

Reproductive biology of *Anthyllis vulneraria* subsp. *vulgaris* (Fabaceae) in northwestern Iberian Peninsula

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In spite of being widely distributed throughout Europe, populations of *Anthyllis vulneraria* are very isolated genetically. As a consequence of processes of independent evolution, some isolated populations have developed characteristics that separate them from the rest. The scope of this study was to investigate whether or not this isolation has affected the reproductive biology. The reproductive biology of an isolated population of *A. vulneraria* subsp. *vulgaris* (Koch) Willk. in northwest Spain was studied, and compared with French populations. In the studied population, insect pollination was necessary for fruit production. Autogamy was precluded by protandry, although geitonogamous pollen transfer might occur. Supplementary pollination did not lead to any increase in fruit-set level, seed-pod weight or seed weight. Mean pollen/ovule ratio (3080) was within the normal range for xenogamous taxa. The first open flowers in capitula lived more than twice as long as later flowers did. During the floral life-span, corolla colour gradually changed from white to partially pink to pink and the nectar production rate appeared to differ between colour stages. Fruit set level in the studied population differed between the four years of study. The breeding system found in this Spanish population differs from French populations. I suggest that classification of this taxon in northwest Iberian Peninsula should be reconsidered, taking into account its breeding system.

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Introduction

Since Müller (1883), many have studied the pollination mechanisms of Fabaceae. However, most works focus on a few species, mainly of economic interest. Thus, the pollination of the majority of Fabaceae is little known. Species of *Anthyllis* have been studied in order to clarify the complicated taxonomic classification of this polymorphic genus (Mardsen-Jones & Turrill 1933a,b; Couderc 1980 and references therein). Mardsen-Jones & Turrill (1933b) stressed the importance of breeding system and intensive field investigations for the eluci-

dation of critical taxonomic problems. Many years later, Couderc (1971a,b; 1980) and Couderc & Gorenflot (1978) continued this *Anthyllis* work in order to address the taxonomic problems in the French taxa. They studied in detail floral morphology and mechanism of pollination of French *Anthyllis* taxa. They found that flowers of most species, excluding those of *A. montana* L. and *A. cytisoides* L., are autogamous by means of a “pump mechanism” where the action of visiting insects is necessary. Couderc & Gorenflot (1978) suggested that *A. vulneraria* has a pollination system similar to that of *Lotus corniculatus* in which heavy insects (usually hon-

eybees and bumblebees) depress the keel in order to probe the floral interior for nectar and thereby mediate the contact between anthers and stigma causing autogamy. Thus, within *Anthyllis* two different systems of reproduction are found: allogamy in woody species and autogamy in herbs (Couderc 1980).

Anthyllis vulneraria L. is a widely distributed herb throughout most of Europe and the Mediterranean Basin east to the Caucasus. It comprises many infraspecific taxa with a controversial classification (Mardsen-Jones & Turril 1933a,b; Couderc 1975). Natural populations are very isolated genetically. Couderc (1975) supports that wild populations of *A. vulneraria* have evolved independently as a consequence of genetic isolation caused by spontaneous autogamy. This fact could explain the high number of observable varieties (i.e. as many as the number of populations). So, Couderc (1975) regards it impossible to treat all these types as different taxa. However, previous studies on this species made in Aquilianos Mountains (Northwest of Spain) showed that flowers of *A. vulneraria* subsp. *vulgaris* did not produce fruits after spontaneous autogamy.

The aims of this work were to investigate the reproductive biology of *Anthyllis vulneraria* subsp. *vulgaris* in the Aquilianos Mountains (Northwest of Spain), and to compare it with its French conspecific. The corolla colour of this taxon changes from white to pink during the life-time of a flower. This character has sometimes been utilized for taxonomic differentiation. Taking into account the importance of floral characters in future studies of the pollination ecology of this species (Navarro in review), I have also described corolla colour changes, nectar production and variation in flower duration. Specifically, I assessed the effects both of bagging and experimental hand pollination on fruit-set, seed-pod weight and seed weight. I also determined the pollen/ovule ratio (sensu Cruden 1977), and investigated the within-inflorescence flowering sequence order, the nectar production rate and changes in corolla colour of flowers of different age.

