ungrazed plants.

2.5. Insect visitation

For each species, insect activity was monitored under natural conditions in all the 8 populations. One day of observation consisted of four 15-min (one per patch/observation unit) periods, repeated twice (AM and PM); each observation unit was monitored by one or two observers. During an observation period, I recorded the starting and ending time of the observation period and, for each individual which entered the observation area, I recorded the residence time within the observation unit, the guild, sex, behavior (nectar or pollen collector), the number of flowers and plants visited (the latter only for *Echium plantagineum* and *Ballota acetabulosa*). Visitors were identified in the field to coarse taxonomic categories (i.e. "functional groups", Fenster et al. 2004). In *Echium plantagineum*, I also recorded, if any, the visits to the co-flowering species.

Each of the 8 populations of *Echium planatgineum* and *Ballota acetabulosa* was visited three times over the peak flowering period (except "Th" and "Ab" *Echium* populations that were visited twice), while all *Ononis masquillierii* populations were visited twice. Overall, a total of 22 days (44 hours), 24 days (48 hours) and 16 days (32 hours) of observation were performed (on *Echium plantagineum*, *Ballota acetabulosa* and *Ononis masquillierii* respectively).

In addition, weather variables were recorded (temperature, relative humidity, lux, wind speed, cloud cover). Observations were not made during non-optimal weather conditions (T<12°C, cloudy or rainy days, strong wind).

After the four 15-min periods, 30 minutes were spent in a random collection of the main visitors within the population. Insects that had just visited an *Echium*, *Ballota* or *Ononis* flower were captured through a net, killed with ethil-acetate and put in separate vials for further identification (identification of insects visiting *Echium plantagineum* is still in progress). All the insects caught on *Ononis* flowers were used for pollen load analysis (see below), while, because of the large number of insects collected, I quantified pollen loads only on a representative subset of *Ballota* insects.

In *Echium plantagineum* and *Ballota acetabulosa*, two aspects of pollinator behavior were considered: the mean number of insect visits per flower per 15', i.e. visitation rate (a measure of pollination quantity), and the number of flowers visited within a plant by each pollinator (a measure of pollination quality). By contrast, in *Ononis masquillierii*, due to the very low visitation rate recorded through the observation intervals across all the 8 populations (mean: 0.0068 ±0.0009 visits/flower*hour), I preferred to use the data relative to the random collection periods as an estimate of the insect abundance and biodiversity in each site. I therefore retained from the observation intervals data on insect behavior and flower handling time.

Insect specimens are conserved at the Laboratory of Biogeography and Cultural Ecology, Department of Geography, University of the Aegean (for *Echium plantagineum* and *Ballota acetabulosa*), and at the BES Department, University of Bologna (for *Ononis masquillierii*).

2.6. Pollinator effectiveness and fidelity

Even if direct measures of pollination effectiveness are generally preferred, such as pollen removal or deposition on stigmas (Herrera 1987, Wilson & Thomson 1991, Fishbein & Venable 1996, Canto-Aguilar & Parra-Tabla 2000) or seed set after single visits (Dieringer 1992, Olsen 1997, Kandori 2002), in wide pollinator assemblages these methods are not reliables, and so pollination effectiveness must be derived through indirect approaches (Lindsey 1984, Petanidou et al. 1995a, Petanidou et al. 1998, Tepedino et al. 1999, Moeller 2005).

The effectiveness of pollinators visiting *Ballota* and *Ononis* flowers was assessed by estimating the likely number of grains deposited on a stigma after a single visit, by visitors from different guilds. For this it is not necessary to determine absolute pollen carrying capacity, but to have a relative measure of pollen from a range of different flower visitors (relative pollinator effectiveness) (Lamborn & Ollerton 2000). Pollen samples were removed by means of small equal-size pieces of fuchsin gel (5 mm ø, Beattie 1971) from all the insects caught in every site during the 30’ random collection (for *Ononis masquillierii*) or from the most representative insect taxa collected in each site (for *Ballota acetabulosa*). Each separate piece of gel was pressed once firmly on the head, thorax and abdomen, in order to localize the part of the body responsible for the pollen transfer. The pollen was then counted and identified under a light microscope; a pollen library was created by collecting specimens of pollen of the most abundant co-flowering species in the area.

In addition, pollinator fidelity was determined by analysing a sample of the scopal pollen of females bees (all bees for *Ononis masquillierii*, only megachilids for *Ballota acetabulosa*).

Overall, 112 *Ononis* and 442 *Ballota* insects were examined.

2.7. Data analysis

For each plant species, the data relative to reproductive success had high non-normal distributions (through the Shapiro test), and transformations couldn’t be succesfully achieved. Non parametric tests were therefore used. Variation in reproductive success after different treatments was tested with Kruskal-Wallis ANOVA (K-S) followed by separate Mann-Whitney *U*-tests (M-W).

In *Echium plantagineum* and *Ballota acetabulosa*, the effects of population size (categorical variable) and among population variation on seed sets were tested through a two-level mixed nested ANOVA design, with populations (random factor) nested within size treatments (fixed factor), and patches as genuine replicates. An analogous nested ANOVA model was used for testing the effects of grazing on productivity values in *Echium plantagineum*. In *Ononis masquillierii*, population size was a continuous variable, and its effect on seed sets was investigated through Pearson's product-moment correlation. In *Echium plantagineum* and *Ballota acetabulosa*, the effect of population size and among population variation on both visitation rate and number of flowers visited within a plant were always tested by means of a mixed nested ANOVA design (three-level), with patch considered in this case as an additional third level (random factor), and rounds as temporal replicates.

Multiple regression analyses (backward procedure) were performed to analyse the effects of different variables on seed sets, visitation rate, and number of flowers visited within a plant (see Results for details); in case of non-normal distributions, variables were conveniently transformed.

Generally, for each response variable, pairwise t-tests multiple comparisons (with Bonferroni correction) were used to detect differences among populations, if any.

In *Ballota acetabulosa*, two-level mixed nested ANOVAs were used to test the effects of population size and among population variation on Shannon indices, and on megachilids female relative frequency and fidelity.

In *Ononis masquillierii*, differences in pollen load, pollen placement and fidelity among groups as well as differences in time spent per flower among groups were analysed with Kruskal-Wallis ANOVA (K-S) followed by separate Mann-Whitney *U*-tests (M-W).

All the regressions referred to Pearson's product-moment correlation.

All calculations were performed using R version 2.3.0. (R Development Core Team 2004).

3a. Results: *Echium plantagineum*

3.1a. Flowering characteristics

The duration of flowering was approximately one month, starting in early-middle April and ending in middle May. The average flower life span was 1.17±0.02 days.

The flower-opening pattern includes morphological and functional changes, flowers are protrandrous. In the first phase (male phase), the stamens protrude from the corolla, while the stigma is short, hidden inside the corolla, with the two stigmatic lobes not spiltted. In this phase a great amount of pollen is presented by anthers. In the second phase (female phase), the style elongates beyond the anthers, and the two stigmatic lobes diverge, meaning that stigma becomes receptive. By contrast, the pollen release is strongly reduced. As new flowers open each day, both sexual phases are simultaneously present in the same plant.

3.2a. Breeding system, pollen limitation and grazing

Only 0.2% of total flowers in the hand-selfing and spontaneous selfing treatments set seeds, while hand crossed flowers significantly set more seeds per flower (M-W *U*-tests; $P<0.001$, Fig.7), indicating that *Echium plantagineum* is not capable of induced or autonomous self-pollination. Bags didn’t affect the production of seeds (M-W *U*-tests; $P>0.05$).

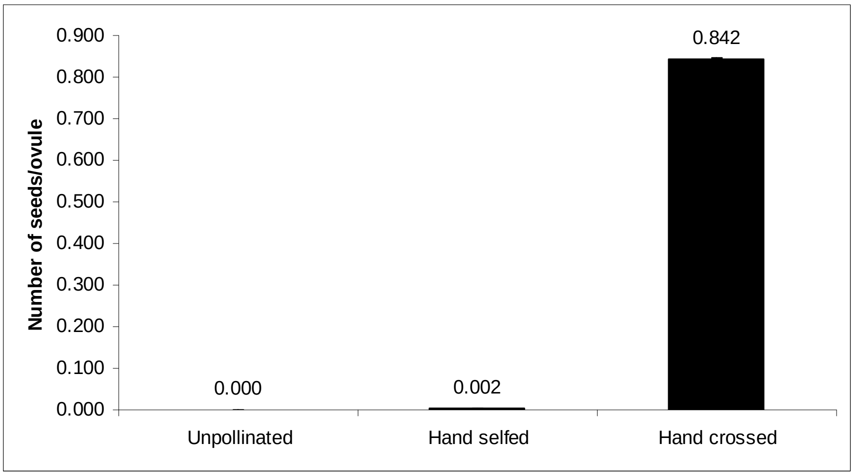


Fig.7. Reproductive success of *Echium plantagineum* in unpollinated, hand selfed and hand crossed flowers conducted at Fl site. Columns represent the mean number of viable seeds per ovule. Mean seed sets are displayed above the bars in the graph.

The number of seeds per flower after hand-crossed pollen addition ranged from zero to four (pooled populations).

Pollen limitation was significantly present in all population except “Mo”, with a mean value of 0.17±0.02 (Table 4). To investigate if any variation in seed set (after hand-crossed and nonsupplemeted treatments, and their difference, i.e. “pollen limitation index”) occurred among populations and between population size categories, a two-level nested mixed ANOVA model was used, in which populations (random factor) were nested within size treatments (fixed factor), and patches considered as genuine replicates. The model was unbalanced as one population (“Th”) was totally lost because of the clearance of the site.

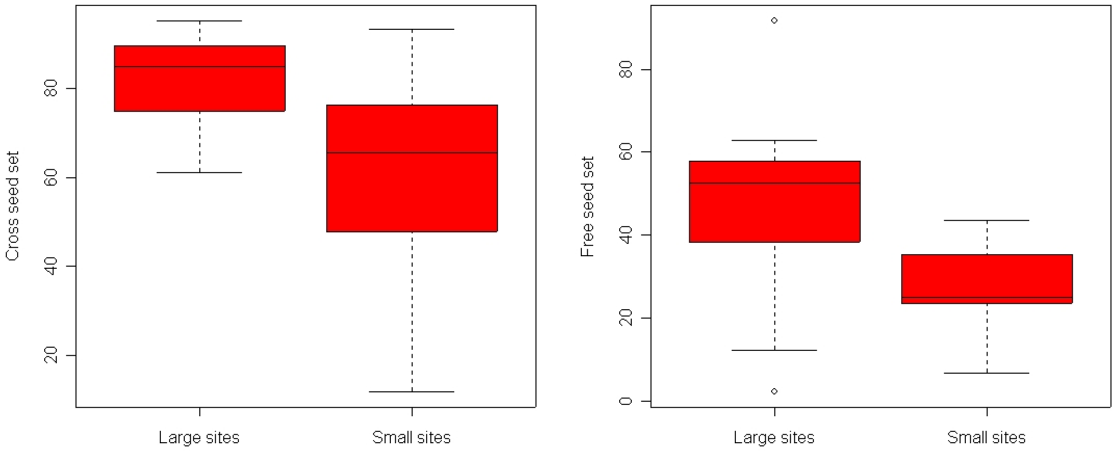
Population size had no significant effect on pollen limitation index (Table 5), indicating that large and small populations were equally subject to pollen limitation, and no significant differences among populations were detected. On the contrary, population size affected the productivity of both hand-crossed and non-supplemented flowers, being large populations significantly more productive than small ones (Fig.8) (Hand-crossed seed set in large Vs small populations: 82.1±2.4 Vs 61.8±6.7; nonsupplemented seed set in large Vs small populations: 47.0±5.3 Vs 27.3±3.1. Mean values, backtransformed data).

Population	Size	Hand crossed	Free pollinated	M-W
Un	large	0.824±0.003	0.476±0.004	***
Ab	large	0.760±0.008	0.21±0.01	***
Pe	large	0.858±0.003	0.591±0.003	***
Ad	large	0.884±0.002	0.513±0.007	***
Pa	small	0.698±0.008	0.30±0.02	*
Mo	small	0.57±0.02	0.26±0.02	ns
Ko	small	0.69±0.01	0.271±0.005	***

Table 4. Results of separated pairwise Mann-Whitney *U*-tests for seed-set in hand-crossed and non-supplemented flowers in 7 populations of *Echium plantagineum*. Mean ± SE are given.
Signif. codes: 0 *** 0.001 ** 0.01 * 0.05, ns= not significant.

Trait	Source	*df*	MS	F
Pollen limitation index	Population size	1	46.9	0.2567 ns
	Population (size)	5	182.7	0.7332 ns
Free seed set	Population size	1	2651.5	6.2981·
	Population (size)	5	421.0	1.5020 ns
Cross seed set	Population size	1	2837.5	6.6096*
	Population (size)	5	429.3	1.7378

Table 5. ANOVA results for the effects of population size and among-population variation on pollen limitation index (hand crossed-nonsupplemented seed set), free (nonsupplemented) seed set, and cross (hand crossed) seed set.
df=degrees of freedom, MS= Mean Square values, F=F-test values.
Signif. codes: 0 *** 0.001 ** 0.01 * 0.05 · 0.1, ns=not significant.



a **b**

Fig.8. Boxplot of productivity after a) hand-pollinated (cross) and b) nonsupplemented (free) treatments in large and small sites (populations). Medians are indicated by the central lines, the lower and upper edges of the box are the 1st and 3rd quartiles respectively. The whiskers extend to a maximum of 1.5 times the inter-quartile range, the points outside the whiskers are potential outliers.

Recovery of the marked flowers was very variable, ranging from complete recovering to less than 50% (“Pa” and “Mo” populations). Livestock grazing was the main responsible for this loss: an analogous nested mixed ANOVA model (see above) was used to compare the effects of population size and among-population variation in the extent of grazing; data revealed how grazing activity for small populations was higher and statistically different (ANOVA, P=0.02) from that for large populations (Table 6, Fig.9). In detail, the mean % of grazed plants in small populations was almost four times higher than in large populations (74.6±9.2; 20.4±7.0 respectively)

Trait	Source	*df*	MS	F
Grazed plants	Population size	1	20177.1	11.7138*
	Population (size)	5	1722.5	2.4935·

Table 6. ANOVA results for the effects of population size and among-population variation on grazing activity (% of grazed plants per 0.25 m²).

df=degrees of freedom, MS= Mean Square values, F=F-test values.

Signif. codes: 0 *** 0.001 ** 0.01 * 0.05 · 0.1, ns=not significant.

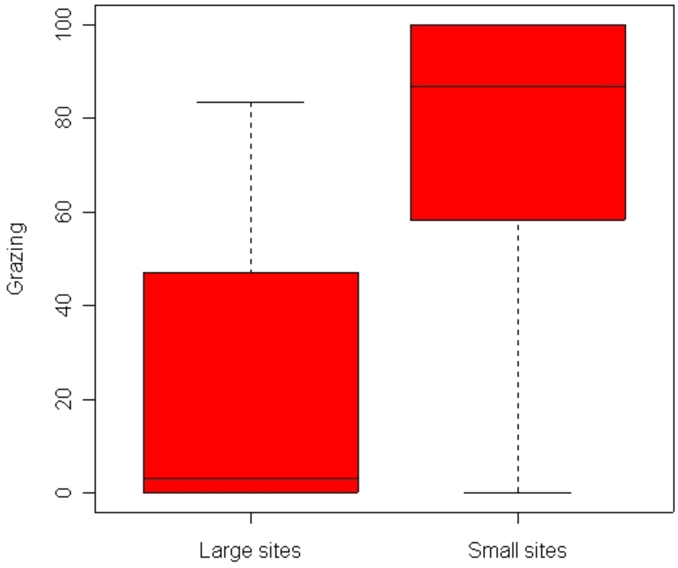


Fig.9. Boxplot of grazing activity (% of grazed plants per 0.25 m²) in large and small sites (populations). Medians are indicated by the central lines, the lower and upper edges of the box are the 1st and 3rd quartiles respectively. The whiskers extend to a maximum of 1.5 times the inter-quartile range, the points outside the whiskers are potential outliers.

In addition, a negative significant correlation was found between grazing activity and patch density (number of *Echium* flowers per area, Log-transformed data), indicating that grazing pressure was stronger in sparser patches than in denser ones ($r = -0.59$, $P<0.001$).

Again, grazing activity (expressed as the % of grazed plants) influenced the seed production of the remaining plants, as a more intense grazing pressure resulted in a lower productivity for both handcrossed and unmanipulated treatment ($r = -0.62$, $P<0.001$; $r = -0.50$, $P<0.01$ respectively).

Summarized for all populations, the multiple regression analysis (backward procedure) performed on the cross-free seed set (pollen limitation index), by taking into account the effects of insect visitation rate (number of visits per *Echium* flower * 15’, Log-transformed data), mean number of visited flowers (per plant per insect *15’, Log-transformed data), patch size (Log-transformed data), patch density (number of *Echium* flowers per 0.25 m^2, Log-transformed data), and spatial distribution (the mean distance to the nearest flowering plant, Log-transformed data), revealed that none of the considered variables was important ($P>0.05$). The same occurred when large and small populations were kept separated.

3.3a. Insect visits

Insect visitation rate (number of visits per *Echium* flower * 15’, Log-transformed data) and mean number of visited flowers (per plant per insect *15’, Log-transformed data) were modelled on population size (fixed factor), population (random factor) and patch (random factor) by means of a three-level nested mixed ANOVA model, to test whether significant effects emerged. Rounds were considered as genuine temporal replicates.

Population size didn’t significantly affect the number of insect visits to *Echium* flowers; by contrast insect visitation rate was somewhat different among populations, but not among patches (Table 7). Pairwise t-tests multiple comparisons (with Bonferroni correction) indicated that “Ko” (small) and “Un” (large) differed from three other populations, being the less visited populations (Fig.10).

Insects visited the same number of flowers per plant in large and small populations, and neither among population nor among patch differences were detected (Table 7).

Trait	Source	*df*	MS	F
Visitation rate	Population size	1	1.670	0.6044 ns
	Population (size)	6	2.763	17.0556***
	Patch (population (size))	24	0.162	0.2819 ns
Flowers/plant*insect	Population size	1	0.107	1.2442 ns
	Population (size)	6	0.086	1.3231 ns
	Patch (population (size))	24	0.065	1.3000 ns

Table 7. ANOVA results for the effects of population size, among-population and among-patch variation on insect visitation rate (number of visits/flower*15’), and number of *Echium* flowers visited/plant*insect. Data from observation trials.

df=degrees of freedom, MS= Mean Square values, F=F-test values.

Signif. codes: 0 *** 0.001 ** 0.01 * 0.05 · 0.1, ns=not significant.

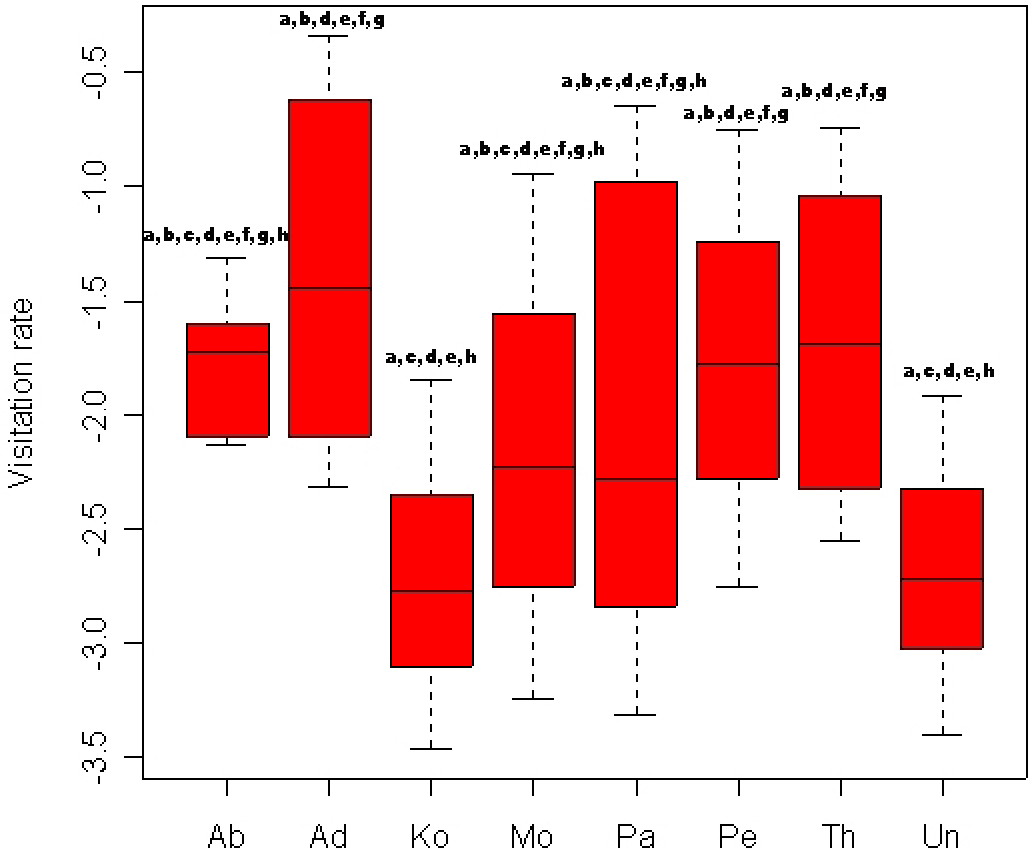


Fig.10. Boxplot of insect visitation rate (number of visits per *Echium* flower * 15’) in 8 populations of *Echium plantagineum* (see text for details on populations). Medians are indicated by the central lines, the lower and upper edges of the box are the 1^{st} and 3^{rd} quartiles respectively. The whiskers extend to a maximum of 1.5 times the inter-quartile range, the points outside the whiskers are potential outliers. Values significantly different (pairwise t-tests multiple comparisons, P<0.05) are indicated by different superscript letters (a=Ab, b=Ad, c=Ko, d=Mo, e=Pa, f=Pe, g=Th, h=Un).

