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C.L. Collin · P. S. Pennings · C. Rueffler · A. Widmer
J.A. Shykoff

Natural enemies and sex: how seed predators and pathogens contribute to sex-differential reproductive success in a gynodioecious plant

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Abstract In insect-pollinated plants flowers must balance the benefits of attracting pollinators with the cost of attracting natural enemies, when these respond to floral traits. This dilemma can have important evolutionary consequences for mating-system evolution and polymorphisms for floral traits. We investigate the benefits and risks associated with flower size and sex morph variation in *Dianthus sylvestris*, a gynodioecious species with pistillate flowers that are much smaller than perfect flowers. We found that this species is mainly pollinated by nocturnal pollinators, probably moths of the genus *Hadena*, that also oviposit in flowers and whose caterpillars feed on developing fruits and seeds. *Hadena* preferred larger flowers as oviposition sites, and flowers in which *Hadena* had deposited eggs bore more pollen on their stigmas, suggesting that *Hadena* is indeed the principle pollinator, or that pollinators and these seed predators employ the same choice criteria for flowers. Globally, perfect flowers suffered more predation by seed predators than did pistillate flowers, suggesting that seed predators play an important role in reproductive system dynamics in this species. On the other hand, female flowers were more likely to be contaminated with spores of another natural enemy, the pathogenic fungus *Microbotryum violaceum*, that are transmitted by pollinating insects. This complex interplay between two natural enemies, one of which, *Hadena*, is also a pollinator and vector of fungal spores, may contribute to the maintenance of the polymorphic reproductive system of the host plant.

Keywords *Dianthus sylvestris* · *Hadena* · *Microbotryum* (=*Ustilago*) *violaceum* · Phenology · Pollination

C.L. Collin (✉) · P.S. Pennings · J.A. Shykoff
Laboratoire d'Écologie, Systématique et Évolution,
Université de Paris-Sud (XI), Bâtiment 362,
91405 Orsay Cedex, France
e-mail: carine.collin@esv.u-psud.fr
Tel.: +33-1-69155664, Fax: +33-1-69157353

C. Rueffler · A. Widmer
Geobotanisches Institut ETH, Zollikerstrasse 107,
8008 Zürich, Switzerland

Introduction

Flowering plants have many different reproductive systems, the most predominant being hermaphroditism, which is found in 72% of all species (Klinkhamer and de Jong 1997). However, unisexuality or dioecy has evolved many times, with gynodioecy – the coexistence of female and hermaphrodite individuals within a species – seen as a possible intermediate state between hermaphroditism and dioecy (Darwin 1888; Thomson and Brunet 1990). Delannay (1978) estimates that 10% of all angiosperm species have this reproductive system, which is widespread in the Lamiaceae, Plantaginaceae (Darwin 1888), and Caryophyllaceae (Desfeux et al. 1996). The genetic determinism of gynodioecy has been found to be nucleo-cytoplasmic in several species, with cytoplasmic male sterility genes and nuclear restorer genes (Charlesworth 1981; Couvet et al. 1986, reviewed in Charlesworth and Laporte 1998). This nucleo-cytoplasmic mechanism is considered to be the mode of sex inheritance of most gynodioecious species (Saumitou-Laprade et al. 1994).

Regardless of the type of genetic determination, females must have a fecundity advantage to become established and maintained. This can be based on production of more, larger, or better seeds than hermaphrodites, i.e. with higher germination or survival success. Female plants themselves can be larger with more flowers than hermaphrodites, or have longer lifespans (van Damme and van Delden 1984). Female advantage through one or more of these components can have different underlying causes. First, females can use resources for seed production that hermaphrodites allocate to pollen production. Secondly, females are always outcross-pollinated and in this way their progeny may be less inbred and may therefore have higher fitness (Darwin 1888). Thirdly, females or hermaphrodites may be differentially pollen limited, with pollen competition stronger in one or the other sex, thereby leading to offspring of different genetic quality (Shykoff 1992). Finally, natural enemies such as predators and pathogens may differentially affect females and

hermaphrodites (Marr 1997; Pettersson 1994; Puterbaugh 1998).

The genus *Dianthus* is found throughout the mountains of non-tropical Eurasia (Friedrich 1979). The rock pink *Dianthus sylvestris* Wulf. (Caryophyllaceae) is a perennial species with a gynomonoecious-gynodioecious reproductive system (Erhardt 1988; Friedrich 1979). Natural populations contain three types of plants: females bearing pistillate flowers, hermaphrodites bearing perfect flowers, and plants with both types of flowers (mixed individuals). This system allows us to compare female and hermaphrodite individuals as well as pistillate and perfect flowers on the same individual. This species is protandrous (Müller 1881) and self-compatible (Erhardt 1988); perfect flowers are in general larger than pistillate flowers but have shorter stigmas (Shykoff et al. 1997).