Material and methods

Natural History

Anthyllis vulneraria is 15 - 30 cm in height with 2-4 shoots. The flowers are 0.7 - 1.2 cm long and arranged in capitula. The base of each capitulum is enclosed by an involucre comprised of two multipartite leaves. Each shoot may bear 2-4 inflorescences (more information in Couderc 1971a,b; Couderc & Gorenflot 1978; Navarro 1996). In the study area, flowering is from mid-March to July. The fruit is a monospermic legume,

which matures approximately two weeks after fertilization. Grazing (largely by goats) causes a significant loss of capitula, shoots and occasionally whole plants (pers. obs.). Voucher specimens are deposited at the herbarium of the Santiago de Compostela University (Santiago de Compostela, La Coruña, Spain).

Study area

The study was carried out in the population of Vilardesilva (Concello de Rubiana, Orense Province) in the Aquilianos Mountains in the northwest Spain where the climate is typically Mediterranean. Here *Anthyllis vulneraria* subsp. *vulgaris* is found in two types of habitat: limestone rock faces and low calcareous grassland. The population in this study grew on the latter habitat at about 600 m.a.s.l.

Data collection

I collected all pollen from 1 to 8 day old bagged flowers ($n = 10$ flowers of each of 8 age classes). Pollen from each flower was immediately transferred to a Petri dish with a sterile solid 30% sucrose medium (Bar-Shalom & Mattson 1977). In a preliminary test this concentration gave the highest germination rate compared to 10, 20, and 40% sucrose solutions. Samples were incubated for four hours. Estimations of germination were done under a stereomicroscope. Grains with a pollen tube longer than the diameter of the grain were regarded as "germinated".

To investigate stigma receptivity, I excised the pistil of 10 flowers from each of 8 age classes and immersed it in a depression on a slide in a solution of benzidine in 60% ethanol, hydrogen peroxide and water. If the stigma is receptive, it breaks the hydrogen peroxide and the oxidation of the benzidine produces a blue colour (Dafni 1992).

Pollen transfer tests were carried out with flowers from different plants on Vilardesilva population in 1993:

- 1.- Insect exclusion: bagged with mosquito net (328 flowers of 22 capitula).
- 2.- Autogamy: pollinated with pollen from the same flower, and then bagged with mosquito net (148 flowers of 10 capitula).
- 3.- Geitonogamy: pollinated with pollen from another flower of the same plant, and then bagged with mosquito net (134 flowers of 10 capitula).
- 4.- Xenogamy: pollinated with pollen from another plant, and then bagged with mosquito net (133 flowers of 10 capitula).
- 5.- Supplementary pollination: pollinated with pollen

from another plant without bagging (632 flowers of 49 capitula).

6.- Control: no treatment (2042 flowers of 164 capitula).

In each treatment pollen transfers were made to all flowers within a capitulum. Subsequently to all treatments, I determined fruit-set level per capitulum, and weight of seed-pods and single seeds. The pollen/ovule ratio was estimated (Cruden 1977) in 1993, from 10 buds (each from different plants) just prior to their opening. In addition, I estimated natural fruit set between 1993-1996 (both 1993 and 1996 included).

To investigate flowering phenology and changes in flower colour over the lifespan of a flower, I monitored eight capitula each from a different plant, i.e. total 110 flowers in 1993. At opening, each flower was marked, with indelible ink varying in colour with flower position in the capitulum. Each day during the flower's lifespan corolla colour was recorded as either "all-white", "partially pink" (white with keel and distal part pink) or "all-pink".

To investigate nectar production, I bagged 16 capitula on 12 plants and, after 24 h, determined nectar volume in each flower with a capillary micropipette and sugar concentration with a portable refractometer from Bellingham & Stanley. On the basis of these data, I calculated the amount of sugar produced by each flower in 24 h (Cruden & Hermann 1983). I also recorded corolla colour (as above) and evidence of nectar robbery for

each flower in relation to nectar production. This part of the study was done in 1993.