Even if the patch effect didn’t result significant in the nested anova model, a multiple regression analysis was performed, by using the same two response variables as before and a series of indipendent variables as covariates. The initial model was started including patch size (Log-transformed data), patch density (Log-transformed data) and spatial distribution (Log-transformed data) in both cases.

The final model (after backward procedure) indicated that only patch density had an effect on visitation rate (+, $P<0.001$, multiple R-squared = 0.39, Fig.11), while spatial distribution had an influence on the mean number of visited flowers per plant (+, $P<0.05$, multiple R-squared = 0.17).

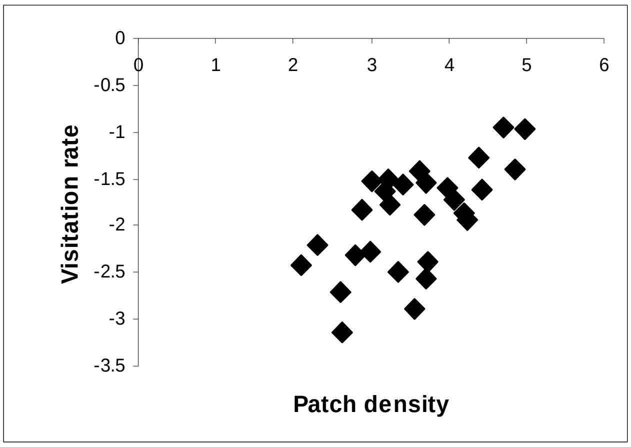


Fig.11. Visitation rate (mean number of visits per *Echium* flower * 15’) as a function of patch density (mean number of *Echium* flowers per 0.25m²) (Log-transformed data). Data are pooled over 8 populations.

Many plant species belonging to different families co-flowered with *Echium plantagineum* (see Appendix 1).The mean proportion of insect visits to these species compared to the visits to *Echium* flowers was in most cases less than 1%, and only visits to *Trifolium nigrescens* accounted for more than 5% of the total visits (8.8%). In addition, *Trifolium nigrescens* was well widespread across the studied *Echium* populations, being present in 7 of 8 sites. For these reasons, it was then considered as the most important co-flowering species. To check whether the presence of this legume led to facilitation, competition or had no influence with respect to *Echium plantagineum* pollinator services, a correlation test between the visitation rate to *Echium* flowers (Log-transformed data) and the occurrence of *Trifolium nigrescens* inflorescence was performed (Log-transformed data). No relationship was found (P>0.05).

3b. Results: *Ballota acetabulosa*

3.1b. Flowering characteristics

Ballota acetabulosa belongs to the group of late-spring/summer flowering species in Greece (Petanidou & Vokou 1993), with an anthesis lasting about one month, from early June to early July. Mean flower duration was 1.4±0.2 days.

Flowers are protrandrous: during the male phase the style is initially curved inwards, hidden among the dehiscent anthers. While reaching the female sexual maturity, the style starts to spread its stigmatic lobes always remaining enclosed within the corolla, and stamens begin to wilt. The dicoghamy is asynchronous, as male and female flower phases occur simultaneously on a given plant. The anthers face downward, providing nototribic pollination.

3.2b. Breeding system and pollen limitation

Recovery of the marked flowers was more than 90%.

Hand crossed flowers in enclusure condition yielded significantly more seeds than both hand selfed and spontaneously selfed flowers (M-W *U*-tests; P<0.001, Fig.12); these last two treatments set a statistically equivalent number of seeds per ovule (M-W *U*-tests; P>0.05), falling nearly to zero. No significant effects of bags were found (M-W *U*-tests; P>0.05).

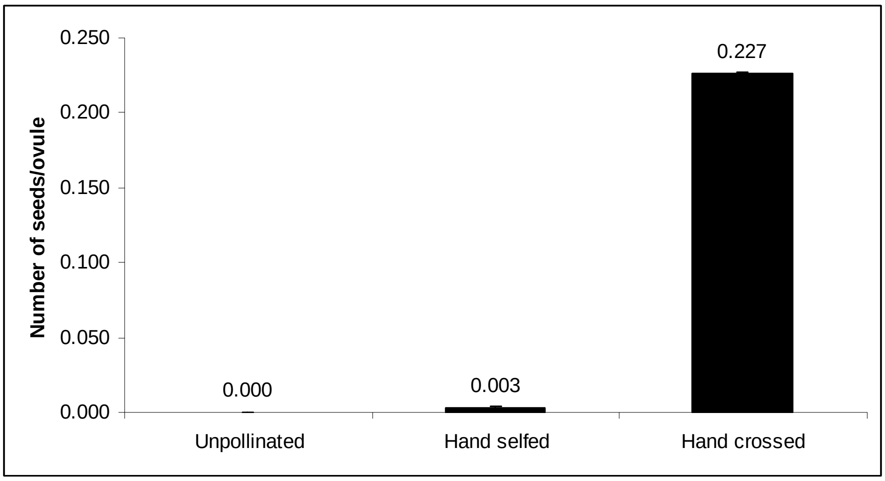


Fig.12 Reproductive success of *Ballota acetabulosa* in unpollinated, hand selfed and hand crossed flowers conducted at Th site. Columns represent the mean number of viable seeds per ovule. Mean seed sets are displayed above the bars in the graph.

Generally, only a small fraction of ovules set seeds, as one seed per flower was the most common output even after pollen hand supplementation.

Overall, hand crossing significantly increased the seed set compared with natural pollination treatment in 6 of 8 populations (Table 8). Spatial components of seed set were investigated by means of a two-level nested mixed ANOVA model, with populations (random factor) nested within size treatments (fixed factor), and patches considered as genuine replicates. The response variables were in turn seed set after hand-crossed and open natural treatments, and seed set resulting from the difference of these two treatments (i.e. “pollen limitation index”).

The intensity of pollen limitation didn’t differ among populations, and populations of different sizes were equally subject to inadequate pollen receipt (Table 9). Mean pollen limitation index for small populations was in fact comparable to large ones (1.5±0.7 and 1.4±0.5 respectively). Seed set resulting from hand-outcrossing was not associated with population size, and was not different among populations. Population size had no effect on open pollinated seed set as well, while significant differences among populations were found, with “Pa” population setting the lowest number of seeds per ovule (0.08±0.01).

Population	Size	Hand crossed	Free pollinated	M-W
Th	large	0.216±0.003	0.13±0.01	*
Va	large	0.216±0.005	0.14±0.01	*
Mo	large	0.222±0.003	0.11±0.01	**
Sk	large	0.206±0.004	0.172±0.007	ns
Pa	small	0.190±0.006	0.08±0.01	**
My	small	0.263±0.005	0.194±0.006	*
Pe	small	0.256±0.002	0.151±0.008	**
Pi	small	0.232±0.003	0.189±0.006	ns

Table 8. Results of separated pairwise Mann-Whitney *U*-tests for seed-set in hand-crossed and non-supplemented flowers in 8 populations of *Ballota acetabulosa*. Mean ± SE are given.
Signif. codes: 0 *** 0.001 ** 0.01 * 0.05, ns= not significant.

Trait	Source	*df*	MS	F
Pollen limitation index	Population size	1	2.13	0.0506 ns
	Population (size)	6	42.08	1.3660 ns
Free seed set	Population size	1	10.52	0.1395 ns
	Population (size)	6	75.39	2.7108*
Cross seed set	Population size	1	22.14	0.8870 ns
	Population (size)	6	24.96	0.8320 ns

Table 9. ANOVA results for the effects of population size and among-population variation on pollen limitation index (hand crossed-nonsupplemented seed set), free (nonsupplemented) seed set, and cross (hand crossed) seed set.
df=degrees of freedom, MS= Mean Square values, F=F-test values.
Signif. codes: 0 *** 0.001 ** 0.01 * 0.05 · 0.1, ns=not significant.

In order to check whether seed set was influenced by covariates, a muliple regression analysis was performed (backward procedure). The tested response variable was the cross-free seed set (pollen limitation index), while insect visitation rate (number of visits per *Ballota* flower * 15’, squareroot-transformed data), mean number of visited flowers (per plant per insect *15’, Log-transformed data), patch size (Log-transformed data), patch density (number of *Ballota* flowering plants per patch area, Log-transformed data) and mean plant size (Log-transformed data) were used as covariates. None of the considered explainatory variables affected seed set (P>0.05, multiple R-squared = 0.25).

3.3b. Insect visits

In order to verify if population size and among-population variation had any influence on insect visitation rate (number of visits per *Ballota* flower * 15’, squareroot-transformed data) and mean number of visited flowers (per plant per insect *15’, Log-transformed data), a three-level nested mixed ANOVA model was built, with population size (fixed factor), population (random factor) and patch (random factor) as discriminant factors, and rounds considered as genuine temporal replicates. In addition, a striking among-population variation in the magnitude of honey bees (*Apis mellifera* L.) abundance was found, which represented the dominant pollinators in some populations (“Sk” and “Mo”) and were nearly absent in others (“Pi”, “Th”, “Va”, “Pa”) (see Appendix 2); it was likely that the presence of beehives near to the populations accounted for these differences, thus biasing the mean values relative to insect visitation rate and number of visited flowers. Thereby, all multiple regressions analyses were performed both considering and not considering the contribution of *Apis mellifera*.

With Apis mellifera

Results are shown in Table 10.

The eight populations of *Ballota acetabulosa* shared a similar insect visitation rate, and population size didn’t result a significant discriminant factor. By contrast, a patch effect was found ($P<0.05$). Thereby, a multiple regression analysis was performed (backward procedure), and the effects of patch size (Log-transformed data), patch density (number of *Ballota* flowering plants per patch area, Log-transformed data) and mean plant size (Log-transformed data) on visitation rate (squareroot-transformed data) were investigated.

None of the variables was significant, and only plant size had a (marginal) positive effect ($P = 0.08$, multiple R-squared = 0.14).

When considering the mean number of flowers visited within a plant per insect, population size was found to have a null influence, and no among-population variation was found. The same multiple regression analysis as before was performed, and no variable resulted statistically significant (multiple R-squared = 0.09).

Without Apis mellifera

Results are shown in Table 10.

In this situation, a stronger among-population variation in visitation rate was found, even if not so marked, while population size always resulted not significant. The two populations that presented the highest visitation rates by honey bees (“Sk” and “Mo”), dropped dramatically in their visitation rate when honey bees were not taken into account, with a loss of 62% and 46% respectively. Multiple comparisons (pairwise t-tests with Bonferroni correction) highlighted how these two populations differed from three other populations, showing the lowest visitation rates ($P<0.05$, Fig.13).

Form multiple regression analysis (multiple R-squared = 0.60), visitation rate differed as a function of patch density (-, $P<0.05$) and plant size (+, $P<0.001$), while patch area influence was less clear (?, $P<0.01$) (Fig.14).

When considering the number of flowers visited within a plant per insect, no differences with the previous situation (with *Apis mellifera*) emerged.

Trait	Source	*df*	MS	F
With Apis mellifera				
Visitation rate	Population size	1	0.007	0.3684 ns
	Population (size)	6	0.019	0.9048 ns
	Patch (population (size))	24	0.021	1.9091*
Flowers/plant*insect	Population size	1	0.411	0.9786 ns
	Population (size)	6	0.420	1.2139 ns
	Patch (population (size))	24	0.346	0.8759 ns
Without Apis mellifera				
Visitation rate	Population size	1	0.107	2.5476 ns
	Population (size)	6	0.042	3.5000*
	Patch (population (size))	24	0.012	1.2000 ns
Flowers/plant*insect	Population size	1	1.281	1.7500 ns
	Population (size)	6	0.732	1.7897 ns
	Patch (population (size))	23	0.409	0.9738 ns

Table 10. ANOVA results for the effects of population size, among-population and among patch variation on insect visitation rate (number of visits/flower*15’), and number of *Ballota* flowers visited/plant*insect, with and without the contribution of *Apis mellifera*. Data from observation trials. df=degrees of freedom, MS= Mean Square values, F=F-test values. Signif. codes: 0 *** 0.001 ** 0.01 * 0.05 · 0.1, ns=not significant.

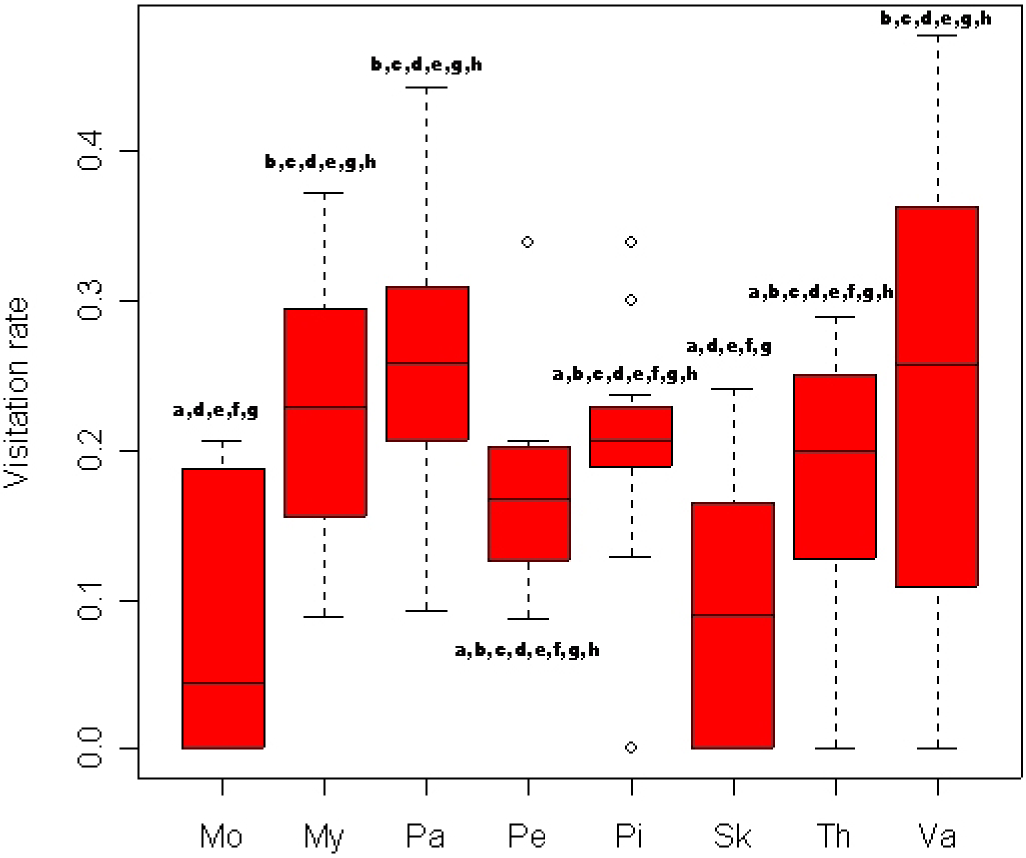


Fig.13. Boxplot of insect visitation rate (number of visits per *Echium* flower * 15’) in 8 populations of *Ballota acetabulosa*. *Apis mellifera* excluded from the analysis. Medians are indicated by the central lines, the lower and upper edges of the box are the 1st and 3rd quartiles respectively. The whiskers extend to a maximum of 1.5 times the inter-quartile range, the points outside the whiskers are potential outliers. Values significantly different (t-tests multiple comparisons, P<0.05) are indicated by different superscript letters (a=Mo, b=My, c=Pa, d=Pe, e=Pi, f=Sk, g=Th, h=Va).

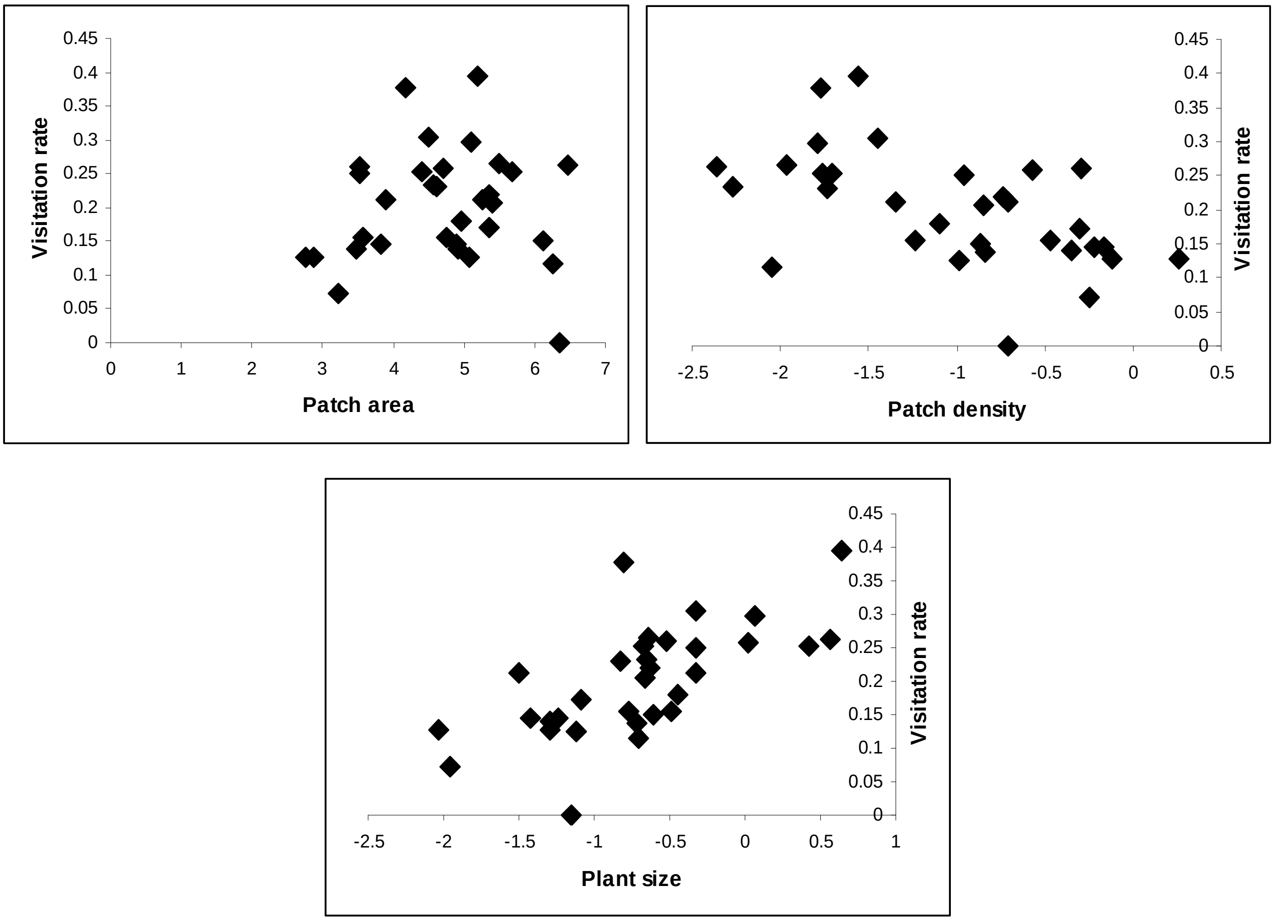


Fig.14. Visitation rate (mean number of visits per *Ballota acetabulosa* flower * 15', squareroot-transformed) as a function of patch area (m², Log-transformed), patch density (number of *Ballota* flowering plants per patch area, Log-transformed) and plant size (m², Log-transformed). *Apis mellifera* excluded from the analysis. Data are pooled over 8 populations.