D. sylvestris has tubular flowers, thus its pollinators must have long probosces (at least 2 cm) to reach the nectar inside the calyx tube. The main pollinators of this species are the nocturnal moth *Hadena compta* Schiff. (Lepidoptera: Noctuidae) and the diurnal European hawk moth *Macroglossum stellatarum* L. (Lepidoptera: Sphingidae). It is also visited by bees and syrphid flies (Erhardt 1988; personal observations). The genus *Hadena* was described as "parasitic-pollinators" of various Caryophyllaceae species (Wahlgren 1924, cited in Pettersson 1992) since females also use the flowers as oviposition sites. They lay eggs while feeding on nectar and pollinating, and the resultant larvae feed on developing seeds and then on mature fruits, eating their way into the fruits through the calyx (Erhardt 1988).

D. sylvestris is also host to the fungus *Microbotryum violaceum* (Pers.) Deml. & Oberw. (=*Ustilago violacea* Pers.) (Basidiomycetes: Ustilaginales), which is widespread in the Caryophyllaceae (Thrall et al. 1993). *M. violaceum* has the dynamics of a sexually transmitted disease (Alexander and Antonovics 1988; Antonovics and Alexander 1992). In infected flowers female function is aborted and fungal spores replace all pollen in the anthers. Pollinators and other visiting insects serve as vectors of the fungus (Jennersten 1983; Roche et al 1995).

In this paper we investigate factors that influence the relative success of female individuals and pistillate flowers in a population of *D. sylvestris* containing mainly hermaphrodites, including plants of mixed sex. Indeed, many gynodioecious species are truly gynomonoecious-gynodioecious but mixed plants are usually excluded (e.g. Puterbaugh 1998; Shykoff 1988; but see Wolff et al. 1988). We compare female, mixed and hermaphrodite individuals for flower and seed production, seed weight, germination and seedling establishment. Further we follow flowering phenology and examine the fruiting success of flowers in relation to their phenology and sex. Because pollinators and seed predators influence fruiting success, we investigated levels of pollen and spore deposition, oviposition by seed predators and seed predation from field observations and an insect exclusion experiment to determine when pollinators and seed predators are most active. We pose the following questions:

1. What are the differences between pistillate and perfect flowers on female, hermaphrodite, and mixed plants for components of reproductive success?
2. Do pollination success, infection or predation risks covary or vary with flower sex or flower size?

Materials and methods

Work was carried out in a natural population of *Dianthus sylvestris* in northern Italy [Grosio, 46°17′24″N and 10°15′11″E] over two flowering seasons in 1999 and 2000. The population size was estimated as 4,200 individuals and covered a large exposed isolated rock outcrop of about 1,500 m^2, at 710 m above sea level. About 79% of the plants studied in 1999 were hermaphrodites (n=1,160) and 5% of the population was infected with *M. violaceum*.

In 1999 two samples of plants were chosen for study: all plants within 2 m along six randomly placed transects (217 m total length) and 112 other plants, randomly chosen among large individuals, bearing at least seven flowers, of the three sexual types (66 hermaphrodites, 19 females and 27 mixed plants). These plants were permanently marked with numbered aluminium tags hammered into the ground. In 2000, 30 additional non-infected plants on two small transects were marked and their sex checked. This second year, at least four randomly chosen flowers were taken from each plant and diverse measurements (see below) made on them. In 2000 we also investigated the relative importance of diurnal and nocturnal insect visitors as pollinators, seed predators, and vectors of fungal disease by conducting an insect-exclusion experiment (see below).

Flower production

From 18 May to 24 July 1999 the plants on the transects were checked every 5 days. For every individual plant the number of open flowers, the sex and the infection status was recorded. All plants bearing at least one pistillate flower were classified "non-hermaphrodite". At the end of the flowering season, all fruits were counted. These data sets allow us to compare flower production over one flowering season between plants that were pure hermaphrodites and plants that bore at least one pistillate flower. In the analysis only healthy plants (i.e. not infected with *M. violaceum*) were considered. Data from the 112 individually marked plants not on the transects were not included in this analysis because these latter plants were chosen to be large, and thus bore more flowers on average than those on the transects (t-test, t=3.026, P=0.003, n=933, for hermaphrodite plants only).

Seed production

Between 21 May and 16 June 1999, the 112 marked individuals were observed every 3 days and all new flowers were individually marked by writing a number on the calyx with a felt tip pen. For each of these flowers the sex was recorded and all the seeds were collected when ripe. Unfortunately some fruits were already open when collected, so some seeds may have been lost. These fruits were excluded from further analysis. All seeds from ripe and undamaged fruits were counted and weighed.

A small experiment was conducted to determine whether the marks on the calyces influenced seed set. On 41 plants one flower was marked with a felt tip pen, and another was marked with a thread at the base of the branch. The number of seeds per fruit was compared within each pair of flowers. No difference was found for the plants on which fruits from both marking methods matured undamaged (paired t-test, mean difference in seed set=1.4,

t=0.282, P=0.784, n=10). Therefore we consider that the marking method did not bias the results by influencing pollinator behaviour.