Data analysis

The possible effects of variation in pollen transfer on fruit set were investigated by an analysis of variance following arcsine transformation of the percentage data with Tukey's test for subsequent multiple pairwise comparisons. Both the variation in flower duration within each capitulum, and the differences in nectar production between flowers of different corolla colour states, were also examined using analysis of variance. All analyses were carried out with Systat v.5 (Systat Inc., Evanston, Illinois, USA). In the text, mean values are cited with their standard deviation.

Results

Pollen germinability and stigma receptivity

The male and female flower phases were well separated in time (Fig. 1). During the first two days of the life of a flower, its pollen grains were not able to germinate. On the third day, the corolla colour changed to the partially pink stage, anthers opened and pollen was released. During day 4-5, when the corolla became partially pink,

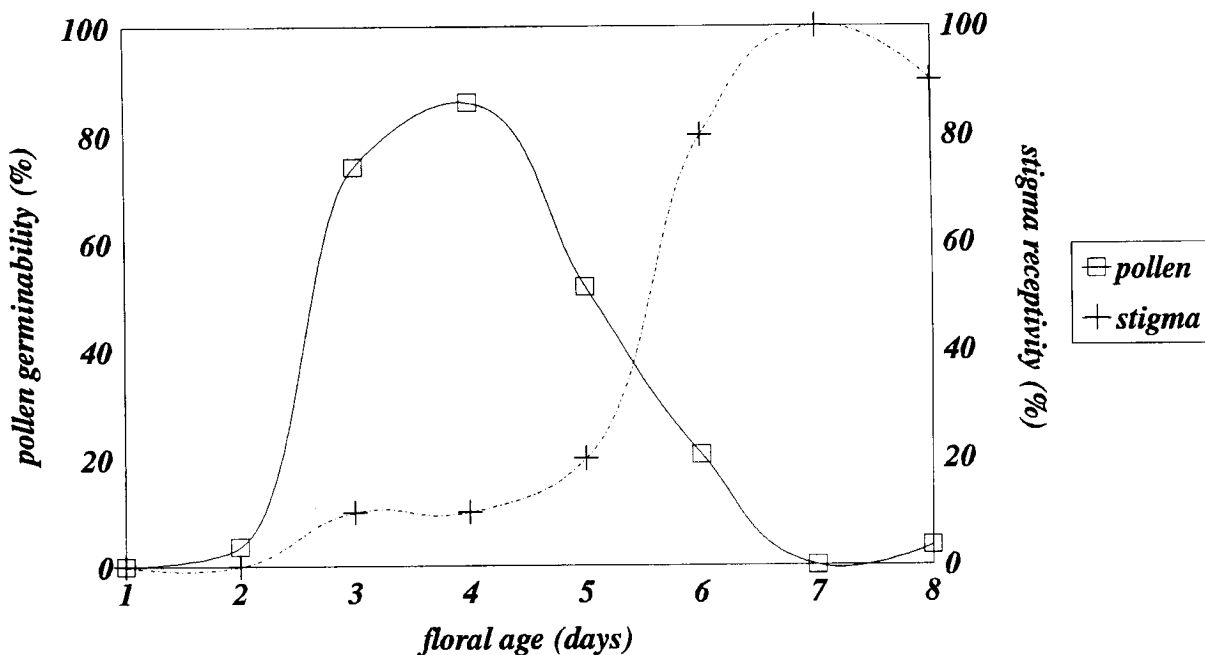


Fig. 1.- Variation in pollen germinability and stigma receptivity with flower age in *Anthyllis vulneraria* subsp. *vulgaris*.

Table 1. Mean (\pm sd) fruit set level per capitulum (%), mean seed-pod weight (μg) and mean seed weight (μg) for *Anthyllis vulneraria* subsp. *vulgaris* under natural conditions (control), and following different pollen transfer experiments. Sample sizes are indicated in brackets. The results of bagging were not included in analyses of variance (ANOVA). Within a column, values with the same superscript did not differ significantly (Tukey's test).