3.4b. Insect diversity, effectiveness and importance

A total of 28 insect genera were collected (through random walks) across the eight populations of *Ballota acetabulosa*, during its entire flowering period. Among these, bees accounted for more than 98%, representing three families (Fig.15). Flies (*Bombylius* spp.), butterflies (*Thymelicus* spp.) and wasps were present at a very low percentage (1.4%, 0.2% and 0.4% respectively) (see Appendix 2).

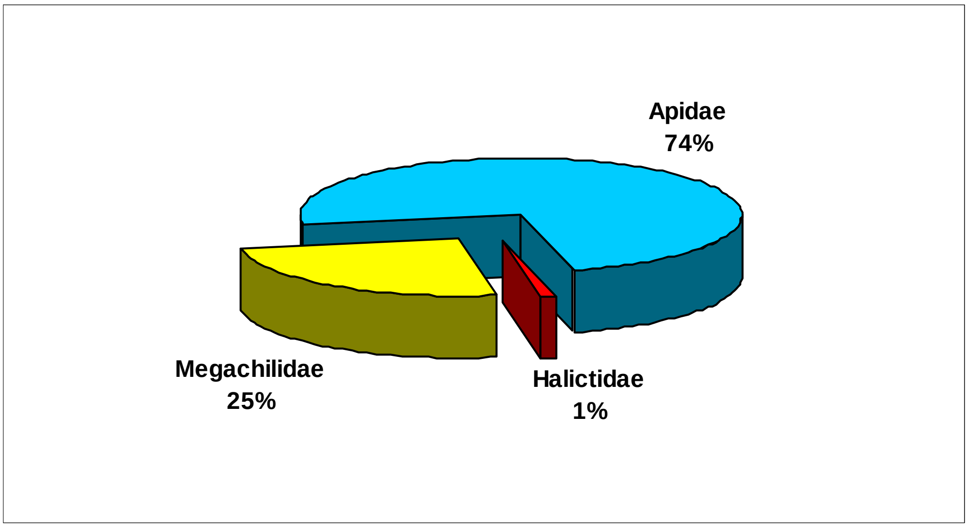


Fig.15. Proportions of insect families visiting *Ballota acetabulosa*. Only Hymenoptera are considered. Data are pooled over 8 populations and 3 rounds.

Apis (mellifera) was the most frequent genus, accounting for 37% of total insects, followed by *Bombus* spp. (18%) and *Chalicodoma* spp. (16%). Females were in general over-represented (64.0%). All bees accidentally or intentionally contacted the flower reproductive structures.

Summarized for all populations, patterns of insect abundance indicated that most of the genera were represented by few individuals (<10) (Fig.16), and the same occurred at population level.

Populations greatly differed in their insect fauna abundance and composition. For example, honey bees clearly dominated the “Sk” and “Mo” populations, whereas they were absent or nearly absent in “Va” and “Pa” populations. The same was true for most of the genera recorded, with the exception of *Chalicodoma* spp., that showed a more homogeneous distribution among populations.
To check if genera collected in few populations were a) widespread across *Ballota*’s distributional range but uncommon, or b) restricted to a small area, for each genus the range size (the maximum distance between occupied sites) was plotted against the number of sites where the insect was recorded. In the first case a positive saturating relationship was expected, otherwise a linear one.
A lack of fit test corroborated the first hypothesis (P<0.001) (Fig.17).

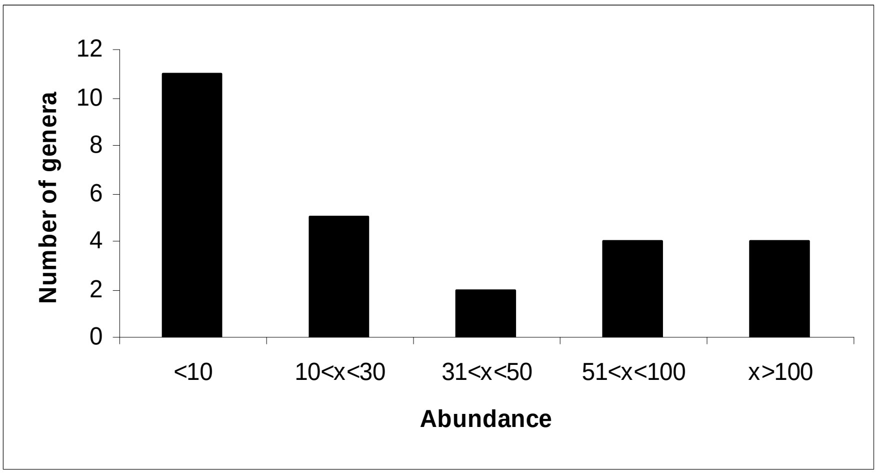


Fig.16. The distribution of abundance classes relative to *Ballota acetabulosa* flower insects (only Hymenoptera). Data refer to insect collection through random walks, and are pooled over 8 populations and 3 rounds.

Fig.17. Range size (the maximum distance between occupied sites by each genus (only Hymenoptera), expressed in Km) Vs the number of sites occupied by each genus in eight populations of *Ballota acetabulosa*. Data refer to insect collection through random walks, and are pooled over 8 populations and 3 rounds.

To compare the diversity of potential pollinators' assemblages among populations and between population size, the Shannon index relative to each population was computed (only bees considered, genus level, from random walks collection). A two-level mixed nested ANOVA model was used, with populations (random factor) nested within size treatments (fixed factor), and rounds considered as genuine replicates. As the presence of beehives could have biased the results, the Shannon indices were calculated both with and without the contribution of *Apis mellifera* (Table 11).

Trait	Source	*df*	MS	F
With Apis mellifera				
Shannon index	Population size	1	0.141	0.2311 ns
	Population (size)	6	0.610	7.625***
Without Apis mellifera				
Shannon index	Population size	1	0.141	0.4184 ns
	Population (size)	6	0.337	4.493**

Table 11. ANOVA results for the effects of population size and among-population variation on Shannon index (only bees considered, genus level), with and without the contribution of *Apis mellifera*. Data from collection through random walks. df=degrees of freedom, MS= Mean Square values, F=F-test values.
Signif. codes: 0 *** 0.001 ** 0.01 * 0.05 · 0.1, ns=not significant.

In both cases small and large populations had a similar insect diversity, while populations markedly differed each other. When honey bees were considered, “Pa” (predominantly visited by *Chalicodoma* spp. and *Amegilla* spp.) and “Sk” (predominantly visited by *Apis mellifera*) population exhibited the lowest Shannon values (0.8±0.2 and 0.77±0.05 respectively), whereas excluding honey bees, “Pa” (where honey bees were nearly absent) resulted the less diverse population (0.7±0.2).

As both observational and catching data showed that *Ballota acetabulosa* flowers were pollinated by numerous insect genera, with various abundance and behavior, it was reasonable to cluster pollinators into different functional groups. Categories were created according to i) insect life-style (i.e. solitary Vs social), ii) broad taxonomic position (i.e. family), and iii) sex. Insect body size didn’t result a good discriminant feature because of the overlapping of most categories.

The following guilds were created:

- ✓ Solitary Megachilidae females bees (Mf);
- ✓ Solitary Megachilidae males bees (Mm);
- ✓ Solitary Apidae females bees (Af);
- ✓ Solitary Apidae males bees (Am);
- ✓ Solitary Halictidae females + males bees (Hfm);
- ✓ Social bees (AB);
- ✓ Others (parasitic bees, butterflies, beetles, Oth).

A list of the insect genera comprised in each functional group is presented in Appendix 2.

Observational and catching data gave equivalent results relative to the frequency of each functional group ($r = 0.77$, $P<0.05$). As I wanted to associate different variables obtained through observations (flower handling time, number of flowers visited) to each guild, the former data were preferred to compute the analyses relative to the abundance and importance of each guild.

The most abundant guild was represented by AB (including *Apis mellifera* and *Bombus* spp), accounting for 34% of total visits, then Mf (32%) and Af (21%). Each guild strongly differed in its relative abundance among populations. For instance, the Mf guild was predominant in “Pa” population (78% of total visits), while it was under represented when the AB guild was prevalent (“Sk” population, 3%). By contrast, the AB guild relative abundance ranged from 84% in “Sk” population to 0% in “Pa” population (Table 12).

	Population							
Functional group	Mo	My	Pa	Pe	Pi	Sk	Th	Va
Mf	21.1	44.7	77.9	20.0	22.5	3.0	58.3	16.0
Mm	0.0	6.4	9.8	8.6	16.9	0.0	18.3	3.2
Af	10.5	11.7	12.3	35.7	31.0	13.4	16.7	35.2
Am	0.0	2.1	0.0	1.4	0.0	0.0	3.3	1.6
Hmf	3.5	2.1	0.0	0.0	14.1	0.0	0.0	1.6
AB	59.6	22.3	0.0	31.4	11.3	83.6	3.3	41.6
Oth	5.3	10.6	0.0	2.9	4.2	0.0	0.0	0.8

Table 12. Relative abundance (% of total visits) of each functional group (see text for details) in each *Ballota acetabulosa* population. Data from observation sessions, pooled rounds.

To compare the guild diversity among populations and population sizes, the Shannon index was calculated, and a two-level mixed nested ANOVA model was used, with populations (random factor) nested within size treatments (fixed factor), and rounds considered as genuine replicates. Again, *Apis mellifera* was in turn taken into account and omitted. Results are presented in Table 13.

Trait	Source	*df*	MS	F
With Apis mellifera				
Shannon index	Population size	1	0.486	1.7802 ns
	Population (size)	6	0.273	4.3333**
Without Apis mellifera				
Shannon index	Population size	1	0.565	1.7937 ns
	Population (size)	6	0.315	3.9375*

Table 13. ANOVA results for the effects of population size and among-population variation on Shannon index, (functional group level), with and without the contribution of *Apis mellifera*. Data from observation trials.
df=degrees of freedom, MS= Mean Square values, F=F-test values.
Signif. codes: 0 *** 0.001 ** 0.01 * 0.05 · 0.1, ns=not significant.

Population size had no effect in both cases (P>0.05), while, as expected, the Shannon index was different among populations (P<0.05). With honey bees not omitted, the same pattern found when insects were grouped into genera was found. “Pa” and “Sk” resulted in fact the less diverse populations (0.64±0.07 and 0.4±0.2 respectively). These two populations remained the last ranked when honeybees were not considered (0.64±0.07 and 0.3±0.2 respectively).

Summarized for all populations, the number of intraplant flowers visited per insect guild ranged from 2.7 (Hmf) to 7.7 (AB, *Apis mellifera* excluded), while flower handling time ranged from 3.1 (Am) to 23 (Hmf) (Table 14).

Functional group	Visitation rate	Flowers visited	Flower handling time
Mf	0.020±0.003	4.6±0.4	6±1
Mm	0.0041±0.0008	3.1±0.4	5.1±0.8
Af	0.013±0.002	5.4±0.7	5.9±0.8
Am	0.0006±0.0002	5±2	3.1±0.6
Hmf	0.0013±0.0005	2.7±0.5	23±9
AB (with *Apis mellifera*)	0.021±0.004	6.0±0.5	5.6±0.4
AB (without *Apis mellifera*)	0.007±0.002	7.7±0.9	4.2±0.6

Table 14. Summary of results of observation sessions. Visitation rate (number of visits per *Ballota acetabulosa* flower * 15'), number of visited flowers/plant*insect (15') and flower handling time (sec) are displayed. Mean ± SE are given. Insects are grouped by functional groups (see text for details), populations and rounds are pooled.

To evaluate the relative importance of each functional group as pollinator, an index of importance was calculated, as the product of mean guild relative abundance and mean pollen load (on head, thorax and abdomen). Both variables were referred to the overall insects scored (pooled populations and rounds).

Mf guild yielded the greatest amounts of *Ballota* pollen, and was present at high frequencies across all populations. This in turn implied it resulted the most effective guild for *Ballota acetabulosa* (Table 15). Honey bees greatly contributed to *Ballota* pollen service especially because of their high relative abundance; when omitted from the analysis, the AB importance index dropped from the second rank position to the fourth one, while Mf remained the most important guild.

In all functional groups (except Af), the thorax resulted the body part mainly deputed to pollen transport, whereas few pollen was placed on the abdomen. In contrast, insects of the Af guild preferentially contacted the anthers with their heads (Fig.18).

Functional group	N	Mean pollen grains per 15mmø sample ± SE	Mean relative frequency	Importance index
With Apis mellifera				
Mf	121	(1073±77) ^{e}	0.333	357.3
Mm	99	(841±106) ^{d,f}	0.071	59.7
Af	103	(347±39) ^{a,b,d}	0.211	73.2
Am	29	(121±15) ^{c,d}	0.010	1.2
Hmf	11	(503±148) ^{a,b,c,d,f}	0.022	11.1
AB	79	(364±50) ^{a,b,d}	0.352	128.1
Without Apis mellifera				
Mf	121	(1073±77) ^{e}	0.439	471.0
Mm	99	(841±106) ^{d,f}	0.094	79.1
Af	103	(347±39) ^{b,d}	0.279	96.8
Am	29	(121±15) ^{a,c,d}	0.013	1.6
Hmf	11	(503±148) ^{a,b,c,d,f}	0.030	15.1
AB	42	(158±29) ^{a,c,d}	0.146	23.1

Table 15. The following data are displayd: sample size of insects used for the pollen load analysis; mean number of *Ballota acetabulosa* pollen grains (per 15ømm sample) adhering to bodies of insect captured on flight (head + thorax + abdomen), after having visited a *Ballota* flower (pooled populations); mean relative frequency of each insect guild (after 48 hours of observations); importance index, i.e the product of mean pollen load and mean relative frequency. Insects are grouped into functional groups (with and without *Apis mellifera*, see text for details). Values significantly different ($P<0.05$) are indicated by different superscript letters (pairwise t-tests with Bonferroni correction, Log-transformed data).

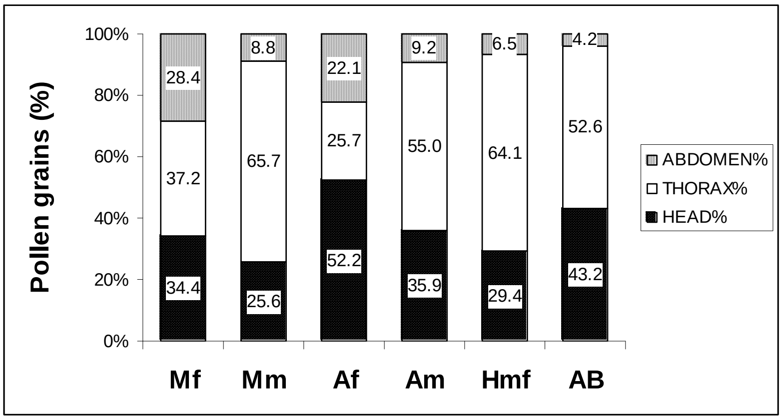


Fig.18. *Ballota acetabulosa* pollen distribution on the three main body parts of insects visiting the plant (pooled populations). Percentage are relative to means. Insects are grouped by functional groups (see text for details).

As Mf resulted the most important guild, its relative abundance and fidelity (the proportions of *Ballota* pollen on ventral scopa) were investigated in relation to both population size and among population variation (two-level nested mixed ANOVA model, with populations (random factor) nested within size treatments (fixed factor), and rounds considered as genuine temporal replicates) (Table 16). As far as fidelity concerns, six populations were considered (three small and three large), as in “Va” and “Pi” populations none or very few insects were examined for pollen composition.

The Mf abundance was never influenced by population size (P>0.05), while a significant among population difference emerged (P<0.05, see Table 12 for details).

Data relative to fidelity showed pronounced percentages of *Ballota* pollen on ventral scopa (mean ± SE: 96.6±0.7); population size had a null effect on it, and populations didn’t differ each other (P>0.05).

Trait	Source	*df*	MS	F
Mf relative frequency (*With Apis mellifera*)	Population size	1	1245.2	2.1637 ns
	Population (size)	6	575.5	3.7197*
Mf relative frequency (*Without Apis mellifera*)	Population size	1	962.3	1.8577 ns
	Population (size)	6	518.0	2.8122*
Mf fidelity	Population size	1	0.0031	1.2400 ns
	Population (size)	4	0.0025	1.6667 ns

Table 16. ANOVA results for the effects of population size and among-population variation on Mf (Megachilidae female bees) relative frequency (proportions of Mf guild on total visits, *Apis mellifera* retained and omitted, angular-transformed data), and Mf fidelity (proportions of *Ballota* pollen, angular-transformed data) .

df=degrees of freedom, MS= Mean Square values, F=F-test values.

Signif. codes: 0 *** 0.001 ** 0.01 * 0.05 · 0.1, ns=not significant.

The two most represented insect families (Apidae and Megachilidae, see Fig.5) were negatively correlated in their absolute abundance (logarithmic relationship, $P<0.05$, Fig.19). The same relationship was found when considering the Mf and AB abundance (with *Apis mellifera* and *Bombus* spp., and only *Apis mellifera* included, $P<0.05$).

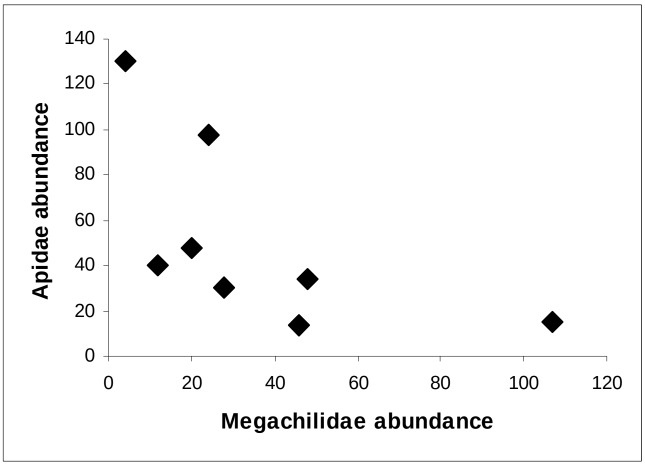


Fig.19. Mean Apidae abundance (overall number of insect visitors recorded in 48 hours of observation) Vs mean Megachilidae abundance (overall number of insect visitors recorded in 48 hours of observation). Populations are pooled.

3c. Results: *Ononis masquillierii*

3.1c. Population characteristics

Population size ranged from 151 to 3575 flowering racemes (average of the two rounds), and density ranged from 0.42 to 7.08 flowering racemes per square meter. Populations fell within the following categories: 3 large populations (>1000 flowering racemes), 3 medium populations (500-1000 flowering racemes) and 2 small populations (<500 flowering racemes) (Table 3).

3.2c. Flowering characteristics and nectar standing crop

The flowering period of *Ononis masquillierii* lasted about one month, from late May to early July. Flower life span was quite short (1.31±0.04 days, N=20).

At the bud stage stigmas are not receptive, and they are often covered by the pollen released from the dehisced anthers; pollen viability tests performed on pollen at this stage showed a positive reaction. Stigmas become receptive when the flowers opened, and they remain in that state until they withered. Pollen is viable in all flower stages.

Nectar standing crop was null in any flower observed.

3.3c. Breeding system and pollen limitation

Recovery of the marked flowers was over 75%.

Seed set after the hand-selfing and spontaneous selfing treatments produced very little seeds, and the two treatments didn't differ in their response (M-W *U*-tests; P>0.05), while manual cross-pollination tests resulted in the highest seed set (M-W *U*-tests; P<0.001, Fig.20).

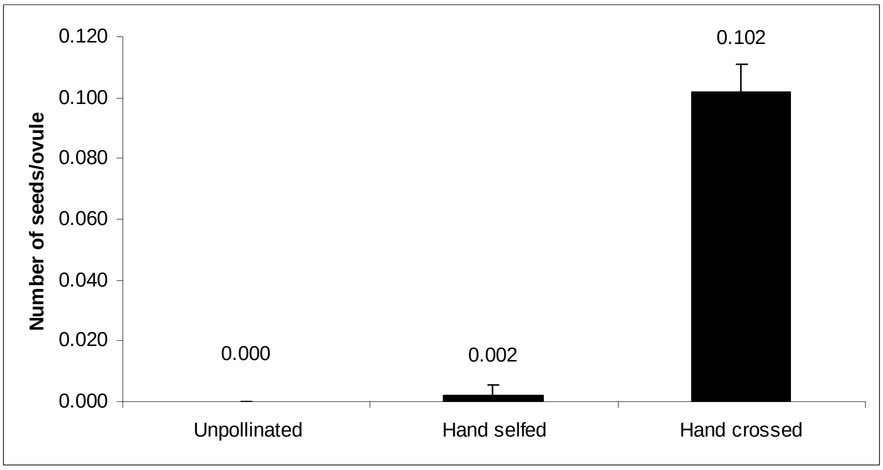


Fig.20. Reproductive success of *Ononis masquillierii* in unpollinated, hand selfed and hand crossed flowers conducted at Fl site. Columns represent the mean number of viable seeds per ovule. Mean seed sets are displayed above the bars in the graph.

