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Male moths provide pollination benefits in the *Silene latifolia–Hadena bicruris* nursery pollination system

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Summary

1. Evolutionary conflicts of interest underlie mutualisms, including plant/pollinator interactions. This is particularly evident in 'nursery pollination', in which the pollinators lay eggs inside the flowers and the offspring of the pollinator consume the developing seeds. Low benefit (pollination service) to cost (seed predation) ratios could destabilize such associations towards parasitism.
2. Although in most of the well-known cases pollen transfer is associated with oviposition, in some systems the males of the seed predator may contribute to pollination, affecting the strength and outcome of the interaction between the plant and their ovipositing pollinators. In addition, in dioecious species male and female plants differ in the direct costs of seed predation and benefits of attracting pollinators, which may lead to sex-specific strategies.
3. We investigated whether pollinator and plant sex affect pollination in the interaction between dioecious plant *Silene latifolia* and its nursery pollinator, *Hadena bicruris* (Noctuidae).
4. Data on visitation behaviour and pollination efficiency in experimental plant patches demonstrate that (i) male moths are equally efficient pollinators as female moths, leading to fruit initiation in around 80% of visits and to fertilization of around 45% of the ovules in one visit; (ii) female and male moths do not preferentially visit flowers of one sex; and (iii) feeding behaviour is sufficient to ensure pollen transfer. However, female moths visited significantly more flowers than male moths.
5. Altogether this suggests that both moth sexes provide a pollination benefit to the plant with no differences in pollination efficiency but that female moths, before seed predation costs are accounted for, seem to provide greater benefits owing to their increased activity. That male moths contribute to seed production likely decreases the plant's dependency on ovipositing moths for pollination.

Key-words: dioecy, evolutionary conflict, fruit set, mutualism, plant/pollinator interactions, seed predation, seed set, white campion

Introduction

Mutualisms involve both benefits and costs for interacting species (Holland & Bronstein 2008) and can be seen as a reciprocal exploitation between partners. From this perspective, mutualism involves a conflict of interests, in which the fitness of one species increases at the expense of the other (Pellmyr & Huth 1994). Traits involved in plant/pollinator interactions may evolve in response to the underlying conflicts, with each of the interacting species being selected to increase its own net benefits (Herre *et al*. 1999). Conflicts of interest in plant/pollinator interactions are evident for nursery pollination systems, in which the pollinators lay their eggs inside the flowers they have pollinated, whereby the larvae feed on the developing seeds or other reproductive structures causing a cost to the plant (Dufaÿ & Anstett 2003).

Nursery pollination is taxonomically widespread (Dufaÿ & Anstett 2003), across different systems that vary in host plant-breeding systems (dioecious, gynodioecious or hermaphroditic), modes of pollination (active/passive) or degree of specialization of the interaction (obligate/non-obligate), with substantial diversification in some clades in which this mode of pollination occurs (Wiebes 1979; Herre 1989; Jürgens, Witt & Gottsberger 1996; Jousselin & Kjellberg 2001; Collin *et al*. 2002; Westerbergh 2004; Kephart *et al*. 2006). In

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most of the well-studied systems, pollen is mainly transferred during oviposition (*Lithophragma parviflorum*/*Greya politella*; Pellmyr & Thompson 1992) or in association with oviposition behaviour, through active pollination (Yucca/Yucca moth, Fig/Fig wasp; Jousselin & Kjellberg 2001; Pellmyr 1997; Pellmyr *et al.* 1996). To date, a contribution of male insects to pollination has only been documented in the association between *Trollius europaeus* and *Chiastocheta* flies (Despres 2003).

As exemplified by the *Trollius*/*Chiastocheta* case, in at least some of these interactions, male and female insects may contribute to pollination. Investigating the role of both insect sexes may be of considerable importance for quantifying benefit and cost functional response curves and thus for the analysis of stability in these systems (Holland, Deangelis & Schultz 2004). In nursery pollination, it is the adult female insect that contributes mostly to the cost of mutualism (deposition of seed-eating offspring). In this perspective, visits by male insects, if they occur, are potentially cost-free (barring costs of nectar feeding) and may modify the strength and outcome of the interaction between the plant and the pollinating, seed-predating insect. To better investigate the importance of pollination by males for the evolution and maintenance of these mutualisms we need more empirical studies, separating pollination benefits from the costs of oviposition and subsequent damage, and covering a broader range of also less-specialized associations.