Treatment	Fruit set level (%)	Seed-pod weight (μg)	Seed weight (μg)
Bagged	0 (22)		
Autogamy	19.4 \pm 21.3 (10) ^a	1185 \pm 199 (27) ^a	137 \pm 33 (27) ^a
Geitonogamy	62.0 \pm 7.7 (10) ^b	1148 \pm 139 (82) ^a	141 \pm 30 (82) ^a
Xenogamy	75.0 \pm 4.8 (10) ^b	1417 \pm 250 (93) ^b	163 \pm 25 (93) ^b
Supplemented	66.9 \pm 8.8 (49) ^b	1319 \pm 164 (164) ^c	153 \pm 23 (164) ^{ab}
Control	65.9 \pm 12.5 (164) ^b	1282 \pm 245 (637) ^{ac}	148 \pm 33 (637) ^a
F-Anova	44.0; $p < 0.0001$	17.7; $p < 0.0001$	7.6; $p < 0.0001$

pollen grains had the highest germinability. From the sixth day and until the flower faded, pollen germinability decreased rapidly. Stigmatic receptivity increased steadily with flower age ($r_s = 0.964$; $p < 0.001$), and peaked on day 7, when pollen germinability was insignificant (Fig. 1).

Breeding system

Each flower contained two ovules and between 5465 and 7346 pollen grains. Mean pollen/ovule ratio was thus 3080 ± 258 .

Under natural conditions (control), mean capitula fruit-set in the year in which the breeding system was

studied (1993) was $65.9 \pm 12.5\%$ (see Table 1). All seed-pods contained only one seed. Mean seed-pod weight was $1282 \pm 245 \mu\text{g}$ and mean seed weight $148 \pm 33 \mu\text{g}$. None of the bagged flowers produced any fruits, indicating that pollinators were needed for fruit set. Fruit set of hand-self pollinated flowers (autogamy treatment) was also significantly lower than fruit set obtained from the other treatments. Seed-pod weight and seed weights from xenogamy were significantly higher than those from the other treatments. Supplementary pollination with nonself pollen did not significantly improve fruit set, seed-pod weight or seed weight compared to fruit set of control plants (Table 1).

Fruit set in the population varied significantly between the four years of study ($\chi^2 = 59.2$; $p < 0.0001$), al-

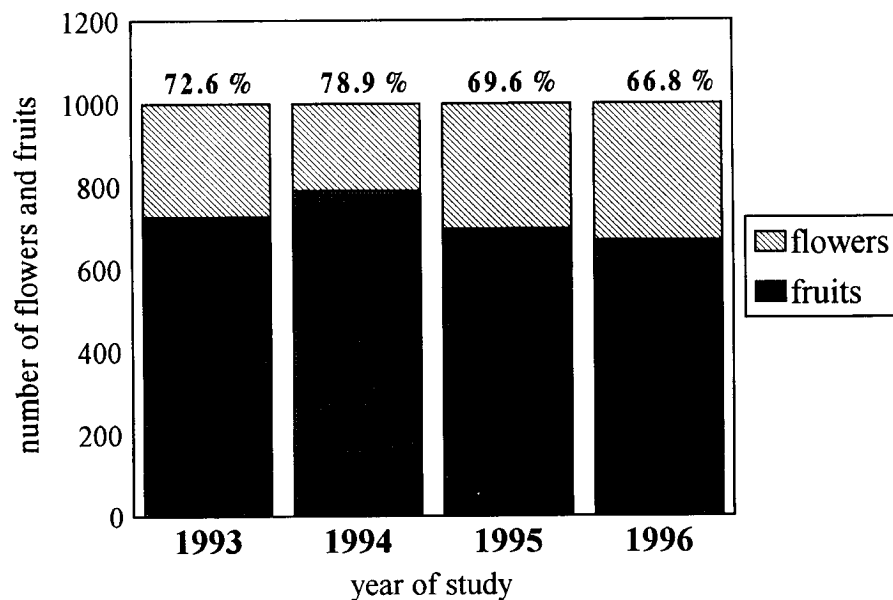
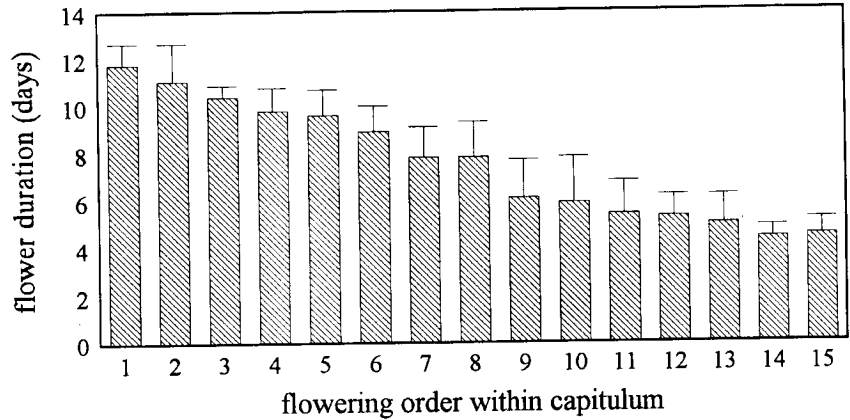