No significant differences in the seed set were found between the bagged and unbagged hand-crossed flowers at "Fl" (M-W *U*-tests; P>0.05), thus excluding any influences of the bags on seed set.

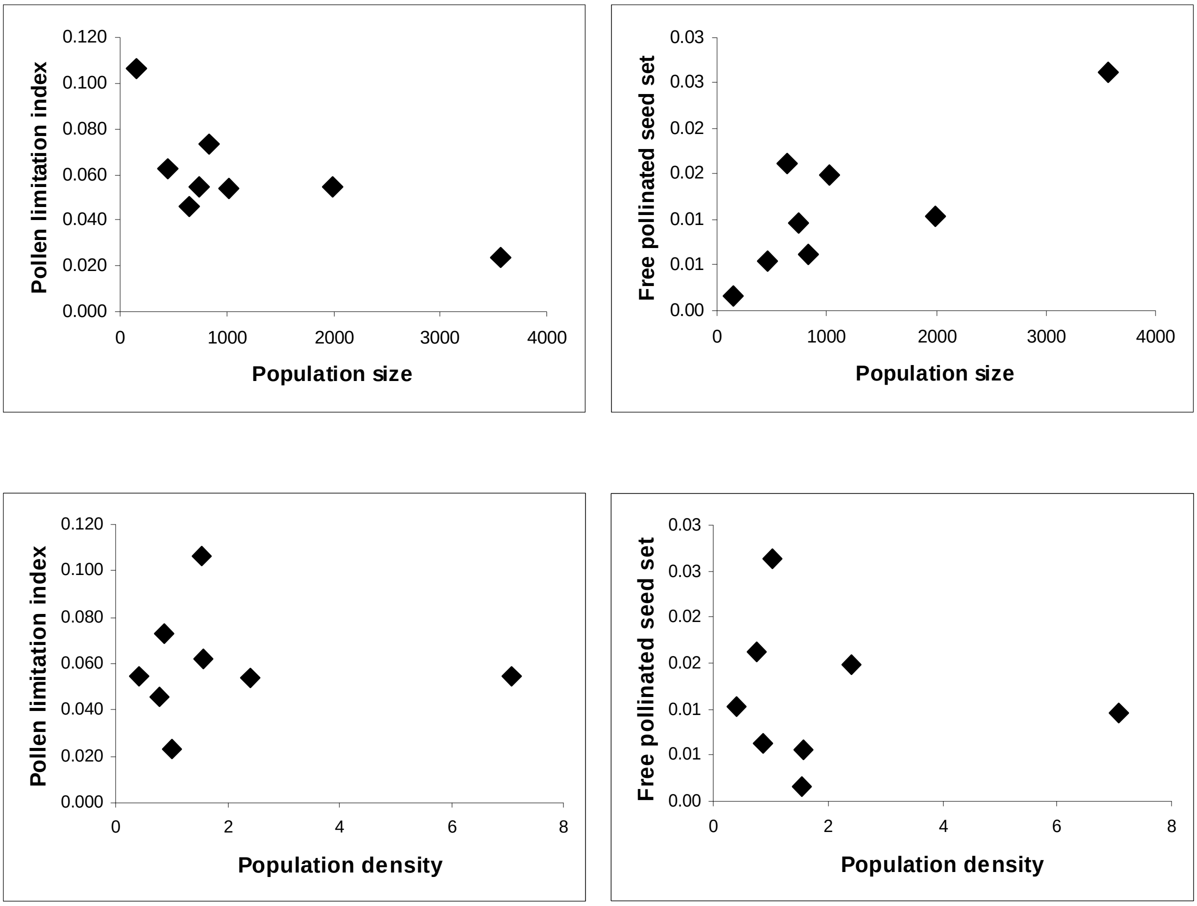
Generally, one or (rarely) two seeds per flower were produced after hand-crossed pollination treatments in all populations.

Pollen limitation was always present, and its magnitude didn't vary among the 8 populations (K-W test, P>0.05). The mean pollen limitation was 0.102 (range 0.074-0.130, N=8) with the minimum value found at "Fl" (the largest population), and the maximum at "Cb" (the smallest population) (Table 17).

Population	Hand crossed	Free pollinated	M-W
Fl	0.10±0.01	0.026±0.008	**
Cc	0.109±0.006	0.010±0.006	***
Er	0.122±0.008	0.015±0.007	***
Mp	0.119±0.007	0.006±0.005	***
Mc	0.11±0.01	0.010±0.006	***
Ab	0.113±0.006	0.016±0.007	***
Cr	0.103±0.007	0.005±0.005	***
Cb	0.132±0.007	0.002±0.003	***

Table 17. Results of separated pairwise Mann-Whitney *U*-tests for seed-set in hand crossed and nonsupplemented flowers in 8 populations of *Ononis masquillierii*. Mean ± SE are given (backtransformed data). Populations are listed in order of size (number of flowering racemes). Signif. codes: 0 *** 0.001 ** 0.01 * 0.05, ns= not significant.

Nevertheless, pollen limitation was negatively correlated with population size (r = -0.73, P=0.04; regression based on population means, Fig.21a), while neither population density nor insect abundance had any influence on pollen limitation (P>0.05). The negative effect of population size on pollen limitation reflected the positive relationship between population size and the seed set of unmanipulated flowers, where large populations yielded greater amounts of seeds than small ones, (r = 0.81, P=0.01, regressions based on population means, Fig.21b), while again population density had a null effect (P>0.05). No patterns emerged when considering the seed set relative to hand crossed flowers (P>0.05).



a **b**

Fig.21. Seed set as a function of population size (mean number of flowering racemes) and density (mean number of flowering racemes/m²). Regressions were calculated for (a) the difference in mean seed set between hand-crossed and unpollinated flowers (i.e. "pollen limitation index"), (b) the mean seed set in unpollinated flowers (Free pollinated seed set). Data refer to the 8 *Ononis masquillierii* populations and are pooled over two rounds.

3.4c. Flower insects

A total of 112 insects were caught in 16 hours of random collection across the eight populations. As the study spanned the full flowering period of the species, I am quite confident to have nearly covered the full spectrum of insect visitors. All insects belonged to Hymenoptera, with 8 genera represented overall (Table 18). On 112 insects no males were caught.

Taxon	Functional group	Population								Total
		Ab	Cc	Cb	Cr	Er	Fl	Mc	Mp	
Hymenoptera										
Andrenidae										
Andrena spp.	M-L	-	3	-	1	1	6	-	1	12
Apidae										
Anthophora sp.	L	-	-	-	-	-	1	1	-	2
Bombus spp.	L	-	7	-	-	4	2	-	-	13
Eucera spp.	M-L	-	3	2	-	4	9	-	6	24
Halictidae										
Lasioglossum spp.	S	4	5	1	-	2	4	4	6	26
Megachilidae										
Anthidium *sensu lato* spp.	S-M	1	-	-	-	4	-	-	1	6
Osmia *sensu lato* spp.	S-M	3	1	1	5	5	2	-	5	22
Megachile spp.	S-M	-	-	1	1	2	-	2	1	7

Table 18. List of insect visitors recorded in 8 populations of *Ononis masquillierii* in 2006. For each population the number of insect caught during a total of 2 h of random collection (over 2 rounds) is indicated. Insect functional groups are given (see text for details).

Three most abundant genera were observed, with a similar relative frequency of approximately 20% (*Lasioglossum* spp, *Osmia* spp, *Eucera* spp), while *Anthophora* spp. accounted for <2%, and could be considered an occasional visitor (pooled populations). Most of the genera were absent in some populations as a consequence of the low insect abundance. For instance, *Anthophora* spp was only recorded in two populations (“Fl” and “Mc”), while *Anthidium* spp and *Bombus* spp in three populations (“Ab”, “Er”, “Mp” and “Cc”, “Er”, “Fl” respectively).

By grouping insects onto functionally similar taxonomic groups, according to both taxon (genus) and size (expressed by the body mass) simultaneously, I obtained the following guilds (in order of size, Table 18):

- ✓ small bees (including *Lasioglossum* spp., “S”);
- ✓ small-medium bees (including *Megachile* spp., *Osmia* spp., *Anthidium* spp., “S-M”);
- ✓ medium-large bees (including *Andrena* spp. and *Eucera* spp., “M-L”);
- ✓ large bees (including *Anthophora* sp. and *Bombus* spp., “L”).

All groups were significant different from each other in terms of body mass (M-W *U*-tests; P<0.001) while genera belonging to the same group were statistically equivalent (M-W *U*-tests; P>0.05). Overall, by pooling all the 8 populations, the four functional groups were nearly equally distributed, except large bees that only accounted for 13,4% of total insects; small bees were represented only by genus *Lasioglossum* but accounted for 23,2% of total insects. No functional group was the most abundant in all populations. For example, medium large bees were overall the most frequent group, but at population level they were the first ranked group only in three populations (“Cb”, “Fl”, “Mp”). Conversely, the large bees group was the less frequent in all populations, but at Cc it was first ranked accounting for 37% of all insects.

The absence of a clear supremacy of the same guild across all populations reflected the great variation in guild composition across populations. In fact only three populations had all the four insect groups represented (“Cc”, “Er” and “Fl”), while two populations had only two functional groups represented (“Ab” and “Cr”). This pointed out a lack of correspondence between the patterns of insect guild abundance in each population and the overall distribution.

Insect abundance was significantly and positively correlated with the logarithm of population size (r = 0.81, P=0.01, Fig.22) but not to population density (P>0.05).

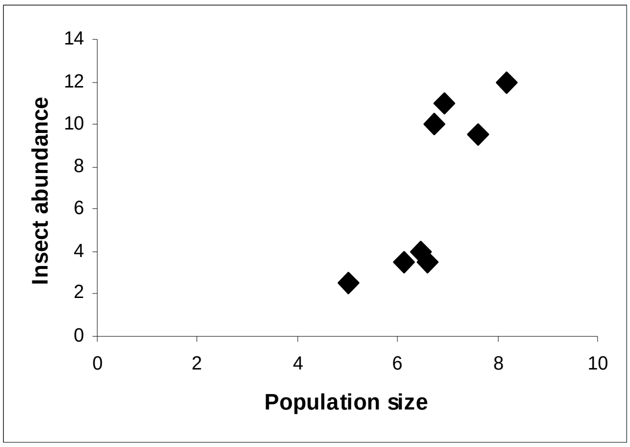


Fig.22. Mean insect abundance (number of *Ononis masquillierii* insect visitors per hour, from random walks collection) Vs mean population size (number of flowering racemes, Log-transformed data).

3.5c. Pollinator effectiveness and importance

From observations on pollinator behavior, it resulted that all insects caught touched the reproductive column, thus effectively contributing to pollen transfer.

Summarized for all populations, functional groups differed in the amount of *Ononis masquillierii* pollen carried per body area (K-W test; P=0.02), even if only large bees statistically differed from the other guilds, carrying the smallest amount of pollen per area (M-W *U*-tests; P<0.05, Table 19).

Functional group	N	Median	Q1	Q3
S	26	232.0 ^{a,b,c}	44.8	626.8
S-M	35	121.0 ^{a,b,c}	27.5	444.0
M-L	36	96.0 ^{a,b,c}	33.3	283.0
L	15	25.0 ^{d}	16.0	39.0

Table 19. Median number of *Ononis masquillierii* pollen grains (per 15ømm sample) adhering to bodies of insect captured on flight (head + thorax + abdomen), after having visited an *Ononis masquillierii* flower (pooled populations). Insects are grouped by functional groups (S= small bees; S-M= small-medium bees; M-L= medium large bees; L= large bees). Sample size, 1st and 3rd quartile are given (N, Q1, Q3 respectively). Values significantly different (P<0.05) are indicated by different superscript letters (a=S, b=S-M, c=M-L, d=L).

A significant inverse correlation between pollen density and insect body mass was found, with small bees that carried on average eight times more pollen per area than the largest bees (r = -0.98, P=0.02, regression based on population means, Fig.23).

Furthermore, differences in flower handling time by the four functional groups were detected, with small bees that spent significantly more time per flower than any other guild (M-W *U*-tests; P<0.001, Fig.24).

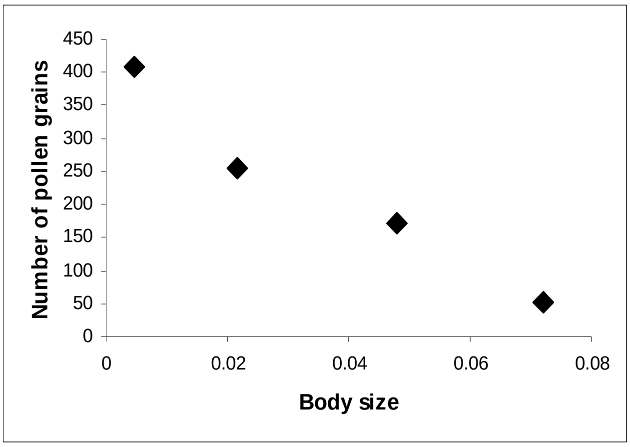


Fig.23. Relationship between mean number of *Ononis masquillierii* pollen grains carried on insect body (head + thorax + abdomen, overall: 15ømm sample) and mean weight (gr) of insect visitors grouped by functional group.

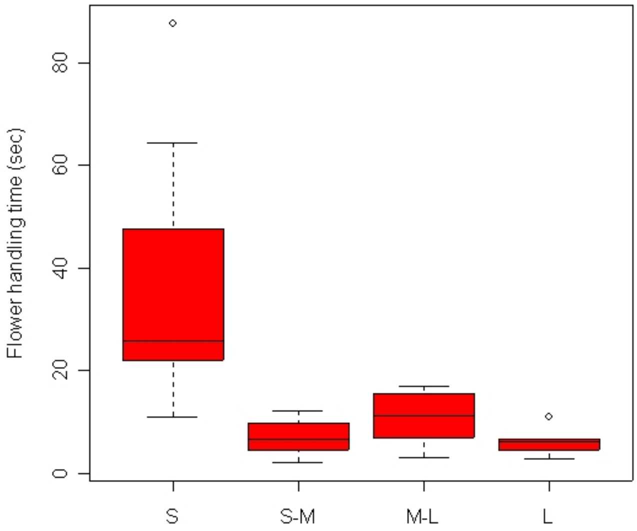


Fig.24. Boxplot of flower handling time (sec) by the different functional groups recorded on *Ononis masquillierii* flowers during 32 hours of observation across 8 populations in 2006 (S= small bees; S-M= small-medium bees; M-L= medium large bees; L= large bees). Medians are indicated by the central lines, the lower and upper edges of the box are the 1st and 3rd quartiles respectively. The whiskers extend to a maximum of 1.5 times the inter-quartile range, the points outside the whiskers are potential outliers.

In addition, insect size affected the pollen placement on the three main body parts (head, thorax, abdomen). No significant pattern of pollen density on head among functional groups was found, while, when considering the pollen adhered to thorax and abdomen, it resulted that large bees tended to transport significantly less pollen than small and medium bees (M-W *U*-tests; $P<0.05$, Table 20). That tendency could be the consequence of a mechanical barrier relative to flower size:

observations revealed that small and medium bees could enter the flower with all their body, and collected pollen by moving freely inside it; conversely large bees were mainly forced to use the head for contacting the reproductive column. This is confirmed by the high proportion of *Ononis* pollen grains carried on head by large bees (41%), compared with the remaining functional groups (Fig.25).

Body part	Functional group	N	Median	Q1	Q3
Head	S	26	$5.5^{a,c,d}$	1.0	28.0
	S-M	35	$23.0^{b,d}$	7.0	92.0
	M-L	36	$6.5^{a,c,d}$	3.0	26.3
	L	15	$11.0^{a,b,c,d}$	2.5	21.5
Thorax	S	26	$50.5^{a,b,c}$	5.75	178.0
	S-M	35	$16.0^{a,b,c}$	5.0	79.0
	M-L	36	$21.0^{a,b,c}$	3.8	63.3
	L	15	5.0^{d}	1.5	8.5
Abdomen	S	26	$40.0^{a,b,c}$	5.3	343.8
	S-M	35	$54.0^{a,b,c}$	9.5	178.0
	M-L	36	$46.5^{a,b,c}$	13.0	119.5
	L	15	6.0^{d}	2.0	13.0

Table 20. Median number of *Ononis masquillierii* pollen grains (per 15ømm sample) carried on different body parts of insects visiting the flowers (pooled populations). Insects are grouped by functional groups (S= small bees; S-M= small-medium bees; M-L= medium large bees; L= large bees). Sample size, 1st and 3rd quartile are given (N, Q1, Q3 respectively). Values significantly different ($P<0.05$) are indicated by different superscript letters (a=S, b=S-M, c=M-L, d=L).

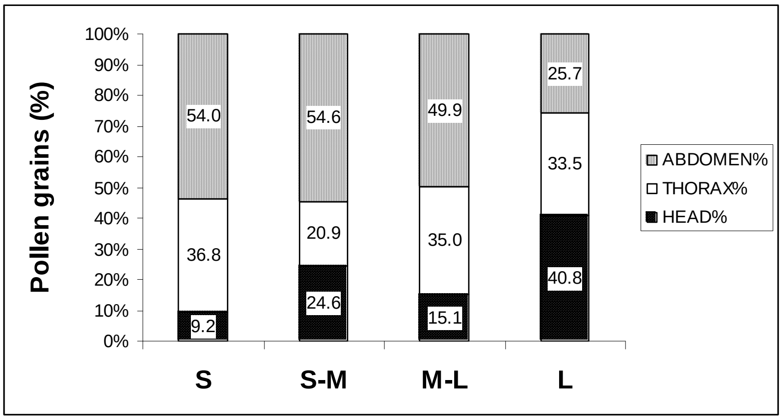


Fig.25. *Ononis masquillierii* pollen distribution on the three main body parts of insects visiting the plant (pooled populations). Percentage are relative to means. Insects are grouped by functional groups (S= small bees; S-M= small-medium bees; M-L= medium large bees; L= large bees).

In order to have an estimate of the contribution of each functional group to the pollination of *Ononis masquillierii*, an index of pollinator importance was calculated as the product of the relative frequency of each functional group and its mean pollen load (head + thorax + abdomen).

The same pattern between pollen density and insect body mass was found, as the importance of each functional group was inversely related to its body size (r = -0.98, P=0.02 regression based on population means, all populations pooled, Fig.26).

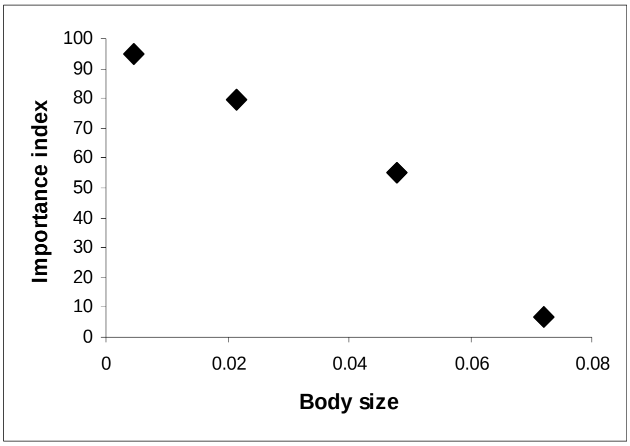


Fig.26. Importance index (mean pollen grains per 15mmø sample (head+thorax+abdomen) x mean relative frequency) Vs mean insect body size relative to insects visiting *Ononis masquillierii* flowers across 8 populations (pooled populations). Insects are grouped by functional groups.

The most important pollinators were small bees (Table 21), with an importance value more than 10 times higher than the less important functional group, represented by the largest insects. This was mainly due to the great amounts of pollen carried on the body of small bees, rather than to their relative frequency (Table 21). By contrast, despite their high relative frequency, medium-large bees ranked only third, as they carried less pollen than small bees.

No insect guild was the most important in all populations, although no statistical elaboration could be performed due to the low number of insects per population.