Interestingly, like *Silene latifolia* (Caryophyllaceae), 5 of 13 host plants involved in known nursery pollination systems are dioecious (Dufaÿ & Anstett 2003). Dioecious host plants are not only comparatively frequent among nursery pollination systems (considering that dioecious species are rare among the Angiosperm; Renner & Ricklefs 1995), but also provide the conditions for such interactions to evolve sex-specific components. First, male and female insects visit flowers for different purposes (e.g. feeding only in males, feeding and ovipositing in females), thus the question arises of whether male and female insects prefer to visit male or female plants. It is noteworthy that such a specialization on one flower sex may compromise pollen transfer. Second, dioecy allows the evolution of sexually dimorphic plant strategies in response to seed predation (that harms female plants) and for pollinator attraction (which may generate greater fitness benefits for male plants). Thus, dioecious systems offer a particularly interesting test case to study the role of male and female insects in the plant/pollinator interaction.

Here, we investigate pollination efficiency of male and female insects and examine whether the sex of a dioecious host plant influences visitation behaviour for the interaction between *S. latifolia* and its pollinator/seed predator, *Hadena bicruris* (Noctuidae; Fig. 1). Male insects may play a role in this system, which is non-obligate and therefore less specialized than other nursery pollination systems that rely on oviposition for pollination (Kephart *et al.* 2006). Moreover, unlike several other dioecious nursery pollination systems in which male tissue is attacked (Dufaÿ & Anstett 2003), in *S. latifolia* the developing moth larvae feed on fruits and developing seeds. Because of the high costs associated with fruit and seed loss, this may increase the relative importance in this system of pollination without costs of seed predation through the male insects. Specifically, we address the following questions: (i) do male and female moths show sex-specific visitation, e.g. do male moths specialize on male flowers that provide richer nectar, and do female moths specialize on female flowers that provide the most suitable oviposition sites? (ii) How efficient are male and female *H. bicruris* moths as a pollinators, i.e. what are the fruit and seed set that result from single visits of male and female moths? Finally, we estimate pollination service across all observed visits.

Materials and methods

STUDY SYSTEM

Silene latifolia Poiret [= *Silene alba* (Miller) Krause] is a short-lived perennial plant, native to Europe that is well-studied in ecology and evolution (Bernasconi *et al.* 2009). Natural patches are often in disturbed habitats (Goulson & Jerrim 1997) and can be small and isolated (Richards, Emery & Mccauley 2003; Elzinga *et al.* 2005). This dioecious plant is sexually dimorphic for several traits including floral size and number (Delph & Meagher 1995), nectar composition and concentration (Shykoff & Bucheli 1995; Witt *et al.* 1999). Flowers are dimorphic for calyx width, calyx length and petal length (Delph, Knapczyk & Taylor 2002). These differences between male and female flowers and plants may provide cues of discrimination for the

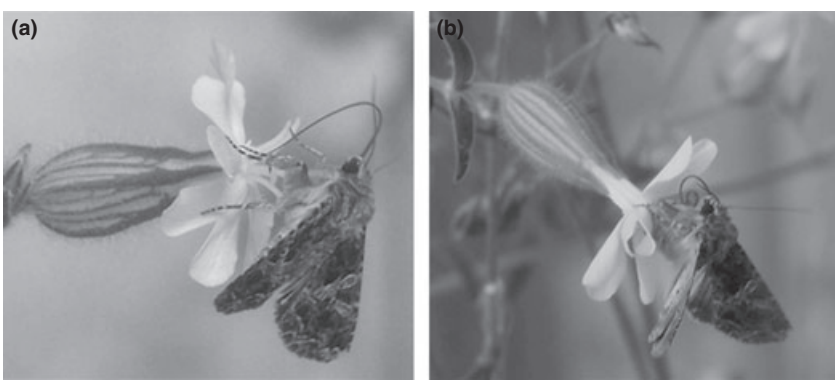

Fig. 1. The noctuid moth *Hadena bicruris* probing (a) and ovipositing (b) on its host, the dioecious plant *Silene latifolia*.