Fig. 2.- Fruit set level in *Anthyllis vulneraria* subsp. *vulgaris* studied 4 years in the Vilardesilva population.

Fig. 3.- Lifespan (in days, mean \pm s.d.) of flowers in eight capitula of *Anthyllis vulneraria* subsp. *vulgaris*. From left to right numbers indicate the flowering order within capitulum.



though neither 1993 and 1994 ($\chi^2 = 0.3$; n.s.), nor 1995 and 1996 ($\chi^2 = 1.7$; n.s.) differed from each other (Fig. 2).

Flowering phenology and flower colour

Flowering order within shoots was always acropetal (i.e., bottom-to-top) and within each capitulum basipetal (i.e., top-to-bottom). Except for the first and sometimes the second flower, all subsequent flowers opened in pairs. Mean flower lifespan was 7.8 ± 2.6 days. However, within each capitulum early-opening flowers lived longer than late-opening flowers, and this pattern was consistent among inflorescences (Kendall's coefficient of concordance = 0.40; $p < 0.001$) (Fig. 3). On average, the corolla was white for the first $30 \pm 9\%$ of its life, partially pink for the next $42 \pm 10\%$, and all-pink for the remaining $28 \pm 6\%$ of the time.

Nectar production

Nectar volume was measured in a total of 84 flowers, 24 of which had been robbed just prior to bagging, and none of them produced any nectar over the subsequent

24 h. Of the remaining flowers, 20 were white, and none of these produced any nectar. The mean nectar volume produced on 24 h was $0.19 \pm 0.10 \mu\text{L}$ in the remaining 40 flowers (20 partially pink, 20 all pink), and the mean sugar concentration was $32.7 \pm 9.8\%$ w/w. Among-flower variation was very high. Both nectar production rate and sugar concentration were significantly higher during the partially pink stage than during the all-pink stage (Table 2).

Discussion

In a study of a set of *Anthyllis* species, Couderc (1980) showed that woody species, in contrast to herbaceous species, are strongly protandrous making autogamy impossible. In some subspecies of *A. vulneraria*, including subsp. *vulgaris*, the precocity of autogamy, as a rule, prevents cross-fertilization, although this could be obtained experimentally (Couderc 1971b). Although the floral morphology of *A. vulneraria* apparently is adapted to cross-pollination by means of its pollen pres-

Table 2. Mean nectar volume (μL), sugar concentration (% w/w) and sugar amount (mg) in all-white, partially pink and all-pink flowers. The values of Student's *t* refer to comparison of the values for partially pink and all-pink flowers ($n = 20$ flowers for each group) (n.s. = not significant at the 5% level; *** = significant at the 0.01 % level).