Seed weight, germination and seedling mortality

For all fruits collected in 1999, mean weight per seed per fruit was determined by bulk weighing all seeds. In mid January 2000 seeds from fruits of randomly chosen mother plants from each of the three types were sown in Petri dishes containing 1% water agar. We used all available (n=21) fruits from 13 female plants, 42 fruits from 21 randomly chosen hermaphrodite plants (i.e. two fruits per plant) and 69 fruits from 26 randomly chosen mixed plants (i.e. two fruits from pistillate flowers and two from perfect flowers, when possible).

The Petri dishes were left for 1 week at 24°C under fluorescent lighting and then put in the greenhouse. In the following weeks germinated seeds were counted, and a minimum of 18 seedlings per fruit for 60 plants were transplanted into small pots (diameter 6 cm, volume about 80 ml) containing a mixture of sand and potting compost (1:1). After 7 weeks under supplementary artificial lighting (2 a.m.–9 a.m., half Na and Hg lamps) and natural lighting, seedling mortality was checked.

Flower and plant phenology

On 24 May 1999, five female and five hermaphrodite plants were randomly chosen and one flower on each plant was followed from when it opened until wilting to determine the timing and duration of the male and female phase of perfect flowers and the lifespan of pistillate flowers.

From 18 May to 24 July 1999, the censuses that were made every 5 days on transects allowed us to determine how many flowering plants there were over the entire season. The 112 individually marked plants had all their flowers followed in order to know their flowering and fruiting dates (see Seed predation).

Seed predation

In 1999, the fate of all flowers on the 112 permanently marked individuals was followed. Flowers could have the following fates: successful fruit production, destruction by predators (when a hole was visible in the calyx or a caterpillar was found in the fruit the predator was considered to be a *Hadena*, all other predators were lumped in an "other predator" class), aborted, malformed or not pollinated. In 2000, flowers were collected in the middle of the season. If *Hadena* eggs or young caterpillars were present we considered the flower as "predated by *Hadena*", presence of other kinds of eggs or fly larvae was considered as "predated by other predators". We tested whether plant sex (hermaphrodite, female, mixed) influenced the proportion of flowers on the plant that were attacked by a predator using a one-way ANOVA on arcsine square-root transformed proportion data.

Infection risk and pollen receipt

On each flower collected in 2000 we measured the corolla diameter using digital callipers. Each measurement was taken twice to allow a repeatability estimate calculated as the intra-class correlation (Zar 1984); corolla measurements were repeatable (ANOVA, $F_{161,162}$=44.968, P<0.0001; intra-class correlation coefficient r_I=0.956). The stigmas were mounted on slides in Alexander's stain (Alexander 1969). For each stigma we counted *Dianthus* pollen and scored the presence of foreign pollen and spores of the fungal pathogen *M. violaceum*. Because pollen was counted by three different individuals, each of them counted 15 slides twice to test for: (1) repeatability of pollen counts, and (2) consistency among individuals. Pollen counts were repeatable (ANOVA, $F_{29,150}$=285.65, P<0.0001, intra-class correlation coefficient r_I=0.993) and we found no difference among counters (ANOVA, $F_{60,90}$=0.84, P=0.764).

Insect exclusion experiment

This experiment was conducted from 8 to 12 June 2000. We constructed cages by removing the bottoms from cardboard boxes and covering them with green nylon mesh. Each box contained at least two plants and treatments were assigned at random. The three sexes of plants were included in the experiment, but not all boxes contained all of them. The four treatments varied in time of availability of the plants to insect visits. Plants were either always available or never available (covered permanently) as two types of controls, or available either throughout the day or throughout the night. For the "day" and "night" treatments boxes were removed and replaced at, respectively, 0900 and 2000, and 2230 and 0430 hours. All treatment plants were therefore covered during dawn and dusk, and plants open to day-flying pollinators were exposed for longer than those open to night-flying pollinators. At the beginning of the experiment, we marked all open flowers on each plant. Each day the newly opened flowers were given identifying marks for the day on which they opened. These flowers were collected on the fourth day of the experiment. All the collected flowers were measured, checked for *Hadena* eggs, and the stigmas were mounted on slides, as described above, to count *Dianthus* pollen grains and note the presence or absence of *M. violaceum* spores.

Statistical analyses

All data were analysed with JMP, version 4.0 (SAS Institute 1997). For all analyses involving multifactor ANOVAs we fitted the full factorial model and reduced the model by backwards removal of the least significant, highest-order interactions first, only removing those for which P>0.1. No main effects were removed from the models. Plant was considered as a random factor in all analyses. ANOVA's assumptions of normality and homoscedasticity of the residuals were met by appropriate transformations. Proportion data were arcsine-square root transformed to approach normality when necessary; other data, such as those for seed weight and percentage of seedling mortality per mother plant, were natural-log transformed, pollen deposition was cubic-root transformed.