moth. A study of scent composition did not unveil any sex-specific scent compounds (Dötterl & Jürgens 2005). Male and female flowers are white and open at dusk. After pollination, the ovary develops into a capsule containing several hundreds of seeds (Jolivet & Bernasconi 2007). Nocturnal (moths) and diurnal (e.g. hoverflies) insects visit the plant (Jürgens, Witt & Gottsberger 1996), whereby the seed predating moth *H. bicruris* Hufn. (Lepidoptera) is considered as the main pollinator of *S. latifolia* (Jürgens, Witt & Gottsberger 1996). Adults of this moth oviposit in *S. latifolia* female flowers (Brantjes 1976a,b; Bopp & Gottsberger 2004), which they appear to prefer over other host plants in the genus *Silene* (Pettersson 1991; Bopp & Gottsberger 2004).

After hatching, the larva enters the fruit (*primary fruit*), it consumes the developing seeds, then (as fourth or fifth instar) leaves this primary fruit and starts feeding on other (*secondary*) fruits. In primary fruits, if the larva develops successfully, all seeds are destroyed, or do not contribute to seed production because the fruit is aborted (Jolivet & Bernasconi 2006). Although to our knowledge this has not been quantified, it may be possible that a fraction of the seeds escapes predation in secondary fruits. *Hadena bicruris* has been recorded in 90% of European *S. latifolia* populations surveyed (Wolfe 2002); attack rates of 25–70% of the fruits are common, indicating an important cost to the plant (Elzinga *et al.* 2005). Plants respond to experimental (Jolivet & Bernasconi 2006) and natural infestation with increased fruit abortion, and abortion rates vary greatly among populations (Elzinga & Bernasconi 2009).

PLANT AND INSECT MATERIAL

We randomly selected plants from a pool of 1120 F2 individuals originated from pollination of F1 plants obtained through crosses between plants raised from seeds (F0) collected in a natural population (Village-Neuf, France, 47°36′25″N 7°33′31″E, Jolivet & Bernasconi 2007, rearing conditions described therein). We reared F1 moths from an F0 generation collected as larvae ($n = 32$) in a natural *S. latifolia* population near Lausanne, Switzerland (46°31′19″N, 6°34′49″E). We reared the larvae individually in vials with vermiculite in a growth cabinet (SANYO MLR-350H; 22–25 °C, 16 : 8 h day : night, 55% RH), fed them on artificial diet (Elzinga, Biere & Harvey 2002) until pupation, and stored them at 4 °C until spring, when we transferred the pupae to the growth chamber. After emergence, we transferred adult moths to cages (1·2 m × 1·4 m × 1 m) sorted by age (1, 2, 3, > 3 days post-emergence) with male and female plants to provide them with nectar, to allow associative learning of differences between plant sexes and floral cues, and to collect eggs to establish a culture. Feeding adult moths on plants in a few cases resulted in pollen carry-over from the rearing flowers to the test flowers (5 of 14 experimental female flowers that were visited first in the experiment developed a fruit); however, given the larger number of visited flowers in the entire experiment, this is unlikely to create a bias (see also Results, showing rapid decline of pollen carry-over). For the experiments, we used moths aged > 3 days post-emergence.