Functional group	Mean pollen grains per 15mmø sample	Mean relative frequency	Importance index
S	409.62	0.232	95.0
S-M	253.97	0.313	79.5
M-L	171.11	0.321	54.9
L	52.73	0.134	7.1

Table 21. Importance index (mean pollen grains per 15mmø sample (head+thorax+abdomen) x mean relative frequency) calculated for each functional group visiting *Ononis masquillierii* flowers (S= small bees; S-M= small-medium bees; M-L= medium large bees; L= large bees).

Data on scopal pollen composition showed very high values of fidelity of all insect guilds, with *Ononis* pollen percentage always over 80% (all populations pooled, Table 22). Despite these high values of fidelity, a great variability among pollinator guild was found (K-W test; P<0.001, Table 22). Small bees carried the highest percentage of *Ononis* pollen (median 100%), while small-medium bees, even if representing the second more important functional group, showed the lowest value of fidelity (median 81%). If taken separately, in 6 of 8 populations the highest values of fidelity came from small bees, even if the very low insect abundance per population prevented the data to be statistically analysed.

Functional group	N	Median (mean) fidelity	Q1	Q3
S	26	1.00 (0.89) ^{a}	0.88	1.00
S-M	35	0.81 (0.69) ^{b}	0.53	0.91
M-L	36	0.98 (0.84) ^{c,d}	0.80	0.99
L	15	0.98 (0.91) ^{c,d}	0.96	0.99

Table 22. Median (mean) fidelity (proportion of *Ononis masquillierii* pollen on scopa) of the four functional groups visiting *Ononis masquillierii* flowers (S= small bees; S-M= small-medium bees; M-L= medium large bees; L= large bees). Sample size, 1st and 3rd quartile are given (N, Q1, Q3 respectively). Values significantly different ($P<0.05$) are indicated by different superscript letters (a=S, b=S-M, c=M-L, d=L).

4a. Discussion: *Echium plantagineum*

The main goal of this study was to test whether population size represented an important factor for *Echium plantagineum* reproductive success. My data suggest to some extent a different behavior of large Vs small populations.

In fact, in the small studied populations, the seed set in open, free pollinated flowers was almost 40% lower than in large ones. This agrees with other studies, where reproductive success was found to be affected by plant population size (Ågren 1996, Hendrix & Kyhl 2000, Kéry et al. 2000, Oostermeijer et al. 2000, Mavraganis & Eckert 2001, Tomimatsu & Ohara 2002, Brys et al. 2004, Kolb 2005, Ward & Johnson 2005, Hensen & Wesche 2006). Surprisingly, the same tendency was found when considering the hand-crossed flowers, being small populations on average 20% less productive than large populations. By contrast, no among–population differences were detected in both “free” and “cross” data.

In addition, for six of seven populations, pollen addition experiments were successful in showing some level of pollen limitation: the average number of seeds per ovule was in fact significantly higher when pollen supplementation occurred than in natural conditions. Nevertheless, small and large populations experienced a similar magnitude of pollen limitation.

Generally, three factors can influence the female reproductive success of a plant species: i) the provision of suitable pollen for fertilization of ovules, ii) the amount of resources required for seed production and development i.e. light, nutrients and water, and iii) the presence of plant enemies (herbivores, pathogens).

Between-population size differences in plant fecundity, calculated as seed set in free pollination treatment, revealed that somehow large populations were slightly more productive than small ones. This difference can be attributed to one or more of the previous listed factors. Even if not directly measured, resources availability seems to be important, as seed output in supplemented flowers (cross seed set) was different between population size classes, being higher for large populations. On the contrary, when considering the pollen limitation index, no pattern emerges, as small and large populations set an equivalent number of seeds. Thus, from productivity data, pollination services didn’t vary in their magnitude between population size categories. This is confirmed by data relative to insect visits: large and small populations received in fact the same amount of insects, and significant differences only occurred at a population level. Again, the foraging behavior of pollinators (in terms of number of flowers visited within a plant) was not influenced by population size.

It is hard to discuss the direct causes of the similar attractiveness to insects of large and small populations, but I can hypothize that the availability of insect nesting sites more than plant population size may have played a major role.

The question of whether resources rather than pollination may represent the principal limiting factor to reproduction success have been already addressed (Stephenson 1981, Lee 1988, Zimmerman & Pyke 1988, Mustajärvi et al. 2001). Nevertheless, the present data need to be treated with some caution, as this study made no attempt to modify the amount of resources, thus not teasing apart the two effects. The strict dicothomy between pollen and resource limitation has been criticized in the past on the ground of both theoretical and experimental manipulation ground, and seed output could be the result of an equilibrium between resource and pollination levels (Galen et al. 1985, Haig & Westoby 1988, Campbell & Halama 1993).

The picture is further complicated by the impact of grazing on *Echium plantagineum* fitness.

Results indicate that in some populations the grazing pressure was so heavy that half the marked seeds were lost, presumably eaten by sheep and goats. Furthermore, small *Echium* populations were more exposed to this type of pastoral practice.

The Mediterranean region has a long history of human-induced perturbations, with fire and grazing playing a major role (reviewed in Petanidou & Lamborn 2005). The effect of grazing on plant-pollinator interactions mainly depends on its intensity: if moderate and not persistent, it can support a richer bee (through an increase of nesting sites) and plant (through a balance between open ground and woody habitat fragments) communities; if intensive or uncontrolled, it can lead to the destruction of bee nesting sites and soil erosion.

In general, grazing practice in the studied sites in Lesvos can be considered threatening for *Echium* populations, as in most cases plants had many damaged cymes, and sometimes after grazing the soil was left completely bare. To what extent grazing pressure may have contributed to determine the size of small *Echium* populations remains unknown, but differences in seed set with large populations (ungrazed or less grazed) suggest a direct effect in causing a potential shortage of seed production for small ones, probably affecting the seedling recruitment in a successive stage. The potential reduction in both seed production and soil seedbank level by grazing is confirmed by studies conducted where *Echium plantagineum* is considered a weedy alien species (Smyth et al. 1997).

Thus, the combined effect of resource limitation and grazing pressure may have significantly shaped the size of *Echium* populations.

When all populations are pooled, further data emerge. As a group in fact, the seven populations of *Echium plantagineum* showed i) a negative relationship between grazing pressure and patch density, and ii) a decline in seed set (both for cross and free treatments) of surviving plants occurring in overgrazing regimes.

On the first point, a possible explanation may lay on a biochemical base. *Echium plantagineum* contains pyrrolizidine alkaloids, toxic to livestock (Culvenor 1956). Even if sheep can graze it for a time, prolonged grazing can provoke serious liver damages. Coupled with the fact that sheep are selecting in their feeding preferences, preferring the more palatable plants (Grant & Armstrong 1993), in the studied populations they presumably tended to avoid *Echium plantagineum* as a first foraging choice, and plants were likely grazed only when the feeding sources provided by the other co-occurring plant species were depleted (pers. obs). It derives that denser *Echium* patches were less grazed than sparser ones, at the expense of co-occurring plant species.

The second point highlights the consequences that intensive grazing had in ungrazed (or partially grazed) plants in terms of seed development. Even if grazing can also have beneficial effects on plants, as defecation and urination can increase the soil's nutrient properties, and competition among large-seeded annual grasses is reduced (Grigulis et al. 2001), overgrazing is likely to stress the detrimental effects. My results suggest that the effects of grazing on productivity were not limited exclusively on a loss of potential seeds (via flower and/or fruit eating), but also on a whatever it might be damage to the ungrazed flowers. Two (non-mutually exclusive) hypotheses can be formulated. The first invokes a possible mechanical damage to *Echium* plants (at the reproductive and/or vegetative structures), and a subsequent loss of vigour. Intensive biting and shearing may cause stem breakage and bruises leaf tissues, while trampling can alter the soil permeability of both air and volume, thus limiting rooting volume (Wells & Dougherty 1997). As plant systems are dominated by trade-offs (Weih 2003), lowered overall resources could imply a reallocation of resources at the expense of the male and/or female reproductive fitness, that is pollen and ovules respectively.

A second explanation can concern the availability of compatible mates in the population. As *Echium plantagineum* resulted fully self-incompatible, and as grazing reduced the number (and/or the vigour) of the flowering conspecifics, legitimate pollen transfer wouldn’t be favoured in overgrazing conditions. A minimum amount of pollen must be available for the fertilization process to occurr (Cruden 2000), therefore a fraction of the egg cells could not have been fertilized. Nevertheless this last hypothesis would not account for the decline in productivity in handcrossed flowers.

Denser patches experienced lower level of grazing than sparser ones, but they also resulted more attractive to insects. The positive relationship between flowering plant density and insect visitation rate has been already documented by other studies (Kunin 1993, 1997), even if not always (Bosch & Waser 1999, 2001, Mustajärvi et al. 2001, Kirchner et al. 2005). The present study shows that denser patches were associated with i) a higher number of visits per flower (with density calculated as the number of flowering individuals per area), and ii) a smaller number of flowers visited within a plant per insect (with density calculated as the spacing between neighbouring individuals). Thus, on a theoretical base, insects visiting denser patches would provide better services both in terms of the quantity (number of pollen grains deposited on stigmas) and the quality (type of pollen grains deposited on stigmas) components of pollination than insects visiting sparser patches. Selfing by geitonogamous pollen may be determined by the behavior of pollinators, and can be enhanced if pollinators visit more flowers within a plant (Utelli & Roy 2000); in self-incompatible species (as *Echium plantagineum*), geitonogamy would imply a waste of pollen and a reduction of the free stigma surface available for the legitimate pollen to germinate.

In conclusion, even though productivity (cross-free seed set) was not associated with patch density, there may be an advantage to organizing *Echium plantagineum* individuals into dense rather than sparse patches because of i) a lower intensity of grazing, and ii) a higher ability to attracting the pollen vectors.

Finally, a puzzling aspect of this study concerns pollen limitation. My findings clearly show that almost all populations occurred in highly pollen limited contexts, even if the extent of pollen limitation didn’t vary with *Echium* population size. More than 75% of the initial ovules after the pollen hand supplementation developed into seeds, while in open natural condition the percentage dropped to 38%. A similar occurrence of pollen limitation was found in another species of the island (*Ballota acetabulosa*). However, it seems hard to detect which factor could have driven this pattern. Generally, common species are expected to enjoy higher visitation rate than rare species, thus are expected to be less prone to pollen limitation. *Echium plantagineum* is a common (even if not dominant) species in the Mediterranean, which represents its native range and is considered a hot spot in terms of plant and bee diversity (Proctor et al. 1996). From my observations, the large, nectariferous flowers attracted a large amout of insects (mainly bees), covering a wide spectrum of families and genera (pers. obs.); thus pollen limitation would not be expected. Moreover, a previous study conducted in Spain showed evidence of a wind-borne transport of *Echium* pollen, that might be important in the among-population pollen exchange (Rodríguez et al. 2005). This could reduce reliance on pollinators, and could account for the similar pollen limitation values found between large and small populations, but again this would contrast with data on pollen limitation. In addition, when it co-flowered with other plant species, *Echium plantagineum* resulted a good competitor, but my study didn’t gather data on insect visitation rate to other flowering species in the absence of *Echium* plants. It seems unlikely that the insect visitation rate to *Echium* flowers was lower than the one to other flowering species, even if in the island spring represents the “boom” period in terms of number of blooming species, and competition for pollinators can be tough (Petanidou et al. 1995b).

Two main hypotheses can be formulated to explain the existence of pollen limitation.

First, plant species evolving in a predictable pollination system may be particularly prone to pollen limitation if some sort of habitat perturbation occurs. The most intuitive effect of recent habitat degradation is that a continuous habitat can be broken up into small fragments, often by some processes derived from anthropogenic sources. Because a mutualistic interaction is involved, it is sufficient that only one component (either plant or pollinators) is plagued by habitat fragmentation for the weakening or disruption of a particular plant-pollinator interaction, hence resulting in pollen limitation. Generally, the surroundings of the sites where *Echium* populations occurred formed a complex and artificial matrix, with olive groves and disturbed open areas often interrupted by roads and villages. In addition, aerial insecticide spraying in olive groves represented a constant threat to bee communities. Even if *Echium plantagineum* is a common species in Lesvos, the degree of habitat fragmentation (both from *Echium* plants and its pollinators perspective) where it occurrs remains unknown, and although tempting (without a complete knowledge of the bee nesting requirements), I cannot exclude the possibility of a pollinator shortage or decline in the area, as a consequence of the low habitat quality. At the level of resolution of my study, the consequences of this loss would have affected *Echium* populations regardless of their size, as bee essential resources were probably *Echium*-indipendent.

Second, pollen limitation may be the result of an adaptive equilibrium in response to stochastic processes that govern pollination systems (Burd 1994, 1995). The unpredictability of pollen receipt in such systems may favour an oversupply of ovules per flower, in order to achieve the maximum seed set in optimal pollination situations, thus it would result adaptive for the species to be chronically pollen limited. The Mediterranean is dominated by tremendous fluctuations in insect abundance and composition between years (Herrera 1988, Petanidou & Ellis 1993, Petanidou & Potts 2006), and variations in the pollination environment coming from human-induced perturbations may exacerbate these fluctuations, putting under risk the pollen transfer also for bee-richer areas and for common plant species.

Therefore, further work (at both spatial and temporal scale) is necessary to untangle the relative role of factors ruling pollen limitation.

4b. Discussion: *Ballota acetabulosa*

There are two major findings of this study: i) natural populations of *Ballota acetabulosa* differing in size equally responded in terms of reproductive success, insect visitation rate, number of flowers visited within a plant per insect and insect diversity; ii) most of populations were strongly subject to pollen limitation, irrespective of population size.

There is a large body of evidence that large population size is positively related to pollination (Jennersten 1988, Aizen & Feinsinger 1994a, Waites & Ågren 2004) or plant fecundity (Ågren 1996, Hendrix & Kyhl 2000, Kéry et al. 2000, Oostermeijer et al. 2000, Mavraganis & Eckert 2001, Tomimatsu & Ohara 2002, Brys et al. 2004, Kolb 2005, Ward & Johnson 2005, Hensen & Wesche 2006). Nevertheless, some other studies indicated that the previous associations are not always valid (Kunin 1997, Molano-Flores & Hendrix 1999, Leimu & Syrjänen 2002); therefore it always results dangerous to draw conclusions from single studies, as pollination and plant reproductive ecology are governed by complex interactions.

From the pollination facet, pollinators can approach large populations at a higher rate than small ones because of their larger floral display (Bell 1985, Valido et al. 2002, Momose 2004). Moreover, in natural plant populations population size and density are often correlated, and large population size can lead to higher rates of outcrossing (Van Treuren et al. 1993). Therefore, insufficient quantity and quality of pollen are commonly considered the most likely explanantions to reduced seed set in small populations (Byers 1995), especially in case of self-incompatible, animal-pollinated species. In this study, the size of *Ballota* populations had no impact on seed set (difference between hand crossed and open pollinated treatments, pollen limitation index), indicating that somehow pollinator services were not prevailing in large populations. Data concerning insect visits confirmed this assessment, as no differences in insect visitation rate between large and small populations were found. Again, with regard to the quality component of pollination, the outcome was that the number of flowers visited by each pollinator within a plant didn’t respond to population size, suggesting that the likelihood of performing geitonogamy was the same in large and small populations.

Therefore, the lack of any differences in seed set between large and small populations can be interpreted in the light of a similar pollen service, as the two patterns go in tandem.

Sometimes, for the plant reproductive output the resources available for reproduction result more important than the pollination services (Stephenson 1981, Lee 1988, Zimmerman & Pyke 1988, Mustajärvi et al. 2001); nevertheless, in *Ballota acetabulosa*, seed set after pollen supplementation was not different between population size groups, suggesting that resources were equally distributed between population size categories.

In addition to a variation in the total amount of visits, some studies have investigated the role of plant population size in determining the composition of the pollinator fauna (Sih & Baltus 1987, Sowig 1989, Levin 2000), while others investigated the role of habitat fragmentation (Aizen & Feinsinger 1994b, Tomimatsu & Ohara 2003). Aizen and collegue showed that, in an Argentine subtropical dry forest, small fragmented habitat units were poorer in native species than continuous fragments, and that some processes of bee guild replacing with non-native bees (*Apis mellifera*) occurred. Again, insect shifts between large and small populations were recorded in a deciduous forest in Japan by Tomimatsu and collegue.

Ballota acetabulosa was mainly pollinated by bees and, even if all insects contacted the flower reproductive column, most of them were uncommon (but widespread across the island) and occurred at a low abundance. This suggests that the web of interactions between *Ballota acetabulosa* and its pollinators was composed by a core of strong interactions, and a network of weak interactions. Both at genus and functional group level, the Shannon index didn’t differ with population size, whereas great differences in insect assemblages among populations existed (whether honyebees were considered or not). Therefore, as it happened with data on visitation rate and geitonogamy, population size didn’t affect the composition and richness of pollinators, as large *Ballota* populations didn’t support a more diverse entomophilous fauna than small ones.

Therefore, the overall results indicate that small populations of *Ballota acetabulosa* were unlikely to be at a reproductive disadvantage, and I feel that some considerations may be relevant in explaining this homogeneity of response.

First, I expect population-related size and density effects on pollination and reproductive success to have a minor influence on common species adapted to grow on different habitats. This is the case of *Ballota acetabulosa*, that can be considered a common species in Lesvos. The eight studied populations fell within a wide range of habitat types, such as open sunny disturbed lands, sparse oak forests or olive groves, often in the proximity of roads and towns. Although large and small populations constituted two clear and distinct categories, I can’t exclude that, at a higher scale, plants of *Ballota acetabulosa* were arranged in more continuous fragments, and that population connectivity was higher than expected. In this case, whether reproduction and pollination in *Ballota* populations showed a significant relationship with size could have depended on the presence of other neighbouring populations, and on whether one or few small populations received great amounts of suitable pollen.

Furthermore, the ecological features characterizing plant-pollinator interactions during the flowering period of the species may provide other insights into the size-related effects on pollination and reproduction of *Ballota acetabulosa*. In an exhaustive 4-years long study carried on the greek phrygana, Petanidou and colleagues (Petanidou & Ellis 1993, Petanidou & Vokou 1993, Petanidou et al. 1995b) examined the temporal patterns of resource selection in this particular plant-pollinator system (Fig.27). It resulted that flowering plant species were very sensitive to the summer climate constraints, and that only few of them managed to overcome the prolonged summer drought, being shrubs the dominant life form. The main flowering period extended from mid-February through May, thus most of the plant species bloomed early in the season. By contrast, insects resulted less affected by summer constraints, as a shift towards summer in the temporal distribution of insect species occurred. Therefore, the overlapped temporal patterns suggested a possible competition among flowering species for insects during the main flowering period, and viceversa (competition among insects for floral rewards) during late spring-summer.

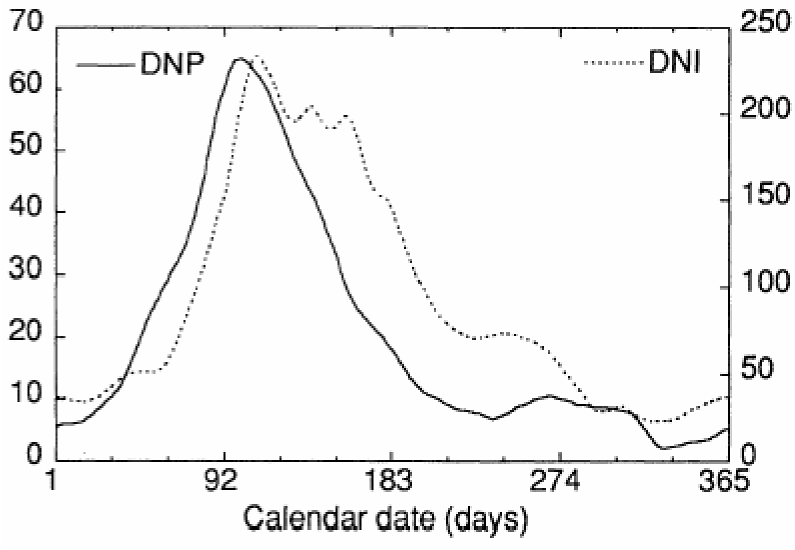


Fig.27. Temporal distribution of the daily number of flowering plant species (DNP) and insect species (DNI) in a Greek phryganic ecosystem. From: Petanidou et al. 1995b.