Results

Reproductive traits

Plants with at least one pistillate flower produced significantly more flowers than plants classified as hermaphrodites (t-test, t=3.221, P=0.001, n=1.006); mean±SE flower number per plant are 10.89±0.77 (n=167) and 8.18±0.34 (n=839), for plants bearing at least one pistillate flower and for hermaphrodites, respectively.

Because data from multiple fruits from the same plant are not independent, plant means were calculated for reproductive traits for which we had multiple measurements per plant. For mixed plants means were calculated for pistillate and perfect flowers separately and thus some pseudo-replication remains since these values are not independent. This provides four categories of flowers: pistillate flowers from female and from mixed plants, perfect flowers from hermaphrodite and from mixed plants.

Table 1 Mean values±SEs (sample size) of five measured characters are represented for the four categories of flowers found in a population of *Dianthus sylvestris*. Means and SE were calculated using mean values per plant. Comparisons for all pairs using Tukey-Kramer honestly significant difference are given (SAS Institute 1997). *n.s.* No significant difference among the sexes

Character	Female plants Pistillate flowers (ff)	Mixed plants		Hermaphrodite plants Perfect flowers (hh)
		Pistillate flowers (fm)	Perfect flowers (hm)	
Seed number per fruit n.s.[a]	41.86±4.49 (11)	41.56±3.18 (22)	38.57±2.92 (26)	39.89±2.13 (49)
Seed weight (mg)[b]	1.11±0.07 (11)	1.01±0.05 (22)	0.95±0.04 (26)	0.87±0.03 (49)
Germination rate (%) n.s.[c]	87.4±4.1 (20)	76.8±3.5 (27)	86.7±3.0 (37)	84.6±2.7 (45)
Mortality of descendants (%) n.s.[d]	15.9±5.0 (21)	16.0±4.3 (28)	16.1±3.8 (37)	16.4±3.5 (43)
Flower size (corolla diameter in mm)[e]	19.65±1.15 (7)	17.80±1.07 (8)	23.43±1.07 (8)	26.65±0.74 (17)

[a] ANOVA, $F_{3,104}$=0.217, P=0.884
[b] ff=fm=hm>hh; ANOVA, $F_{3,104}$=4.402, P=0.006
[c] See Table 2
[d] ANOVA, $F_{3,62}$=0.055, P=0.983
[e] ff=fm<hm=hh; ANOVA, $F_{3,36}$=18.97, P<0.0001

Table 2 Analysis of covariance (ANCOVA) of the percentage of seed germination per fruit. We used means per plant and sex, therefore some pseudo-replication remains from fruits of pistillate and perfect flowers on mixed plants. The four categories of flowers are: pistillate flowers from female and mixed plants, perfect flowers from hermaphrodite and mixed plants. Mean seed number and weight are covariates. *MS* Mean squares

Source	*df*	MS (×10⁶)	*F*	*P*
Flower category	3	0.8	1.709	0.173
Mean seed number	1	6.4	1.383	0.244
Mean seed weight	1	31.9	6.881	0.011
Error	70	4.6		
R^2=0.165 (P=0.024)				

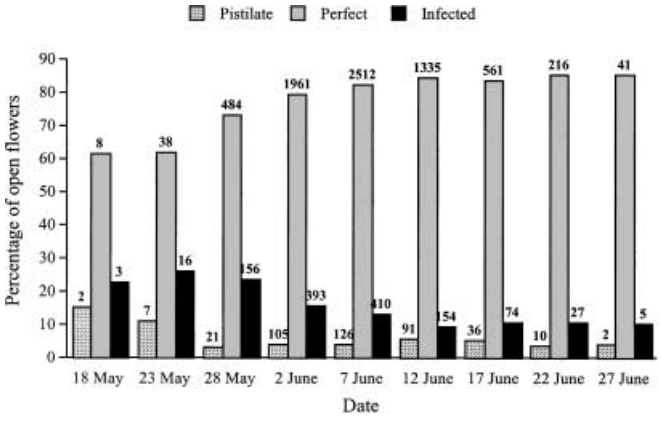

Fig. 1 Percentage of open flowers of *Dianthus sylvestris* belonging to three categories, pistillate, perfect and infected found on the transects in 1999. *Numbers at the top of the bars* represent the number of flowers in each category. *Pistillate* Includes pistillate flowers of both female and mixed plants, *Perfect* includes perfect flowers of hermaphrodite and mixed plants

There was no significant difference in mean seed number per fruit among the four types of flowers (Table 1); the overall mean seed number per fruit was 40.11±1.42 (mean±SE). We found a significant effect of flower category on mean seed weight per fruit (Table 1), with pistillate flowers from females producing heavier seeds than perfect flowers from hermaphrodites. A similar though non-significant tendency was observed for mixed plants (Table 1). Heavier seeds had a higher percentage of germination (r=0.295, P<0.01, n=76), but this was independent of flower category (Tables 1, 2); flower category did not affect seedling mortality either (Table 1). Pistillate flowers had smaller corollas than perfect flowers, analysed using the means of flower size per plant and sex (Table 1).