Flower phase	Nectar volume (μL)	Sugar concentration (% w/w)	Sugar content (mg)
All-white	0	0	0
Partially pink	0.27 ± 0.07	31.8 ± 9.8	0.075 ± 0.028
All-pink	0.11 ± 0.04	33.6 ± 10.0	0.030 ± 0.011
Student's <i>t</i>	8.7 ***	0.6 n.s.	6.7 ***

entation mechanism of shooting stamens and stigmas, Couderc & Gorenflot (1978) showed that the shooting mechanism also promotes autogamy. The bagging and pollen transfer results from my study showed that *A. vulneraria* subsp. *vulgaris* was pollinator-dependent, but not strictly xenogamous. However, the strong protandry normally prevented the autogamy. This conclusion is supported by both the low fruit set obtained in plants that were subjected to autogamy, and the estimated pollen/ovule ratio. According to Cruden (1977) plants with spontaneous autogamy tend to have lower pollen/ovule ratio than pollinator-dependent plants. Thus, the value of the ratio is a useful indicator of the breeding system of a plant species. A value of about 3000 obtained in the present study is within the range cited by Cruden (1977) for xenogamous taxa. Despite this pollinator-dependence, pollen/pollinator availability was not a factor limiting female reproductive success during the study period, as shown by supplementary pollination experiments. This is true whether reproductive success is quantified as fruit-set, seed-pod weight or seed weight.

Pollinators of this species in the study population are *Anthophora* bees, and the nectar-robbing bumblebees *Bombus terrestris* and *B. jonellus* (Navarro in review). The behaviour of the two latter ones is similar to that described by Müller (1883). The pollen mass, which is found in the extremes of the floral wings, is expelled against the body of the visitors, when these alight directly on the capitulum and crawl across it in a "heavy-footed" fashion depressing the floral keel (Navarro in review). This behavior mediates pollen transfer between flowers of a capitulum. As flower phenology within the capitulum is asynchronous, pollen grains from "partially pink flowers", with a high capacity to germinate, may be transferred to "pink flowers" where the stigma is receptive. Thus, geitonogamous fruits may be produced.

Nectar characteristics (production rate, sugar concentration, and standing crop of nectar throughout the day) are of high importance to the plant-pollinator interaction (Mitchell & Paton 1990). Flowers of *A. vulneraria* subsp. *vulgaris* plants produced small quantities of nectar of a very high sugar concentration, as is typical to bee-pollinated flowers (Baker & Baker 1983, Cruden et al. 1983). Nectar production in 24 hours varied considerably among flowers. My data suggest that this variability is attributable to differences in nectar secretion rate at different stages in the flower's lifespan (Zimmerman & Pyke 1986, and references therein). The nectar production pattern is similar to that observed in other studies (e.g. Feinsinger 1978; Muraoka & Watanabe 1994). These have suggested that a higher production during male phase would increase pollen export (Mitchell 1993). Since the flower is one-seeded,

the female function may not need as many visits as the male function in order to be at its maximum.

In a number of species, it has been shown that nectar extraction induces further production of nectar (Thomson et al. 1989, Kadmon 1992). I found that robbed flowers stopped their nectar production, suggesting that extraction did not induce further secretion. Similar results have been obtained for *Mirabilis jalapa* (Martínez del Río & Búrquez 1986). Cruden et al. (1983) have suggested that species in which nectar extraction does not induce nectar secretion tend to require only one pollinator visit per flower in order to achieve maximal seed set.

In conclusion, the results reported from my study of a population of *A. vulneraria* subsp. *vulgaris* at the north-west Iberian peninsula, showed that, in disagreement with the results obtained by Couderc (1971b) and Couderc & Gorenflot (1978) for some French populations of *A. vulneraria* species, autogamy was precluded by a strong protandry, although fertilization with self-pollen can happen through geitonogamy. Couderc (1980) distinguished between two breeding systems in *Anthyllis*: herbaceous species with autogamous, and woody ones with protandry precluding autogamy. The results from this population identified as *Anthyllis vulneraria* subsp. *vulgaris* place this herbaceous subspecies within the latter group. The conflicting results and conclusions from the French and Spanish may be partly solved by a reconsideration of the taxonomy of the species. The results of this study illustrate the importance, as already stressed by Marsden-Jones & Turril (1933b), of including field investigations of breeding systems to elucidate critical taxonomic problems.