In my year of study, *Ballota acetabulosa* was in anthesis from early June to early July, immediately after the main flowering period of the plant community, when flowering plants are scarce (then competition among plants is low, highlightened by the high values of Mf guild fidelity for *Ballota* pollen) and insects comparatively abundant. Moreover, like all late-flowering Labiatae, the flowers of *Ballota* offer nectar as the main reward (Petanidou & Vokou 1993) and, in conjunction with this, they provide the main source of water for bees. Again, even if Megachilidae (females) represented the most important guild (strongly favoured by their high summer foraging activity levels), the wide spectrum of pollinators indicates that *Ballota acetabulosa* is a generalist species, thus should be less exposed to problems with reduced visitation rates in small populations (Waser et al. 1996).

For these reasons, *Ballota acetabulosa* would represent a key species for bees during summer, and pollinators would visit it despite of its population size, with the result of a similar performance of large and small populations.

By contrast, the differences in both visitation rate (with honey bees omitted) and Shannon index (both for genera and functional groups) among populations suggest that random or unknown environmental events might have interacted with bee communities in complex ways and at a lower scale. When it has been possible to perform the multiple regression analyses at patch level, the variables used (patch size, density and plant size) had a general low explainatory power, even if in one case (when honey bees were omitted) higher insect visitation rate was positively associated with plant size (consequently, negatively with plant density), as previously found in other studies (Schemske 1980, Klinkhamer et al. 1989). However, it results tempting to unravel these interactions, unless investigations on bee habitat preferences are done.

Some additional data coming from honey bee abundance suggest a cautionary note. Generally, honey bees were relevant to pollen transfer in *Ballota acetabulosa* especially for their higher abundance, as they accounted for 37% of total visits. Despite this high but dishomogeneous abundance (likely to be attributed to the occurrence and nearness of apiaries), whether including or not honey bees in the analyses led to similar patterns of responses of the considered variables. Nevertheless, in this study, an increase in honey bees meant a decrease in the females of megachilids, that represented the most important functional group in the *Ballota* insect spectrum. Honey bees are assumed to be superior competitors than wild solitary bees especially beacuse of their high exploitation of floral rewards for the maintenance of large colonies, and because of the complex dance-language used for communicating to other individuals the position of flowering patches representing high-quality food resources (Visscher & Seeley 1982). There is evidence of a mutual avoidance between honey bees and wild bees, suggesting a competition for flower resources (Roubik 1983), but the density of honey bees colonies is not always associated with a decrease in wild bee species richness or abundance (Steffan-Dewenter & Tscharntke 2000). It remains unclear if the significant correlation between honey bee and megachilid abundance found in this study is biologically meaningful; if so however, the presence of beehives near the populations could have distorted all the conclusions concerning *Ballota* population size effects through an interspecific inhibitory effect.

The answer to the question of if *Ballota acetabulosa* populations of different sizes differently responded in terms of reproduction and pollination services is no. Yet, like in *Echium plantagineum*, this study has further shown that most of the populations strongly suffered from pollen limitation. This is unexpected, as like *Echium plantagineum*, *Ballota acetabulosa* is a common species in Lesvos, offered nectar as a bait for pollinators, and was visited by many insect genera (28, representing different insect families of different body sizes and sexes), suggesting it's a generalist plant species. In addition, as already said, the competition with other co-flowering species was expected to be low.

The two hypotheses mentioned for *Echium plantagineum* seem to cover the main possibilities for justifying the occurrence of pollen limitation in *Ballota acetabulosa*, but still, this study provides no empirical evidence for these. It appears problematic to define the distribution of *Ballota* populations as fragmented, without a complete mapping of the island; nonetheless, even in the presence of a high population connectivity, the habitat could be fragmented from the pollinators' perspective, and a general pollinator decline could allow the existence of pollen limitation. In addition, for the self-incompatible *Ballota acetabulosa*, another problem could be represented by a high degree of improper pollen transfer, as each plant had many flowers open at a time.

Again, I suggest that detailed studies on pollen limitation over different seasons and on ecological attributes of bee community would be worthwhile to clarify these interactions.

4c. Discussion*: Ononis masquillierii*

Ononis masquillierii resulted fully self-incompatible, and although capable of vegetative propagation, it appeared dependent on pollen vectors for its reproductive success.

Reproduction appeared to be pollen limited in all the eight populations. The fact that hand-outcrossings significantly increased seed set in all populations, without significant differences in %, suggests that pollen limitation is a natural condition of the species. Moreover, even if the magnitude of pollen limitation didn't vary among populations, large populations were associated by a better seed output.

Many other studies reported how plant reproductive success could be sensitive to plant population size and/or density (Kunin 1993, 1997, Ågren 1996, Bosch & Waser 1999, Hendrix & Kyhl 2000, Kéry et al. 2000, Oostermeijer et al. 2000, Mavraganis & Eckert 2001, Tomimatsu & Ohara 2002, Brys et al. 2004, Waites & Ågren 2004, Kirchner et al. 2005, Kolb 2005, Ward & Johnson 2005, Hensen & Wesche 2006). The two most tangible interpretations of the influence of population attributes on the degree of pollen limitation involve the quantity and the quality of pollinator service to the plants. About the quantity facet, large and dense populations are supposed to be more attractive to pollinators, showing increased number of visits per flower (Jennersten 1988, Kunin 1997) and higher reproductive success. In *O. masquillierii*, the strong correlation found between insect abundance and the logarithm of population size ($r = 0.81$, $P=0.01$ Fig. 22) corroborates this hypothesis. The finding of the negative correlation between pollen limitation index and population size clearly shows the importance of pollen services in determining the seed set; in addition, the positive correlation between plant fecundity (expressed as the seed set in open natural condition) and population size disappears when considering the seed set of experimentally hand-crossed flowers, as we would expect if seed output was limited by pollinator activity levels.

Moreover, the quality of pollinator services can play an additional and important role. Van Treuren and colleagues (1993) documented a positive relationship between outcrossing rate and plant density in four populations of the bumblebee-pollinated *Salvia pratensis*, that could underlie a different pollinator foraging behavior in response to different plant population densities.

One of the hypotheses was that higher levels of outcrossing found in denser populations were caused by a higher frequency of interplant flights; this could lead insects to visit more flower per plant in small or sparse populations, thus promoting geitonogamy (De Jong et al. 1993, Karron et al. 1995). In this study I couldn't demonstrate this, as it resulted impossible to distinguish separate plants because of the vegetative propagation of this species. Anyhow, population density was found to have little effects in terms of productivity, and this can be the consequence of i) the occurrence of clonal growth, that may have shaded the effects of density through the arrangement of genets into more or less clumped distributions (Charpentier 2002), ii) the type of experimental design, as this study made no attempt to modify population densities. Therfore, density experimental manipulations would be necessary to investigate the potential role of plant density in contributing to the Allee effect.

In contrast to my expectations, even if pollen limitation tended to be negatively related to insect abundance, it only approached statistical significance. This can be mainly due to the exceptional low number of insect collected per census, that prevented the 30'-long catching sessions to be sufficient to obtain a more powerful correlation. Therefore, exceedingly large sampling efforts would be advisable in pollination systems characterized by low and unconstant pollination services (Larson & Barrett 1999, Baker et al. 2000).

Finally, another interesting feature of *O. masquillierii* reproductive biology is represented by its widespread reduced female fecundity. *Ononis masquillierii* had an ovary containing on average six ovules, but generally one (rarely two) seed was produced, regardless of the pollination treatment, thus even in presence of great amounts of compatible pollen. In hand-crossed flowers in fact, only 11% of the initial ovules developed into seeds, while the percentage dropped to 1.1% in open pollinated flowers. One of the factor responsible to this loss can be represented by a limitation in the available resources needed to develop all ovules, as the particular habitat where the species occurrs is particularly subject to soil dissolution, water runoff and drought.

Even if population size represented an important extrinsic factor regulating the magnitude of pollen limitation (which affected small populations to a bigger extent than large ones), it remains that the pollination environment where the species occurred was characterized by a widespread inadequate pollination. Thus, the problem of detecting the principal causes of the occurrence of pollen limitation can be solved by examining the intrinsic reproductive traits of the species.

My findings demonstrate that *Ononis masquillierii* displays several characteristics of its reproductive biology that can account for the presence of pollen limitation.

a. Plant specialization & competition

Flower specialization may play an important role in determining the extent of pollen limitation, and pollinators may not equally contribute to the pollen transfer dynamics.

The spectrum of insects visiting *O. masquillierii* was taxonomically and morphologically diverse. A similar number of genera has been documented for other legumes growing in the same area (Galloni et al., in press), and, at functional group level, insects ranged in size from very small bees (*Lasioglossum* spp.) to large ones (*Bombus* spp.). All insects recorded touched the reproductive structures. Not surprisingly, great differences in insect assemblage (both at genus and functional group level) were found among populations. Previous studies highlighted spatially variable plant-pollinator associations (Herrera 1988, Fenster & Dudash 2000), mainly due to variations in plant community backgroud (Moeller 2005), availability of nesting sites (Cane 1991, Steffan-Dewenter & Tscharntke 1999, Michener 2000, Potts et al. 2005) and landcape fragmentation (Aizen & Feinsinger 1994b). In *O. masquillierii*, neither population size nor density resulted effective in explaining the shift in insect fauna composition across populations, but populations were located in slightly different landscape contexts, with populations in turn delimited by roads, surrounded by agricultural matrices or close to forest fragments. Variations in insect fauna at a small spatial scale have been already found (Herrera 1988, Moeller 2005), and heterogeneity of environmental factors between small spatial sectors can play a major role in shaping the structure of insect assemblages. For this reason, deeper investigations on the biotic and abiotic characteristics of the environment where the species occurs would be advisable.

Despite this variation in insect guild abundance (and importance) across populations, it resulted that small bees were the most important pollinators for *O. masquillierii*, and that there was a clear pattern of variation of importance with insect size. Previous studies have documented the effects of forager body size on pollinator behavior and plant reproductive success (Tepedino et al. 1999, Stout 2000), and mechanical constraints on pollen harvesting seemed to represent in *O. masquillierii* the major cause of the negative relationship between insect importance and body size. Shall I have to consider the plant as specialized? *O. masquillierii* flowers show the typical “bee-pollinated syndrome”(Fægri & van der Pijl 1979), with small zigomorphic flowers, but at the same time they were promiscuously visited by different insect taxa. Recently, the concept that a plant could be successfully pollinated only by a narrow subset of pollen vectors has been criticized after field studies showed widespread plant-pollinator interactions of high generality (Waser et al. 1996). Anyhow, Fenster and colleagues (2004) proved that the majority of the generalist interactions obtained by organizing pollinators into species became instead specialized when pollinators were organized into functional groups. In this case, insect body size and taxonomic position seemed to represent the more adapt ecological features to discriminate the set of *Ononis* pollinators; therefore, small *O. masquillierii* flowers resulted specialized on small bees (represented only by *Lasioglossum* spp.). A correspondence between the pattern of pollen density Vs insect body size and pollinator importance Vs insect body size was found. This means that the insect relative frequency had less influence in determining the importance of the various functional groups in comparison with the amount of pollen carried on insect bodies, although data could suffer from the low insect abundance widespread across the populations. Thus, small bees resulted the most important pollinators especially because of their great pollen load, while their relative frequency was not particularly high. Because of their small size, they managed to enter the flowers with the entire body, and, by working flowers at a lower speed than the other guilds, they gathered more pollen and they contributed to a great extent to the pollen transfer. Their high fidelity to *Ononis* pollen strengthens their prominent role in the assurance of the pollen service.

Zygomorphy is expected to be associated with specialized pollinators, and with a higher quality of the pollination mechanism (Kunin & Shmida 1997, Neal et al. 1998). This could in theory imply a smaller probability of facing pollen limitation for specialized flowers (but see Larson & Barrett 2000, Knight et al. 2005a). Nevertheless, two factors can reverse this consideration.

First, the ecological advantages of having a complex flower architecture can be counterbalanced by a lack of flower attractiveness. Without the proper rewards or advertising, flowers are likely to be avoided by pollinators, bringing benefits to other more attractive co-flowering species.

The overall number of *Ononis* visitors caught in 16 hours of random collection across the eight populations represents a very low value if compared with insect abundance on other legumes growing in the same habitat (*Hedysarum coronarium*, pers.obs).

Higher insect visitation rate has been reported for large-flowered Vs small-flowered species (Valido et al. 2002, Momose 2004), and for nectariferous Vs nectarless species (Motten 1986, Thomson 1986, Klinkhamer & De Jong 1990), and higher seed output has been reported for nectariferous Vs nectarless orchids (Johnson & Bond 1997, Neiland & Wilcock 1998) and in general for 224 species of animal-pollinated flowering plants (Larson & Barrett 2000). *O. masquillierii* coexisted with other bee pollinated species well adapted to the selective substrate of the gullies, primarily other Leguminosae (*Hedysarum coronarium, Dorycnium hirsutum, Trifolium* spp.), or it was surrounded by arid-meadowed Labiatae (*Ajuga genevensis* and *Stachys officinalis* for example). These species attracted in general more insects than *O. masquillierii* (pers.obs), and even if *O. masquillierii* and its main co-flowering species shared a similar flower structure (in respect of size and shape), profound differences in nectar production were documented, *O. masquillierii* being completely nectarless, contrary to the majority of its neighbouring species. This could in theory translate in an interspecific facilitative interaction via shared pollinators (Oostermeijer et al.1998, Moeller 2004), as facilitation has been often documented when nectarless species are surrounded by rewarding species ("the magnet species effect", Thomson 1978, Johnson et al. 2003). Nevertheless my observations contrast with this expectation, as the two more frequent insect taxa visiting the main co-flowering species (*Apis* sp. and *Bombus* ssp., pers.obs.) were either absent (*Apis* sp.*)* or accounted for only 12% of total insects (*Bombus* spp.) in the *Ononis* spectrum. In addition, during the observation sessions, some insects belonging to other taxa (*Chalicodoma* spp. and *Xilocopa* sp. for example) were noted to approach the flowers of *Ononis masquillierii* at first, even if they avoided any contact when they were closer to them, as optimal foraging models assume (Goulson 2000). It could be possible that some insect taxa were shared in the plant community, as it often happens when flowering times of different species overlap (Gross et al. 2000), but a dicothomy in terms of pollination agents between *Ononis masquillierii* and its co-flowering species seemed to exist, and the competition hypothesis appears more reasonable.

The high level of fidelity of the different insect guilds visiting *O. masquillierii* seems to strengthen this hypothesis. Among these, the insects that were supposed to be the less adapt to *Ononis* flowers because of their large size (*Anthophora* sp. and *Bombus* spp.) resulted the second ranked pollinators, suggesting a selective preference for *Ononis* flowers as a pollen source.

Again, as the only reward offered by *Ononis* flowers was pollen, no insect males were recorded across all the eight populations; to what extent this can have contributed to pollen limitation remains unknown, and sex-ratio investigations relative to the main insect taxa would be necessary.

Second, plant species specialized on a narrow subset of pollinators may experience reproductive failure in unpredictable pollination systems. Pollinator abundance and composition may fluctuate tremendously across years and sites (Herrera 1988, Petanidou & Ellis 1993, Tepedino et al. 1999, Petanidou & Potts 2006), hence it can happen that the proper pollinator could not be always present. Therefore, the low number of suitable pollen vectors makes specialized plants less flexible to environmental stochasticity than generalist species (Bond 1994, Waser et al. 1996).

Even if this study spanned only one year, the heterogeneity in insect composition and frequency among the eight populations of *Ononis masquillierii* suggests that this risk can be real.

b. Breeding system & asexual propagation

Data relative to the breeding system showed that the species was fully self-incompatible, leading to the conclusion that *Ononis masquillierii* was incapable of producing seeds in the absence of pollinators. Previous studies supported the hypothesis of a lower degree of pollen limitation in self-compatible species compared with self-incompatible ones (Larson & Barrett 2000, Knight et al. 2005a). The most quoted hypothesis to justify this pattern was provided by Burd (1994), who invoked the self pollen as an additional source of pollen received by stigmas in self-compatible species.

My data indicate that all the eight populations of *Ononis masquillierii* were deeply subject to pollen limitation; if the same pattern occurred across years, the plant would be chronically pollen-limited. Plant species occurring in chronically pollen-limited systems may be subject to evolutionary pressures, and the evolution of self-fertilization can result as an adaptation for the assurance of reproduction (Motten 1986, Bond 1994). This is true despite pollen discounting and inbreeding depression may contrast the evolution towards autogamy, as in pollen-limited environments smaller amounts of self-pollen are needed to promote selfing (Porcher & Lande 2005). Therefore, if pollen limitation in *Ononis masquillierii* were a natural condition of the species, I would expect a long-term modification of its breeding system, with the breakdown of self-incompatibility (Vallejo-Marín & Uyenoyama 2004).

Furthermore, *Ononis masquillierii* is capable of vegetative propagation through rhizomes. Different ecological advantages are associated with clonal reproduction, and trade-offs in allocation to asexual Vs sexual reproduction can help the plant to face changing and heterogeneous environments (Ronsheim & Bever 2000). The most intuitive advantage of asexual reproductive mode is the (partial) independence of the plant from pollination mechanisms, particularly important in case of self-incompatible entomophilous species. In addition, clonal growth may contribute to the floral display through the increase of the flowering shoots, thus enhancing pollinator attractiveness. Despite these advantages, vegetative propagation implies negative aspects as well. For example, clonal growth can increase the rate of geitonogamy, hence reducing the plant fitness because of a lack of compatible pollen (Charpentier 2002). Hence, even if it’s true that vegetative propagation allows the species to persist even in scarce pollinator environments, it can also lower the quality of the few pollinator services. In my study, it’s possible that the higher values of pollen limitation found in small populations were the consequences of higher levels of geitonogamy, as small populations were likely represented by few genets (Eckert & Barrett 1993).

To summarize, *Ononis masquillierii*’s reproductive biology presented several characteristics that made it a poor competitor. The small, nectarless, short-living flowers attracted very few insects than the co-flowering species, and among them, small bees seemed to matter most, thus representing the main pollen vectors. Although pollen limitation may not always mean an immediate conservation threat because of the stochastic nature of plant-pollinator systems (Burd 1994, 1995), the reduced reproductive success associated with the size of *Ononis masquillierii* populations may lead to dangerous demographic consequences for small populations. Rare, self-incompatible unattractive and specialized plant species are more vulnerable to this risk (Larson & Barrett 2000), and, when possible, have to rely more on asexual reproduction for their persistence; this in turn can provoke a reduction in plant fitness, and in the ability to buffer the effects of environmental stochasticity.

5. Literature cited

Ågren J. 1996. Population size, pollinator limitation, and seed set in the self-incompatible herb *Lythrum salicaria*. *Ecology* **77**:1779-1790

Aizen M.A., Feinsinger P. 1994a. Forest fragmentation, pollination, and plant reproduction in a Chaco dry forest, Argentina. *Ecology* **75**: 330-351

Aizen M.A., Feinsinger P. 1994b. Habitat fragmentation, native insect pollinators, and feral honey bees in Argentine “chaco serrano”. *Ecological Applications* **4**:378-392

Andrén H. 1994. Effects of habitat fragmentation on birds and mammals in landscapes with different proportions of suitable habitat: a review. *Oikos* **71**:355-366

Arroyo M.T.K. 1981. Breeding systems and pollination biology in Leguminosae. In: **Polhill R.M., Raven P.H.**, eds. *Advances in Legume Systematics*. II, pp. 723-769. The Royal Botanical Gardens, Kew.

Baker A.M., Barrett S.C.H., Thompson J.D. 2000. Variation of pollen limitation in the early flowering Mediterranean geophyte *Narcissus assoanus* (Amaryllidaceae). *Oecologia* **124**:529-535

Batra S.W.T. 1982. Biological control in agroecosystems. *Science* **215**:134-139

Beattie A.J. 1971. A technique for the study of insect-borne pollen. *Pan-Pacific Entomoogist* **47**:82

Bell G. 1985. On the Function of Flowers. *Proceedings of the Royal Society of London. Series B, Biological Sciences* **224**.223-265

Bond W.J. 1994. Do mutualisms matter? Assessing the impact of pollinator and disperser disruption on plant extinction. *Philosophical Transactions of the Royal Society of London: Biological Sciences* **344**:83-90

Bosch M., Waser N.M. 1999. Effects of local density on pollination and reproduction in *Delphinium nuttallianum* and *Aconitum columbianum* (Ranuncolaceae). *American Journal of Botany* **86**:871-879

Bosch M., Waser N.M. 2001. Experimental manipulation of plant density and its effect on pollination and reproduction of two confamilial montane herbs. *Oecologia* **126**:76-83

Brown B.J., Mitchell R.J. 2001. Competition for pollination: effects of pollen of an invasive plant on seed set of a native congener. *Oecologia* **129**:43-49

Brys R., Jacquemyn H., Endels P., Van Rossum F., Hermy M., Triest L., De Bruyn L., Blust G.D.E. 2004. Reduced reproductive success in small populations of the self-incompatible *Primula vulgaris*. *Journal of Ecology* **92**:5-14

Buchmann S.L., Nabhan G.P. 1996. The Forgotten pollinators. Washington DC: Island press

Burd M. 1994. Bateman's principle and plant reproduction: the role of pollen limitation in fruit and seed set. *Botanical Review* **60**:83-139

Burd M. 1995. Ovule packaging in stochastic pollination and fertilization environments. *Evolution* **49**:100-109

Burley N., Willson M.F. 1983. Mate Choice in Plants: Tactics, Mechanisms and Consequences. Princeton, NJ, USA: Princeton University Press.