Flower and plant phenology

Pistillate flowers remained open only about one day (mean±SE=23.2±5.4 hours, n=5) and perfect flowers for about five days (mean=110.8±18.3 hours, n=5). Pistillate flowers opened with elongated receptive stigmas, and for some flowers the stigmas extended beyond the petals even before the flowers opened. Perfect flowers opened bearing pollen in a first whorl of up to five anthers and the second whorl of anthers exposed pollen subsequently. After 2 days the stigmas elongated and began to appear mature and receptive. Perfect flowers had a phase of stigma growth that lasted about 1 day before the female phase, and they remained in the female phase for about 2 days (mean=43.2±14.6 h, n=5), which was significantly longer than the entire life span of female flowers (t-test, t=2.87, P=0.02, n=10). Population flowering phenology is depicted for pistillate, perfect, and infected flowers in Fig. 1. Female and infected flowers were slightly more abundant at the beginning of the flowering season than later, but hermaphrodite flowers were always the majority. The flowering peak occurred about 3 weeks after the initiation of flowering. Seed production decrease over the season is presented in Fig. 2.

Seed predation, infection risk, and pollen receipt

Predation by unknown predators in 1999 and by *Hadena* in 2000 differed for the three types of plants (Fig. 3). Hermaphrodites generally suffered more predation pressure than females, with mixed plants being intermediate, except for *Hadena* predation in 1999. *Hadena* females appeared to prefer large flowers, since larger flowers

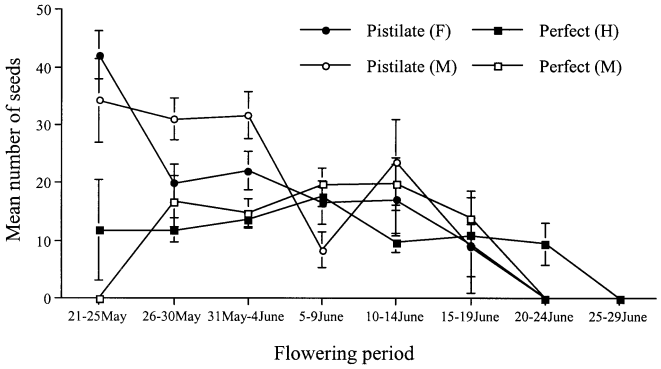

Fig. 2 Number (mean±SE) of seeds produced by the four flower categories as a function of their flowering dates. The mean seed set was calculated as an average of all flowers that flowered in a period, including predated ones. *Filled symbols* depict plants of pure sex [female (*F*) and hermaphrodite (*H*)], *open symbols* depict mixed plants (*M*). ✧, □ represent pistillate flowers and ♦, ■ represent perfect flowers

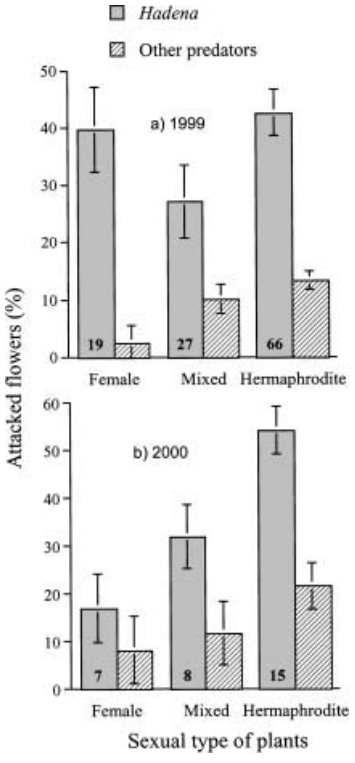

Fig. 3a, b Percentage of flowers (mean±SE) per plant predated either by *Hadena* or "other predators". **a** In 1999 predation by *Hadena* moths did not differ significantly among the three types of plants (ANOVA, $F_{2,109}$=2.613, *P*=0.078), whereas other predators attacked significantly more mixed and hermaphrodite than female plants (ANOVA, $F_{2,109}$=7.605, *P*<0.001). **b** In 2000 predation by *Hadena* moths was significantly higher for hermaphrodite than female or mixed plants (ANOVA, $F_{2,27}$=10.007, *P*<0.001), but no difference in predation by the other predators among the three types of plants was found (ANOVA, $F_{2,27}$=1.224, *P*=0.31). Sample sizes presented in the *grey bars* are the same for both kinds of predators

Table 3 ANCOVA with corolla diameter as covariate of the amount of pollen on stigmas of dissected flowers. The four categories of flowers are: pistillate flowers from female and mixed plants, perfect flowers from hermaphrodite and mixed plants

Source	*df*	MS	*F*	*P*
Corolla diameter	1	36.48	3.859	0.060
Flower category	3	3.814	0.404	0.752
Corolla×flower category	3	2.693	0.285	0.836
Error	27	9.452		
R^2=0.312 (*P*=0.140)				