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References

- Baker, H. C. & Baker, I. 1983. Floral nectar sugar constituents in relation to pollinator type. – In: Jones, C. E. & Little, R. J. (eds), Handbook of experimental pollination ecology. Van Nostrand Reinhold Co. Inc., New York, pp. 117-141.
- Bar-Shalom, D. & Mattson, O. 1977. Mode of hydration, an important factor in the germination of trinucleate pollen

- grains. – Bot. Tidsskr. 71: 245-251.
- Couderc, H. 1971a. Sur la biologie florale de l' *Anthyllis vulneraria* L. – C. R. Acad. Sc. Paris 272: 1256-1259.
- 1971b. Etude expérimentale de la reproduction de l' *Anthyllis vulneraria* L. – Bull. Soc. Bot. France 118: 359-374.
- 1975. Contribution à la révision des taxons infra-spécifiques de l' *Anthyllis vulneraria* L. en France. – Revue Generale de Botanique 82: 93-118.
- 1980. Biologie florale de quelques espèces du genre *Anthyllis* L. et notamment de l' *A. montana* L. – Bull. Soc. Bot. France 127: 139-149.
- & Gorenflot, R. 1978. Adaptation de la fleur entomophile de l' *Anthyllis vulneraria* L. à l'autogamie. – Bull. Soc. Bot. France 125: 369-378.
- Cruden, R. W. 1977. Pollen-Ovule ratio: A conservative indicator of breeding systems in flowering plants. – Evolution 31: 32-46.
- & Hermann, S. M. 1983. Studying nectar? Some observations on the art. – In: Bentley, B. & Elias, T. S. (eds), The biology of nectaries. Columbia University Press. New York, pp. 223-241.
- , Hermann, S. M. & Peterson, S. 1983. Patterns of nectar production and plant-pollinator coevolution. – In: Bentley, B. & Elias, T. S. (eds), The biology of nectaries. Columbia University Press. New York, pp. 80-125.
- Dafni, A. 1992. Pollination ecology. A practical approach. – Oxford University Press, Oxford, UK.
- Feinsinger, P. 1978. Ecological interactions between plants and hummingbirds in a successional tropical community. – Ecol. Monographs 48: 269-287.
- Kadmon, R. 1992. Dynamics of forager arrivals and nectar renewal in flowers of *Anchusa strigosa*. – Oecologia 92: 552-555.
- Mardsen-Jones, E. M. & Turrill, W. B. 1933a. Studies in variation of *Anthyllis vulneraria*. – J. Genetics 27: 261-285.
- & Turrill, W. B. 1933b. Notes on the taxonomy of the British material of *Anthyllis vulneraria*. – J. Bot. Brit. Foreign. 71: 207-213.
- Martínez del Río, C. & Búrquez, A. 1986. Nectar production and temperature dependent pollination in *Mirabilis jalapa* L. – Biotropica 18: 28-31.
- Mitchell, R. J. 1993. Adaptative significance of *Ipomopsis aggregata* nectar production: observation and experiment in the field. – Evolution 47: 25-35.
- & Paton, D. C. 1990. Effects of nectar volume and concentration on sugar intake rates of Australian honeyeaters (Meliphagidae). – Oecologia 83: 238-246.
- Müller, H. 1883. The fertilization of flowers. – London.
- Muraoka, K. & Watanabe, M. 1994. A preliminary study of nectar production of the field cress, *Rorippa indica*, in relation to the age of its flowers. – Ecol. Res. 9: 33-36.
- Navarro, L. 1996. Fruit-set and seed weight variation in *Anthyllis vulneraria* subsp. *vulgaris* (Fabaceae). – Plant Syst. Evol. 201: 139-148.
- (in review). Pollination ecology of *Anthyllis vulneraria* subsp. *vulgaris* (Fabaceae): nectar robbers-like pollinators.
- Thomson, J. D., McKenna, M. A. & Cruzan, M. B. 1989. Temporal patterns of nectar and pollen production in *Aralia hispida*: implications for reproductive success. – Ecology 70: 1061-1068.
- Zimmerman, M. & Pyke, G. H. 1986. Reproduction in *Polemonium*: patterns and implications of floral nectar production and standing crops. – Amer. J. Bot. 73: 1405-1415.