Byers D.L. 1995. Pollen quantity and quality as explanations for low seed set in small populations exemplified by *Eupatorium* (Asteraceae). *American Journal of Botany* **82**:1000-1006

Campbell D.R. 1985. Pollinator Sharing and Seed Set of *Stellaria pubera*: Competition for Pollination. *Ecology* **66**:544-553

Campbell D.R., Motten A.F. 1985. The mechanism of competition for pollination between two forest herbs. *Ecology* **66**:554-563

Campbell D.R., Halama K.J. 1993. Resource and pollen limitations to lifetime seed production in a natural plant population. *Ecology* **74**:1043-1051

Cane J. H. 1991. Soils of ground-nesting bees (Hymenoptera: Apoidea): texture, moisture, cell depth and climate. *Journal of Kansas Entomological Society* **64**: 406-413.

Cane J. H. 2001. Habitat fragmentation and native bees: a premature verdict? *Conservation Ecology* **5(1)**: 3. URL: http://www.consecol.org/vol5/iss1/art3/

Canto-Aguilar M.A., Parra-Tabla V. 2000. Importance of conserving alternative pollinators: assessing the pollination efficiency of the squash bee, *Peponapis limitaris* in *Cucurbita moschata* (Cucurbitaceae). *Journal of Insect Conservation* **4**:203-210

Caruso C.M. 1999. Pollination of *Ipomopsis aggregata* (Polemoniaceae): effects of intra- vs. interspecific competition. *American Journal of Botany* **86**:663-668

Caruso C.M. 2001. Differential selection on floral traits of *Ipomopsis aggregata* growing in contrasting environments. *Oikos* **94**:295-302

Caruso C.M. 2002. Influence of plant abundance on pollination and selection on floral traits of *Ipomopsis aggregata*. *Ecology* **83**:241-254

Charpentier A. 2002. Consequences of clonal growth for plant mating. *Evolutionary Ecology* **15**:521-530

Corbet S.A., Delfosse E.S. 1984. Honeybees and the nectar of *Echium plantagineum* L. in southeastern Australia. *Australian Journal of Ecology* **9**:125-139

Corbet S.A., Williams I.H., Osborne J.L. 1991. Bees and the pollination of crops and wild flowers in the European Community. *Bee World* **72**: 47-59

Cruden R.W. 2000. Pollen grains: why so many? *Plant Systematics and Evolution* **222**:143-165

Culvenor C.C.J. 1956. The alkaloids of *Echium plantagineum* L. Echiumine and Echimidine. *Australian Journal of Chemistry* **9**:512-520

Cunningham S.A. 2000. Depressed pollination in habitat fragments causes low fruit set. *Proceedings of the Royal Society of London. Series B* **267**:1149-1152

Dafni A., Pacini E., Nepi M. 2005. Pollen and stigma biology. In **Dafni A., Kevan P.G.**, Husband B.C., eds. *Practical Pollination Biology*. pp. 83-145. Environquest, Ltd, Canada.

Davis H.G., Taylor C.M., Lambrinos J.G., Strong D.R. 2004. Pollen limitation causes an Allee effect in a wind-pollinated invasive grass (*Spartina alterniflora*). *Proceedings of the National Academy of Sciences USA* **101**:13804-13807

De Jong T.J., Waser N.M., Klinkhamer P.G.L. 1993. Geitonogamy: the neglected side of selfing. *Trends in Ecology and Evolution* **8**:321-325

Dieringer G. 1992. Pollinator effectiveness and seed set in populations of *Agalinis strictifolia* (Scrophulariaceae). *American Journal of Botany* **79**:1018-1023

Dudash M.R., Fenster C.B. 2000. Inbreeding and outbreeding depression in fragmented populations. In: **Young A.G., Clarke G.M.** eds. *Genetics, Demography and Viability of Fragmented Populations*. pp. 35-53. Cambridge University Press, Cambridge.

Dukas R. 2001. Effects of perceived danger on flower choice by bees. *Ecology Letters* **4**:327-333

Dukas R., Morse D.H. 2003. Crab spiders affect flower visitation by bees. *Oikos* **101**:157-163

Eckert C.G., Barrett S.C.H. 1993. Clonal reproduction and patterns of genotypic diversity in *Decodon verticillatus* (Lythraceae). *American Journal of Botany* **80**:1175-1182

Eckhart V.M. 1991. The effects of floral display on pollinator visitation vary among populations of *Phacelia linearis* (Hydrophyllaceae). *Evolutionary Ecology* **5**:370-384

Ellstrand N.C., Elam D.R. 1993. Population genetic consequences of small population size: Implications for Plant Conservation. *Annual Review of Ecology and Systematics* **24**:217-242

Fægri K, van der Pijl L, 1979. The Principles of Pollination Ecology. New York: Pergamon Press.

Fausto J.A., Eckhart V.M., Geber M.A. 2001. Reproductive assurance and the evolutionary ecology of self-pollination in *Clarkia xantiana* (Onagraceae). *American Journal of Botany* **88**:1794-1800

Feinsinger P., Tiebout H.M., Young B.E. 1991. Do tropical bird-pollinated plants exhibit density-dependent interactions? Field experiments. *Ecology* **72**:1953-1963

Fenster C.B., Dudash M.R. 2000. Spatiotemporal variation in the role of hummingbirds as pollinators of *Silene virginica*. *Ecology* **82**:844-851

Fenster C.B., Armbruster W.S., Wilson P., Dudash M.R., Thomson J.D. 2004. Pollination Syndromes and Floral Specialization. *Annual Review of Ecology, Evolution and Systematics* **35**:375-403

Fischer M., Matthies D. 1998. RAPD variation in relation to population size and plant fitness in the rare *Gentianella germanica* (Gentianaceae). *American Journal of Botany* **85**:811-819

Fishbein M., Venable D.L. 1996. Diversity and temporal change in the effective pollinators of *Asclepias tuberosa. Ecology* **77**: 1061-1073

Forsyth S.A. 2003. Density-dependent seed set in the Haleakala silversword: evidence for an Allee effect. *Oecologia* **136**:551-557

Franceschinelli E.V., Bawa K.S. 2000. The effect of ecological factors on the mating system of a South American shrub species (*Helicteres brevispira*) *Heredity* **84**:116-123

Galen C., Plowright R.C., Thomson J.D. 1985. Floral Biology and Regulation of Seed Set and Seed Size in the Lily, *Clintonia borealis*. *American Journal of Botany* **72**:1544-1552

Galen C., Gregory T. 1989. Interspecific pollen transfer as a mechanism of competition: consequences of foreign pollen contamination for seed set in the alpine wildflower, *Polemonium viscosum*. *Oecologia* **81**:120-123

Galloni M., Podda L., Vivarelli D., Quaranta M., Cristofolini G. Visitor diversity and pollinator specialization in Mediterranean Legumes. *Flora*, in press.

Goulson D. 2000. Why do pollinators visit proportionally fewer flowers in large patches? *Oikos* **91**:485-492

Grant, S.A., Armstrong, H.M. 1993. Grazing ecology and the conservation of heather moorland: the development of models as aids to management. *Biodiversity and Conservation* **2**: 79-94

Grigulis K., Sheppard A.W., Ash J.E., Groves R.H. 2001. The comparative demography of the pasture weed *Echium plantagineum* between its native and invaded ranges. *Journal of Applied Ecology* **38**:281-290

Groom M.J. 2001. Consequences of subpopulation isolation for pollination, herbivory, and population growth in *Clarkia concinna concinna* (Onagraceae). *Biological Conservation* **100**:55-63

Gross R.S., Werner P.A. 1983. Relationships among flowering phenology, insect visitors, and seed-set of individuals: experimental studies on four co-occurring species of goldenrod (*Solidago*: Compositae). *Ecological Monographs* **53**:95-117

Gross C.L. 1996. Is resource overlap disadvantageous to three sympatric legumes? *Australian Journal of Ecology* **21**:133-143

Gross C.L., Mackay D.A., Whalen M.A. 2000. Aggregated flowering phenologies among three sympatric legumes. *Plant Ecology* **148**:13-21

Haig D., Westoby M. 1988. On limits to seed production. *The American Naturalist* **131**:757-759

Hendrix S.D., Kyhl A.J.F. 2000. Population size and reproduction in *Phlox pilosa. Conservation Biology* **14**:304-313

Hensen I., Oberprieler C. 2005. Effects of population size on genetic diversity and seed production in the rare *Dictamnus albus* (Rutaceae) in central Germany. *Conservation Genetics* **6**:63-73

Hensen I., Wesche K. 2006. Relationships between population size, genetic diversity and fitness components in the rare plant *Dictamnus albus* in Central Germany. *Biodiversity and Conservation* **15**:2249-2261

Herrera C.M. 1987. Components of pollinator “quality”: comparative analysis of a diverse insect assemblage. *Oikos* **50**:79-90

Herrera C.M. 1988. Variation in mutualisms: the spatio-temporal mosaic of a pollinator assemblage. *Biological Journal of the Linnean Society* **35**:95-125

Holsinger K.E. 2000. Demography and extinction in small populations. In: **Young A.G. and Clarke G.M.** eds. *Genetics, demography and viability of fragmented populations*. pp. 55-74. Cambridge Univ. Press.

Irwin R.E., Brody A.K., Waser N.M. 2001. The impact of floral larceny on individuals, populations, and communities. *Oecologia* **129**:161-168

Iwaizumi M.G., Sakai S. 2004. Variation in flower biomass among nearby populations of *Impatiens textori* (Balsaminaceae): effects of population plant densities. *Canadian Journal of Botany* **82**:563-572

Jennersten O. 1988. Pollination in *Dianthus deltoides* (Caryophyllaceae): Effects of Habitat Fragmentation on Visitation and Seed Set. *Conservation Biology* **2**:359-366

Johnson S.D., Bond W.J. 1997. Evidence for widespread pollen limitation of fruiting success in Cape wildflowers. *Oecologia* **109**:530-534

Johnson S.D., Peter C.I., Nilsson L.A., Ågren J. 2003. Pollination success in a deceptive orchid is enhanced by co-occurring rewarding magnet plants. *Ecology* **84**:2919-2927

Johnson S.D., Collin C.L., Wissman H.J., Halvarsson E., Ågren J. 2004. Factors contributing to variation in seed production among remnant populations of the endangered daisy *Gerbera aurantiaca*. *Biotropica* **36**:148-155

Kandori I. 2002. Diverse visitors with various pollinator importance and temporal change in the important pollinators of *Geranium thunbergii* (Geraniaceae). *Ecological Research* **17**:283-294

Karron J.D., Thumser N.N., Tucker R., Hessenauer A.J. 1995. The influence of population density on outcrossing rates in *Mimulus ringens*. *Heredity* **75**:175-180

Kearns C.A., Inouye D.W., Waser N.M. 1998. Endangered mutualisms: the conservation of plant-pollinator interactions. *Annual Review of Ecology and Systematics* **29**:83-112

Kéry M., Matthies D., Spillmann H.-H. 2000. Reduced fecundity and offspring performance in small populations of the declining grassland plants *Primula veris* and *Gentiana lutea*. *Journal of Ecology* **88**:17-30

Kirchner F., Luijten S.H., Imbert E., Riba M., Mayol M., González-Martínez S.C., Mignot A., Colas B. 2005. Effects of local density on insect visitation and fertilization success in the narrow-endemic *Centaurea corymbosa* (Asteraceae). *Oikos* **111**:130-142

Klinkhamer P.G.L., De Jong T.J., De Bruyn G.-J. 1989. Plant size and pollinator visitation in *Cynoglossum officinale*. *Oikos* **54**:201-204

Klinkhamer P.G.L., De Jong T.J. 1990. Effects of plant size, plant density and sex differential nectar reward on pollinator visitation in the protandrous *Echium vulgare* (Boraginaceae). *Oikos* **57**:399-405

Knight T.M. 2003. Floral density, pollen limitation, and reproductive success in *Trillium grandiflorum*. *Oecologia* **137**:557-563

Knight T.M., Steets J.A., Vamosi J.C., Mazer S.J., Burd M., Campbell D.R., Dudash M.R., Johnston M.O., Mitchell R.J., Ashman T.-L. 2005a. Pollen limitation of plant reproduction: pattern and process. *Annual review of ecology, evolution, and systematics* **36**:467-497

Knight T.M., McCoy M.W., Chase J.M., McCoy K.A., Holt R.D. 2005b. Trophic cascades across ecosystems. *Nature* **437**:880-883

Kolb A. 2005. Reduced reproductive success and offspring survival in fragmented populations of the forest herb *Phyteuma spicatum*. *Journal of Ecology*. **93**:1226-1237

Kunin W.E. 1993. Sex and the single mustard: population density and pollinator behavior effects on seed-set. *Ecology* **74**:2145-2160

Kunin W.E. 1997. Population size and density effects in pollination: pollinator foraging and plant reproductive success in experimental arrays of *Brassica kaber*. *Journal of Ecology* **85**:225-234

Kunin W.E., Shmida A. 1997. Plant reproductive traits as a function of local, Regional, and Global Abundance. *Conservation Biology* **11**:183-192

Lamborn E., Ollerton J. 2000. Experimental assessment of the functional morphology of inflorescences of *Daucus carota* (Apiaceae): testing the “fly catcher effect”. *Functional Ecology* **14**: 445-454

Larson B.M.H., Barrett S.C.H. 1999. The ecology of pollen limitation in buzz-pollinated *Rhexia virginica* (Melastomataceae). *Journal of Ecology* **87**:371-381

Larson B.M.H., Barrett S.C.H. 2000. A comparative analysis of pollen limitation in flowering plants. *Biological Journal of the Linnean Society* **69**:503-520

Lee T.D. 1988. Patterns of fruit and seed production. In **Lovett-Doust J. & Lovett-Doust L.** eds. *Plant reproductive ecology: patterns and strategies*. pp. 179-202. Oxford University press, New York, USA.

Leimu R., Syrjänen K. 2002. Effects of population size, seed predation and plant size on male and female reproductive success in *Vincetoxicum hirundinaria* (Asclepiadaceae). *Oikos* **98**:229-238

Levin D.A. 2000. The origin, expansion, and demise of plant species. Oxford University Press.

Lienert J., Fischer M., Schneller J., Diemer M. 2002. Isozyme variability of the wetland specialist *Swertia perennis* (Gentianaceae) in relation to habitat size, isolation, and plant fitness. *American Journal of Botany* **89**:801-811

Lindsey A.H. 1984. Reproductive biology of Apiaceae. I. Floral visitors to *Thapsium* and *Zizia* and their importance in pollination. *American Journal of Botany* **71**:375-387

Linhart Y.B., Feinsinger P. 1980. Plant-hummingbird interactions: effects of island size and degree of specialization on pollination. *Journal of Ecology* **68**:745-760

Liu H., Koptur S. 2003. Breeding system and pollination of a narrowly endemic herb of the Lower Florida Keys: impacts of the urban-wildland interface. *American Journal of Botany* **90**:1180-1187

López J., Rodríguez-Riaño T., Ortega-Olivencia A., Devesa J.A., Ruiz T. 1999. Pollination mechanisms and pollen-ovule ratios in some Genisteae (Fabaceae) from Southwestern Europe. *Plant Systematics and Evolution* **216**:23-47

Mavraganis K., Eckert C.G. 2001. Effects of population size and isolation on reproductive output in *Aquilegia canadensis* (Ranunculaceae). *Oikos* **95**:300-310

Menges E.S. 1992. Stochastic modeling of extinction in plant populations. In: **Fiedler P.L., Jain S.K**. eds., *Conservation biology: the theory and practice of nature conservation, preservation and management*. Chapman and Hall, New York, USA pp 253-275

Michener C.D. 2000. The Bees of the world. Baltimore: The Johns Hopkins University Press.

Moeller D.A. 2004. Facilitative interactions among plants via shared pollinators. *Ecology* **85**:3289-3301

Moeller D.A. 2005. Pollinator community structure and sources of spatial variation in plant-pollinator interactions in *Clarkia xantiana* ssp.*xantiana*. *Oecologia* **142**:28-37

Molano-Flores B., Hendrix S.D. 1999. The effects of population size and density on the reproductive output of *Anemone canadensis* L. (Ranunculaceae). *International Journal of Plant Sciences* **160**:759-766

Momose K. 2004. Plant reproductive interval and population density in aseasonal tropics. *Ecological research* **19**:245-253

Moody-Weis J.M., Heywood J.S. 2001. Pollination limitation to reproductive success in the Missouri evening primrose, *Oenothera macrocarpa* (Onagraceae). *American Journal of Botany* **88**:1615-1622

Morgan J.W. 1999. Effects of population size on seed production and germinability in an endangered, fragmented grassland plant. *Conservation Biology* **13**:266-273

Morgan M.T., Wilson W.G., Knight T.M. 2005. Plant Population Dynamics, Pollinator Foraging and the selection of Self-Fertilization. *The American Naturalist* **166**:169-183

Mothershead K., Marquis R.J. 2000. Fitness impacts of herbivory through indirect effects on plant-pollinator interactions in *Oenothera macrocarpa*. *Ecology* **81**:30-40

Motten A.F. 1986. Pollination ecology of the spring wildflower community of a temperate deciduous forest. *Ecological Monographs* **56**:21-42

Muñoz A.A., Arroyo M.T.K. 2004. Negative impacts of a vertebrate predator on insect pollinator visitation and seed output in *Chuquiraga oppositifolia*, a high Andean shrub. *Oecologia* **138**:66-73

Murphy S.D. 2000. Field testing for pollen allelopathy: a review. *Journal of Chemical Ecology* **26**:2155-2172

Mustajärvi K., Siikamäki P., Rytkönen S., Lammi A. 2001. Consequences of plant population size and density for plant-pollinator interactions and plant performance. *Journal of Ecology* **89**:80-87

Neal P.R., Dafni A., Giurfa M. 1998. Floral symmetry and its role in plant-pollinator systems: Terminology, Distribution, and Hypotheses. *Annual Review of Ecology and Systematics* **29**.345-373

Neiland M.R.M., Wilcock C.C. 1998. Fruit set, nectar reward and rarity in the Orchidaceae. *American Journal of Botany* **85**:1657-1671

Olsen K.M. 1997. Pollination effectiveness and pollinator importance in a population of *Heterotheca subaxillaris* (Asteraceae). *Oecologia* **109**:114-121

Oostermeijer J.G.B., Luijten S.H., Křenová Z.V., Den Nijs H.C.M. 1998. Relationships between Population and Habitat Characteristics and Reproduction of the Rare *Gentiana pneumonanthe* L. *Conservation Biology* **12**: 1042-1053

Oostermeijer J.G.B., Luijten S.H., Petanidou T., Kos M., Ellis-Adam A.C., Den Nijs H.C.M. 2000. Pollination in rare plants: is population size important? In: **Totland Ø., Armbruster S., Fenster C., Molau U., Nilsson A., Olesen J.M., Ollerton J., Philipp M., Ågren J.,** eds. *The Scandinavian Association for Pollination Ecology honours Knut Fægri*. pp. 201–213. Oslo: The Norwegian Academy of Science and Letters.

Oostermeijer J.G.B. 2003. Threats to rare plant persistence. In: Brigham C.A., Schwartz M.W. eds. *Population Viability in Plants*. pp. 17-43. Springer, New York.

O'Toole C. 1993. Diversity of native bees and agroecosystems. In: **LaSalle J., Gauld I.D.,** eds. *Hymenoptera and biodiversity*. pp. 169-196. CAB International, London.