Table 4 ANCOVA of the number of *Dianthus sylvestris* pollen grains found on the stigmas of flowers in the pollinator-exclusion experiment; number of *Hadena* eggs was the covariate. The four treatments were: flowers always or never available for pollinators and flowers available by day or by night only. Pollen deposition and number of *Hadena* eggs were positively related: slope (±SE)=0.638 (±0.312). *Plant* was considered as a random factor; denominator *df* and MS were calculated by Satterthwaite approximations in JMP (SAS Institute 1997)

Source	*df* (n, d)	MS	*F*	*P*
Treatment	3, 67	1.048	1.135	0.341
Box (treatment)	9, 13	0.810	0.543	0.819
Plant (box, treatment)	20, 58	1.259	1.642	0.073
Hadena eggs	1, 58	3.217	4.194	0.045
Error	58	0.767		
R^2=0.451 (*P*=0.111)				

contained more *Hadena* eggs or young caterpillars (*r*=0.47, *P*=0.002, *n*=41).

Among the 225 randomly chosen dissected flowers (pistillate and perfect), 52 contained both *Dianthus* pollen and spores of *M. violaceum* and 54 had nothing on their stigmas. Most of the flowers bore only *Dianthus* pollen (*n*=115) whereas only four flowers had just *M. violaceum* spores. Data from field observations and the "always available" treatment from the insect-exclusion experiment were not significantly different by heterogeneity χ^2 (χ^2=0.48, *df*=1, n.s.; Zar 1984). Combining the two, pollen and spore receipt were not independent (χ^2=13.5, *df*=1, *P*<0.001), with spores found almost only on stigmas that had also received pollen. The four flowers that received spores but no *D. sylvestris* pollen did bear pollen of other insect-pollinated plants, such as *Thymus vulgaris*, on their stigmas. The sexual type of the plant influenced the proportion of flowers per plant that received spores of *M. violaceum* (ANOVA, $F_{2,27}$=3.923, *P*=0.032, *n*=30), with females receiving spores on most flowers and hermaphrodites receiving them on the lowest number of flowers, mixed plants being intermediate [mean±SE percentage of flowers with spores per plant: 61.1±12.5 (*n*=7), 24.1±8.5 (*n*=15), and 54.1±11.7 (*n*=8) respectively]. Indeed smaller flowers had a greater probability of receiving spores in our sample (correlation between mean flower size per plant and the proportion that received spores; *r*=–0.43, *P*=0.018, *n*=30). We found no effect of flower category on the amount of pollen on stigmas [Table 3; mean±SE number of pollen grains on

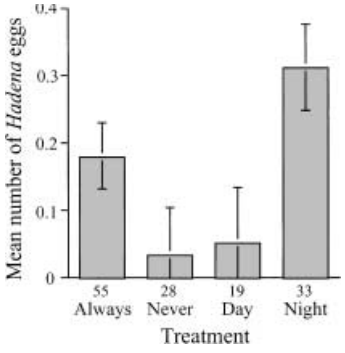

Fig. 4 Mean number (±SE) of *Hadena* eggs per plant for the four treatments of the insect-exclusion experiment. There were significantly more eggs in flowers available to nocturnal insects (contrasts method, night>always=never=day; see Table 5). *Numbers under the bars* denote sample sizes

Table 5 ANOVA of the number of *Hadena* eggs in the flowers of *D. sylvestris* submitted to the pollinator-exclusion experiment. There were significantly more eggs in the flowers available to the nocturnal pollinators (Contrast method: "night" treatment versus all other treatments, t=2.565, P<0.01, n=134) even if treatment is not significant in the full model. *Plant* was considered as a random factor; denominator *df* and MS were calculated by Satterthwaite approximations in JMP (SAS Institute 1997). See Fig. 4

Source	*df* (n, d)	MS	*F*	*P*
Treatment	3, 71	0.315	2.413	0.074
Box (treatment)	9, 16	0.283	1.660	0.180
Plant (box, treatment)	30, 91	0.149	1.362	0.134
Error	91	0.109		
R^2=0.459 (P=0.008)				

stigmas: female plants 65.19±14.32 (n=7), mixed plants 41.66±13.39 (n=8) and hermaphrodite plants 31.18±9.78 (n=15)] although larger flowers received more *Dianthus* pollen (r=0.522, P=0.001, n=35). In the pollinator-exclusion experiment significantly more pollen was present on the stigmas of flowers that contained *Hadena* eggs (35.19±9.34, n=13) than on those without (9.08±3.79, n=79); see Table 4. Further we found most *Hadena* eggs in the "night" treatment, when plants were exposed only to nocturnal visitors (Fig. 4 and Table 5).

Discussion

We found differences between female and hermaphrodite plants in flower size, flower production, seed weight, and predation and infection risk. Females produced smaller flowers and heavier seeds (Table 1), as is general for gynodioecious plants (Ågren and Willson 1991; Dulberger and Horovitz 1984; Gigord et al. 1999; Puterbaugh et al. 1997; Wolfe and Shmida 1997). In addition, plants with at least one female flower bore more flowers than pure hermaphrodites. However, we may have missed very small female plants because pistillate flowers remained open for an average of 1 day and the transects were inspected only every 5 days. As perfect flowers remained open longer, female plants with only one or few flowers were more likely to have been completely missed than hermaphrodite plants with very few flowers.

Heavier seeds germinated better than the lighter ones, but there was no effect of sex on germination success (Table 2) despite the significant effect of sex on seed weight. Higher germination success of seeds from females is a general but not universal finding for gynodioecious species (J. A. Shykoff and S. O. Kolokotronis, unpublished manuscript). Seeds of higher phenotypic or genetic quality should have a higher percentage of germination. In general, female plants should require more resources, saved from pollen production, as well as being completely outcrossed, and thus may better provision seeds (but see Delph et al. 1999) as well as produce seeds of higher genetic quality. In *D. sylvestris* hermaphrodite plants perform more selfing than females (Collin et al., in prep), but the rate of self-fertilization is nonetheless very low compared with other gynodioecious (Molina-Freaner and Jain 1992; Sakai et al. 1997; Wolff et al. 1988) or hermaphrodite species (Karron et al. 1995). Thus, though most seeds produced by hermaphrodite plants result from outcrossing events and should be therefore of comparable genetic quality to the seeds of females, seeds produced by hermaphrodite plants could experience inbreeding depression. Experiments investigating inbreeding depression in this species are currently underway.

Female advantage for reproductive traits is a common result in studies of gynodioecious species (J. A. Shykoff and S. O. Kolokotronis, unpublished manuscript). The role of natural enemies in moderating the relative success of females and hermaphrodites is a new and exciting aspect of the long-standing question of the maintenance of females (Puterbaugh 1998). In *D. sylvestris* females suffered lower predation risk by predispersal seed predators, caterpillars of the genus *Hadena* (Lepidoptera: Noctuidae), than did hermaphrodites, but were more likely to receive spores of the pathogenic fungus *M. violaceum*. The interaction between *D. sylvestris* and *Hadena* is multifaceted, because these moths pollinate and transmit fungal spores, and oviposit in and on the flowers, their larvae serving as seed predators (Erhardt 1988).

Hadena is known as a seed predator and pollinator of several species of Caryophyllaceae (Biere and Honders 1996; Brantjes 1976a, 1976b; Erhardt 1988; Pettersson 1994). In gynodioecious species, unlike in dioecious species where only females produce fruits, all flowers may produce fruits, so there is no a priori reason for ovipositing moths to discriminate on the basis of gender (Pettersson 1992). However, pistillate flowers have higher probabilities of producing fruits in many gynodioecious species (J. A. Shykoff and S. O. Kolokotronis, unpublished manuscript), so seed predators should choose female flowers since these represent a more certain substrate for the developing larvae (Brody 1992b). It appears, however, that *Hadena* females pollinate while ovipositing (Table 4), so they ensure a high-quality resource for their larvae, even though this is not a case of

active pollination, since specific morphological structures and behavioural adaptations are lacking (Pellmyr 1997). Furthermore, *Hadena* larvae spend only the first three larval instars within the flower where the egg is deposited, consuming the flower parts and ovary with either ovules or young developing seeds. The larva then exits from its natal flower and consumes many other fruits before completing its development (Brantjes 1976a, 1976b; Erhardt 1988). Therefore pollination of the flower on which the egg is laid is not essential for successful larval development (see also Jousselin and Kjellberg 2001). Regardless, our findings suggest either that *Hadena* females pollinate while ovipositing, and choose large flowers as oviposition sites (Brody 1992a), or that ovipositing *Hadena* and other nocturnal pollinators choose flowers by a similar criterion (Brody 1992b), in this case large size.

If large flowers suffer greater risk of predation, selection for enemy avoidance should act to reduce flower size (Galen 1999). Flower size should evolve to the point where natural selection through physiological costs and predation risk is balanced by sexual selection for increased mating opportunities. This optimum may be different for pistillate and perfect flowers, since investment in attractive structures is thought to benefit male more than female function in hermaphroditic flowering plants (Bell 1985; Delph et al. 1996). Indeed in gynodioecious species, small pistillate flowers are suggested to result from release from the pressure of sexual selection to maintain large costly flowers for the purpose of pollen export (Queller 1983), though a genetic or developmental correlation between petal and stamen production may also play a role (Darwin 1888; Stanton and Young 1984). We suggest here that additional selection by natural enemies such as predispersal seed predators may further drive the evolution of sexual size dimorphism by increasing the cost of large flowers. This idea is testable by comparing the degree of sexual size dimorphism in populations or species with and without natural enemies that differentially attack large flowers.