Ouborg N.J., Van Treuren R., Van Damme J.M.M. 1991. The significance of genetic erosion in the process of extinction. II. Morphological variation and fitness components in populations of varying size of *Salvia pratensis* L. and *Scabiosa columbaria* L. *Oecologia* **86**:359-367

Paini D.R. 2004. Impact of the introduced honey bee (*Apis mellifera*) (Hymenoptera: Apidae) on native bees: a review. *Austral Ecology* **29**:399-407

Parker M.A. 1987. Pathogen impact on sexual vs. asexual reproductive success in *Arisaema triphyllum*. *American Journal of Botany* **74**:1758-1763

Parker I.M. 1997. Pollinator limitation of *Cytisus scoparius* (Scotch broom), an invasive exotic shrub. *Ecology*, **78**:1457-1470

Paton D.C. 1993. Honeybees *Apis mellifera* in the Australian environment. Does *Apis mellifera* disrupt or benefit native biota? *BioScience* **43**:95-103

Petanidou T., Ellis W.N. 1993. Pollinating fauna of a phryganic ecosystem: composition and diversity. *Biodiversity Letters* **1**:9-22

Petanidou T., Vokou D. 1993. Pollination Ecology of Labiatae in a Phryganic (East Mediterranean) Ecosystem. *American Journal of Botany* **80**:892-899

Petanidou T., den Nijs J.C.M., Oostermeijer J.G.B., Ellis-Adam A.C. 1995a. Pollination ecology and patch-dependent reproductive success of the rare perennial *Gentiana pneumonanthe* in The Netherlands. *The New Phytologist* **129**:155-163

Petanidou T., Ellis W.N., Margaris N.S., Vokou D. 1995b. Constraints on Flowering Phenology in a Phryganic (East Mediterranean Shrub) Community. *American Journal of Botany* **82**:607-620

Petanidou T., Ellis W.N. 1996. Interdependence of native bee faunas and floras in changing Mediterranean communities. In: **Matheson A., Buchmann S.L., O'Toole C., Westrich P., Williams I.H.,** eds. *The conservation of bees*. pp. 201-226. London, Academic Press.

Petanidou T., Ellis-Adam A.C., den Nijs J.C.M., Oostermeijer J.G.B. 1998. Pollination ecology of *Gentianella uliginosa*, a rare annual of the Dutch coastal dunes. *Nordic Journal of Bot*any **18**:537-548

Petanidou T., Goethals V., Smets E. 2000. Nectary structure of Labiatae in relation to their nectar secretion and characteristics in a Mediterranean shrub community – Does flowering time matter? *Plant Systematics and Evolution* **225**:103-118

Petanidou T., Lamborn E. 2005. A land for flowers and bees: studying pollination ecology in Mediterranean communities. *Plant Byosystems* **139**:279-294

Petanidou T., Potts S.G. 2006. Mutual use of resources in Mediterranean plant-pollinator communities: how specialized are pollination webs? In: **Waser N.M., Ollerton J.,** eds. *Plant-pollinator interactions: from specialization to generalization*. pp. 220-244. Chicago: University of Chicago Press.

Piggin C.M., Sheppard A.W. 1995. *Echium plantagineum* L. In: **Groves R.H., Shepherd R.C.H., Richardson R.G.,** eds. *The Biology of Australian Weeds,* Vol 1. pp. 87-110. Richardson, Melbourne, Australia.

Porcher E., Lande R. 2005. The evolution of self-fertilization and inbreeding depression under pollen discounting and pollen limitation. *Journal of Evolutionary Biol*ogy **18**:497-508

Potts S.G., Dafni A., Ne'eman G. 2001. Pollination of a core flowering shrub species in Mediterranean phrygana: variation in pollinator diversity, abundance and effectiveness in response to fire. *Oikos* **92**:71-80

Potts S.G., Vulliamy B., Dafni A., Ne'eman G., O'Toole C., Roberts S., Willmer P. 2003. Response of plant-pollinator communities following fire: changes in diversity, abundance and reward structure? *Oikos* **101**:103-112

Potts S.G., Vulliamy B., Roberts S., O'Toole C., Dafni A., Ne'eman G., Willmer P. 2005. Role of nesting resources in organising diverse bee communities in a Mediterranean landscape. *Ecological Entomology* **30**:78-85

Price M.V., Waser N.M. 1998. Effects of experimental warming on plant reproductive phenology in a subalpine meadow. *Ecology* **79**:1261-1271

Proctor M., Yeo P., Lack A. 1996. The natural history of pollination. London: Harper Collins

Psaras G.K., Rhizopoulou S. 1995. Mesophyll structure during leaf development in *Ballota acetabulosa*. *New Phytologist* **131**:303-309

Rambuda T.D., Johnson S.D. 2004. Breeding systems of invasive alien plants in South Africa: does Baker's rule apply? *Diversisty and Distribution* **10**:409-416

Rathcke B.J. 1983. Competition and facilitation among plants for pollination. In: **Real L.** ed. *Pollination Biology*. pp. 305-329. Academic, Orlando, Florida, USA.

R Development Core Team 2004. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-00-3, URL http://www.R-project.org.

Regal P.J. 1982. Pollination by wind and animals: ecology of geographic patterns. *Annual Review of Ecology and Systematics* **13**:497-524

Rodríguez A.F.M., Molina R.T., Palacios I.S., Corchero A.M., Muñoz J.T. 2005. Airborne behaviour of *Echium* pollen. *Aerobiologia* **21**:125-130

Ronsheim M.L., Bever J.D. 2000. Genetic variation and evolutionary trade-offs for sexual and asexual reproductive modes in *Allium vineale* (Liliaceae). *American Journal of Botany* **87**:1769-1777

Roubik D.W. 1983. Experimental community studies: time-series tests of competition between African and neotropical bees. *Ecology* **64**:971-978

Sargent R.D. 2004. Floral symmetry affects speciation rates in angiosperms. *Proceedings of the Royal Society of London* B **271**: 603-608

Schemske D.W. 1980. Evolution of floral display in the orchid *Brassavola nodosa*. *Evoultion* **34**:489-493

Scribailo R.W., Barrett S.C.H. 1994. Effects of prior self-pollination on outcrossed seed set in tristylous *Pontederia sagittata* (Pontederiaceae). *Sexual Plant Reproduction* **7**:273-281

Shore J.S., Barrett S.C.H. 1984. The effect of pollination intensity and incompatible pollen on seed set in *Turnera ulmifolia* (Turneraceae). *Canadian Journal of Botany* **62**:1298-1303

Sih A., Baltus M.S. 1987. Patch size, pollinator behaviour and pollinator limitation in catnip. *Ecology* **68**:1679-1690

Smyth M.J., Sheppard A.W., Swirepik A. 1997. The effect of grazing on seed production in *Echium plantagineum. Weed Research* **37**:63-70

Snow A. 1986. Evidence for and against pollen tube competition in natural populations. In: **Mulcahy D.L., Mulcahy G.B., Ottaviano E.,** eds. *Biotechnology and Ecology of Pollen*. pp. 330-338. New York, USA: Springer.

Sowig P. 1989. Effects of flowering plant's patch size on species composition of pollinator communities, foraging strategies, and resource partitioning in bumblebees (Hymenoptera: Apidae). *Oecologia* **78**:550-558

Stanton M.L., Preston R.E. 1988. Ecological consequences and phenotypic correlates of petal size variation in wild radish, *Raphanus sativus* (Brassicaceae). *American Journal of Botany* **75**:528-539

Steets J.A., Ashman T.-L. 2004. Herbivory alters the expression of a mixed-mating system. *American Journal of Botany* **91**:1046-1051

Steffan-Dewenter I., Tscharntke T. 1999. Effects of habitat isolation on pollinator communities and seed set. *Oecologia* **121**:432-440

Steffan-Dewenter I., Tscharntke T. 2000. Resource overlap and possible competition between honey bees and wild bees in central Europe. *Oecologia* **122**:288-296

Steffan-Dewenter I., Tscharntke T. 2002. Insect communities and biotic interactions on fragmented calcareous grasslands- a mini review. *Biological Conservation* **104**:275-284

Stephens P.A., Sutherland W.J., Freckleton R.P. 1999. What is the Allee effect? *Oikos* **87**:185-190

Stephenson A.G. 1981. Flower and fruit abortion: proximate causes and ultimate functions. *Annual Review of Ecology and Systematics* **12**:253-279

Stout J.C. 2000. Does size matter? Bumblebee behaviour and the pollination of *Cytisus scoparius* L (Fabaceae). *Apidologie* **31**:129-139

Strauss S.Y., Conner J.K., Rush S.L. 1996. Foliar herbivory affects floral characters and plant attractiveness to pollinators: implications for male and female plant fitness. The *American Naturalist* **147**:1098-1107

Sugden E.A., Pyke G.H. 1991. Effects of honey bees on colonies of *Exoneura asimillima,* an Australian native bee. *Australian Journal of Ecology* **16**:171-181

Suttle K.B. 2003. Pollinators as mediators of top-down effects on plants. *Ecology Letters* **6**:688-694

Tepedino V.J., Sipes S.D., Griswold T.L. 1999. The reproductive biology and effective pollinators of the endangered beardtongue *Penstemon penlandii* (Scrophulariaceae). *Plant Systematics and Evolution* **219**:39-54

Tomimatsu H., Ohara M. 2002. Effects of Forest Fragmentation on Seed Production of the Understory Herb *Trillium camschatcense*. *Conservation Biology* **16**:1277-1285

Tomimatsu H., Ohara M. 2003. Floral visitors of *Trillium camschatcense* (Trilliaceae) in fragmented forests. *Plant Species Biology* **18**:123-127

Thomson J.D. 1978. Effects of stand composition on insect visitation in two-species mixtures of *Hieracium*. *American Midland Naturalist* **100**: 431-440

Thomson J.D. 1986. Pollen transport and deposition by bumble bees in *Erythronium*: influences of floral nectar and bee grooming. *Journal of Ecology* **74**:329-341

Thomson J.D. 2001. Using Pollination Deficits to Infer Pollinator Declines: Can Theory Guide Us? *Conservation Ecology* **5(1)**: 6. URL: http://www.consecol.org/vol5/iss1/art6/

Utelli A.B., Roy B.A. 2000. Pollinator abundance and behavior on *Aconitum lycoctonum* (Ranuncolaceae): an analysis of the quantity and quality components of pollination. *Oikos* **89**:461-470

Valido A., Dupond Y.L., Hansen D.M. 2002. Native birds and insects, and introduced honey bees visiting *Echium wildpretii (Boraginaceae*) in the Canary Islands. *Acta Oecologica* **23**:413-19

Vallejo-Marín M., Uyenoyama M.K. 2004. On the evolutionary costs of self-incompatibility: incomplete reproductive compensation due to pollen limitation. *Evoultion* **58**:1924-1935

Van Treuren R., Bijlsma R., Ouborg N.J., Van Delden W. 1993. The effects of population size and plant density on outcrossing rates in locally endangered *Salvia pratensis*. *Evolution* **47**:1094-1104

Vaughton G., Ramsey M. 1998. Floral display, pollinator visitation and reproductive success in the dioecious perennial herb *Wurmbea dioica* (Liliaceae). *Oecologia* **115**:93-101

Visscher P.K., Seeley T.D. 1982. Foraging strategy of honey bee colonies in a temperate deciduous forest. *Ecology* **63**:1790-1801

Waites A.R., Ågren J. 2004. Pollinator visitation, stigmatic pollen loads and among-population variation in seed set in *Lythrum salicaria*. *Journal of Ecology* **92**:512-526

Walsh N.E., Charlesworth D. 1992. Evolutionary interpretations of differences in pollen tube growth rates. *Quarterly Review of Biology* **67**:19-36

Ward M., Johnson S.D. 2005. Pollen limitation and demographic structure in small fragmented populations of *Brunsvigia radulosa* (Amaryllidaceae). *Oikos* **108**:253-262

Warren M.S. Hill J.K., Thomas J.A., Asher J., Fox R., Huntley B., et al. 2001. Rapid responses of British butterflies to opposing forces of climate and habitat change. *Nature* **414**:65-69

Waser N.M. 1983. The adaptive nature of floral traits: ideas and evidence. In: **Real L.** ed. *Pollination Biology*. pp. 241-285. New York: Academic Press.

Waser N.M., Chittka L., Price M.V., Williams N.M., Ollerton J., 1996. Generalization in pollination systems, and why it matters. *Ecology* **77**: 1043-1060

Weidema I.R., Siegismund H.R., Philipp M. 1996. Distribution of genetic variation within and among Danish populations of *Armeria maritima*, with special reference to the effects of population size. *Hereditas* **124**:121-129

Weih M. 2003. Trade-offs in plants and the prospects for breeding using modern biotechnology. *New Phytologist* **158**: 7-9

Wells K.L., Dougherty C.T. 1997. Soil Management For Intensive Grazing. *Soil Science News & Views* Vol 18, No 2

Westerkamp C. 1997. Keel blossoms: bee flowers with adaptations against bees. *Flora* **192**:125-132

Whitehead D.R. 1983. Wind pollination: some ecological and evolutionary perspectives. In: **Real L.** ed., *Pollination Biology*. pp. 97-108. New York: Academic.

Widén B. 1993. Demographic and genetic effects on reproduction as related to population size in a rare, perennial herb, *Senecio integrifolius* (Asteraceae). *Biological Journal of the Linnean Society* **50**:179-195

Wilcove D.S., McLellan C.H., Dobson A.P. 1986. Habitat fragmentation in the temperate zone. In: **Soulé M.E.** ed. *Conservation Biology*. pp. 237-256. Sunderland, MA: Sinauer.

Wilson P., Thomson J.D. 1991. Heterogeneity among floral visitors leads to discordance between removal and deposition of pollen. *Ecology* **72**:1503-1507

Wolf A.T., Harrison S.P. 2001. Effects of habitat size and patch isolation on reproductive success of the serpentine morning glory. *Conservation Biology* **15**:111-121

Wolfe B.E., Husband B.C., Klironomos J.N. 2005. Effects of a belowground mutualism on an aboveground mutualism. *Ecology Letters* **8**:218-223

Young A., Boyle T., Brown T. 1996. The population genetic consequences of habitat fragmentation for plants. *Trends in Ecology and Evolution* **11**:413-418

Zimmerman M., Pyke G.H. 1988. Reproduction in *Polemonium*: assessing the factors limiting seed set. *The American Naturalist* **131**:723-738

6a. Appendix 1

Plant species	Family	Population
Anagallis arvensis L.	Primulaceae	Ab, Ad, Ko, Mo, Pe
Anchusa hybrida Ten.	Boraginaceae	Ab
Anthemis spp.	Compositae	Ab, Ad, Ko, Mo, Pa, Pe, Th
Brassica nigra (L.) Koch	Cruciferae	Ab, Un
Bunias erucago L.	Cruciferae	Ko, Pe
Calendula arvensis L.	Compositae	Ko, Mo, Pa, Un
Carduus pycnocephalus L.	Compositae	Ad, Ko, Mo, Pa
Chrysanthemum coronarium L.	Compositae	Th, Un
Chrysanthemum segetum L.	Compositae	Mo
Crepis spp.	Compositae	Ab, Ad, Ko, Mo, Pa, Pe, Un
Erucastrum sp.	Cruciferae	Ko, Mo, Pa, Pe
Geranium molle L.	Geraniaceae	Ko, Pa, Mo
Hedypnois cretica (L.) Willd.	Compositae	Ab, Ko, Mo, Pa , Th
Linaria pelisseriana (L.) Miller	Scrophulariaceae	Ko, Mo, Pe
Lotus sp.	Leguminosae	Un
Matricaria chamomilla L.	Compositae	Ab, Ad, Pa
Moenchia mantica (L.) Bartl.	Caryophyllaceae	Ad, Pa, Pe
Ornithogalum narbonense L.	Liliaceae	Ab, Ko, Mo
Papaver argemone L.	Papaveraceae	Ab, Ad, Pe
Petrorhagia (Raf.) G.López & Romo	Caryophyllaceae	Mo, Pa, Pe
Prasium majus L.	Labiatae	Un
Ranunculus sp.	Ranunculaceae	Ad
Tolpis barbata (L.) Gaertner	Compositae	Pe
Tordylium apulum L.	Umbelliferae	Ko, Mo
Trifolium campestre Schreber	Leguminosae	Ab, Ad, Ko, Mo, Pa, Pe
Trifolium echunatum Bieb.	Leguminosae	Un
Trifolium nigrescens Viv.	Leguminosae	Ab, Ad, Ko, Mo, Pa, Pe, Th
Trifolium tomentosum L.	Leguminosae	Ab, Pa,, Pe, Th, Un

The 28 plant species co-flowering with *Echium plantagineum* in the 8 studied *Echium* populations in 2005. For each plant, the species, family and the population where it was recorded are given (see text for details).

6b. Appendix 2

Taxon	Functional group	Population								Total
		Th	Va	Mo	Sk	Pa	My	Pe	Pi	
Hymenoptera										
Apidae (Xylocopinae)										
Ceratina spp.(females)	Af	1	2	1	-	5	-	-	4	13
Ceratina spp.(males)	Am	2	-	1	-	-	-	1	-	4
Xilocopa spp.(females)	Af	5	5	6	1	-	6	9	19	51
Xilocopa sp.(males)	Am	-	-	-	-	-	-	1	-	1
Apidae (Nomadinae)										
Biastes spp.(females)	Oth	-	4	-	-	-	-	-	-	4
Apidae (Apinae)										
Amegilla spp.(females)	Af	8	12	6	6	12	15	2	16	77
Amegilla spp.(males)	Am	3	1	1	1	1	1	2	-	10
Anthophora spp.(females)	Af	3	2	5	7	1	1	6	-	25
Anthophora spp.(males)	Am	1	1	3	10	2	3	14	-	34
Apis mellifera (females)	AB	5	-	227	407	1	11	49	5	705
Bombus spp.(females)	AB	12	67	56	-	-	92	-	115	342
Eucera spp.(females)	Af	-	17	3	17	-	1	31	15	84
Eucera spp.(males)	Am	-	16	-	2	-	-	1	-	19
Melecta spp.(females)	Oth	-	-	-	2	-	-	-	-	2
Tetralonia spp.(females)	Af	1	4	3	-	-	8	-	6	22
Thyreus spp.(females)	Oth	1	1	1	2	-	-	2	1	8
Thyreus spp.(males)	Oth	1	-	3	5	-	-	3	-	12
Halictidae										
Halictus spp.(females)	Hfm	-	-	-	-	-	8	-	-	8
Halictus spp.(males)	Hfm	-	-	-	-	-	3	-	-	3
Lasioglossum spp.(females)	Hfm	-	2	-	-	-	-	-	-	2
Lasioglossum spp.(males)	Hfm	-	-	2	-	-	-	-	1	3
Pseudapis spp.	Hfm	-	-	1	1	-	-	1	-	3
Megachilidae										
Anthidium spp.(females)	Mf	17	4	2	-	-	9	1	-	33
Anthidium spp.(males)	Mm	11	2	5	-	-	15	-	-	33
Chalicodoma spp.(females)	Mf	37	11	29	31	61	26	10	9	214
Chalicodoma spp.(males)	Mm	21	5	12	3	8	18	22	2	91
Coelioxys sp.(males)	Mm	1	-	-	-	-	-	-	-	1

(continued)

Creightonella spp.(females)	Mf	-	-	1	-	-	-	-	1	2
Creightonella spp.(males)	Mm	-	-	1	1	1	-	1	-	4
Eoanthidium spp.(females)	Mf	2	-	-	-	-	-	-	-	2
Eoanthidium sp.(males)	Mm	1	-	-	-	-	-	-	-	1
Hoplitis spp.(females)	Mf	-	-	-	-	-	4	-	4	8
Hoplitis spp.(males)	Mm	-	-	1	-	-	4	-	-	5
Icteranthidium sp.(females)	Mf	-	-	1	-	-	-	-	-	1
Lithurgus spp.(males)	Mm	-	1	-	-	-	-	1	-	2
Megachile spp.(females)	Mf	3	9	-	3	1	3	1	3	23
Megachile spp.(males)	Mm	-	3	1	-	4	1	-	2	11
Osmia spp.(females)	Mf	-	-	2	-	-	2	1	2	7
Osmia spp.(males)	Mm	1	-	-	-	-	-	1	-	2
Rhodanthidium spp.(females)	Mf	-	-	-	-	-	2	-	-	2
Rhodanthidium spp.(males)	Mm	2	-	1	-	-	1	-	27	31
Stelis sp.(females)	Mf	-	-	-	-	-	-	1	-	1
Other Hymenoptera (wasps)	-	2	1	4	-	-	-	-	-	7
Diptera	-	1	14	1	7	1	-	1	3	28
Lepidoptera	-	-	-	1	-	-	3	-	-	4

List of insect visitors recorded in 8 populations of *Ballota acetabulosa* in 2005. For each site the number of insect caught during a total of 3 h of random collection is indicated (overall: 24 h). Functional groups are given in the second column (see text for details).