In this population *Hadena* is not the only natural enemy that may differentially affect plants bearing flowers of different sizes and thereby influence both the relative success of female and hermaphrodite plants and sexual dimorphism. We found a higher proportion of flowers on female and mixed-sex plants than on hermaphrodites receiving spores of the pathogenic fungus *M. violaceum*, and overall smaller flowers were more likely to receive spores, in contrast with previous findings for the same species (Shykoff et al. 1997). Sex can influence spore receipt in dioecious *S. latifolia*, with females, which bear larger flowers than males, receiving more spores per flower in some (Bucheli and Shykoff 1999) but not all cases (Alexander 1990; Antonovics and Alexander 1992; Bucheli and Shykoff 1999; Roche et al. 1995). Previous studies have found that many flowered (Thrall and Jarosz 1994) and large flowered plants receive more spores (Elmqvist et al. 1993; Shykoff et al. 1997). Here we did not quantify spore receipt, but noted only their presence, since, in principle, a single spore can lead to infection. Females, with their longer, stickier stigmas (Dulberger and Horovitz 1984; Shykoff 1992; Shykoff et al. 1997) may be more prone to pick up spores from a contaminated insect. Thus it appears that in this population one natural enemy, *Hadena*, favours females by preferentially predating hermaphrodites while the other, *M. violaceum*, imparts greater risk to small-flowered females. Indeed spatially varying selection pressure, with small flowers being at lower infection risk in some populations (Shykoff et al. 1997) and higher in others can contribute to the maintenance of variation for fitness-related traits such as flower size.

Not all plants that receive spores become infected with *M. violaceum* in this system (e.g. Bucheli and Shykoff 1999). Plants may vary for resistance (Alexander 1989; Kaltz et al. 1999), and rapid flower senescence in males can reduce susceptibility (Kaltz and Shykoff 2001). In *D. sylvestris*, flowers that receive spores are likely to be pollinated at the same time. Therefore contaminated flowers will be retained on the plant while fruits develop. If, however, most pollination activity is enacted by *Hadena* that also oviposit in the flowers, young *Hadena* caterpillars may consume these flowers and thus break their physiological connection with the plant, effectively preventing invasion of the fungus. Indeed, though in general pollinators prefer healthy over diseased plants (Jennersten 1988; Shykoff and Bucheli 1995), *Hadena* visit diseased plants and sometimes even oviposit in diseased flowers (Collin CL, Shykoff JA, personal observation; Biere A, personal communication). The *Hadena* suffer no negative effects by transporting spores, because even if the plant on which they oviposit becomes infected, infection appears only the subsequent year and caterpillars develop in healthy fruits (Biere and Honders 1996). For the plant, although it may lose its current fruit crop to predation by *Hadena* caterpillars, this is a minor cost when measured against permanent systemic sterilizing infection.

The flowering season in this population was short, occurring over only 6 weeks, with females and diseased plants over-represented at the onset of flowering (Fig. 1). These early flowering females enjoyed the greatest fecundity advantage relative to hermaphrodites (Fig. 2). The observed decrease in seed set is due to higher predation by *Hadena*. Indeed, along the season, there is an increased number of caterpillars (third and fourth instar) eating more and more fruits. The negative effects of natural enemies in this population varied over the season, with the lowest risk of predation by *Hadena* but the highest frequency of diseased flowers (Fig. 1) at the onset of flowering. Infected plants are known to flower earlier than healthy ones in several species (Alexander 1990; Jennersten 1988). By monitoring this *D. sylvestris* population over several years we shall be able to determine whether early flowering females are more likely to become infected with *M. violaceum* although they are less predated by *Hadena*. Plant phenology should evolve in response to variation in reproductive success over the

season, whether this is due to differential pollination success, differential attack rates, or abiotic factors, as long as there is heritable genetic variation for this trait (Brody 1997). It is impossible to say which of these natural enemies provides stronger selection pressure on flowering time in this population, but it is tempting to suggest that the highly synchronous flowering schedule results from high predation risk to later flowering plants, particularly since other related species in the same environment, such as *Gypsophila repens* (J. A. Shykoff, personal observation), have a far longer flowering season.

Flowering plants face a dilemma. They must attract pollinators but avoid enemies and minimize physiological costs of flowers (Galen 1999). In *D. sylvestris* pistillate flowers are smaller than perfect ones and suffer different risks from predators and pathogens. They may also be differentially pollen limited, which can contribute to reproductive system evolution (Maurice and Fleming 1995). Our attempt to experimentally investigate pollen limitation in this species was not successful, because of high levels of fruit predation, but further experiments are underway. Indeed the presence of spores of *M. violaceum* can also influence pollen limitation (Alexander 1987) because spores can interfere with pollen germination (Marr 1998). To sum up, *D. sylvestris* faces a complex biotic system including pollinating seed predators and pathogen vectors that can influence the evolution of flower size, floral sexual dimorphism, and plant reproductive system. More generally, our findings suggest that biotic interactions with natural enemies may be as important as those with pollinators in influencing the relative success of pistillate versus perfect flowers in sex-polymorphic reproductive systems.

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