EXPERIMENTAL ANALYSIS OF SEX-SPECIFIC POLLINATION

To test for sex differences in pollination behaviour and efficiency, we exposed individual moths to male and female plants in a large cage (3·5 m × 2·1 m × 1 m). For each replicate, we placed 42 flowering plants (21 males, 21 females) on equidistant cells on a grid. Plants were assigned randomly to positions, with the restriction to alternate males and females. Nectar production varies substantially between flowers (Shykoff & Bucheli 1995). Estimates range from a mean volume of about 1·7 and 1·3 μL in female and male flowers respectively (Shykoff & Bucheli 1995), to a maximum of 3–4 μL in female flowers (Shykoff 1997; Witt *et al.* 1999), and consumption by the moth of about 23 μL nectar in 2 h (Brantjes 1976a). As our trials lasted ≤40 min from the first visit (see below), our setup (with 42 flowers never exposed to pollinators before) should meet the foraging requirements of the moth, even if it foraged only on one sex, thus allowing unbiased assessment of sex-specific preferences. That moths were satiated in this setting is consistent with the observation that during the 40 min of observation the moths visited only *c.* half of the available plants (see Results). Each experimental plant had only one flower (all additional flowers or buds were removed) to isolate the effect of flower sex on visitation from the potential effects of variation in floral display (Harder & Johnson 2005). Although we did not measure flower size, male flowers were clearly smaller than female flowers as has been reported for several other natural populations (Delph, Knapczyk & Taylor 2002). Physical interspersion, random assignment to position (within sex), and standardization of floral display aimed at avoiding confounding variables. We tested 38 moths, 20 females and 18 males. As far as possible, we tried to use new, independent plants for each trial. When we reused non-visited plants from a previous trial, we waited until new flowers were formed.

Each moth was used for a single trial only. We isolated moths in the afternoon before the trial in a small cage (Bugdorm-1®, Megaview Science Co. Ltd., Taichung, Taiwan) without food. At dusk, we released the moth in the experimental cage and followed it with a red light for 40 min after it landed on its first flower, recording every visited flower and the observed behaviour (landing, feeding, or ovipositing). We chose this observation time based on preliminary observations indicating that after *c.* 30 min, male moths ceased flying, whereas females often continued flying but towards the walls of the cage, and did not visit any flower thereafter. After 40 min, we removed the moth. The next morning, we labelled all visited flowers and removed all eggs. Removing eggs had two goals: (i) to assess in how many cases the observed insertion of the abdomen in the corolla tube resulted in egg-laying; and (ii) to allow normal fruit development so as to measure pollination efficiency of pollinator visits. We bagged the flowers to be sure to recover the fruit in case of abscission. After 10 days, we preserved the developing fruits in formaldehyde-acetic acid solution (Bernasconi, Lang & Schmid 2007).

For each replicate, we recorded the *visitation sequence* (a list with the identity of the visited plant and the order of visitation), the *total number* and the *proportion of male and female flowers visited*, the *alternation between plant sexes*, the *occurrence of feeding and oviposition behaviour* and the *total activity time* (time between the first and the last flower visited). For each individual flower, we determined a number of potential correlates of pollen import and export: in addition to behaviour (feeding and oviposition), the *time spent on each flower*, the *number of visits to the same flower* (single or repeated visits), the *number of male and female flowers visited prior to the focal flower*, and the '*distance' to the last male flower* previously visited (the number of female plants visited between the focus female plant and the last-visited male plant).

To ensure that pollination was exclusively performed by the experimental moths, we equipped our greenhouses with an insect-proof netting (GVZ Bolltec AG, Zurich, Switzerland), we kept male and female plants in two separate greenhouses before experiments, and checked that no floral pests (e.g. thrips) were present in our plant rearings.

MEASUREMENT OF POLLINATION EFFICIENCY

For each moth, we dissected three (for two male moths, two) randomly chosen fruits, with the restrictions that the fruit arose from a single visit and that this visit was preceded by at least one visit to a male flower. We dissected developing fruits under a dissecting microscope (Leica MZ 95, 10 × 0·63 magnification; Leica Microsystems AG, Heerbrugg, Switzerland) in Petri dishes containing 70% ethanol, and separated developing seeds (fertilized) and unfertilized ovules (Fig. 2). We evaporated the ethanol and captured three images of the Petri dish (Fig. 2), which was shaken between images. We counted fertilized and unfertilized ovules on an enlarged print of each image. The three counts were highly repeatable (not shown) and we used the average for analysis. In total, we counted fertilized and unfertilized ovules in 103 fruits, 54 female-visited and 49 male-visited.

STATISTICAL ANALYSIS

To test for visitation preference of plants of a given sex, we compared the proportion of female flowers visited by male and female moths using *G*-test of heterogeneity (Sokal & Rohlf 1995). We first tested separately for female and male moths whether the proportion of female flowers visited departed from the expectation of 50% (GP), and we controlled for homogeneity among replicates (GH). Then, we tested whether the proportion of visited female flowers differed between male and female moths using generalized linear model (GLM, binomial error distribution), and whether male and female moths visited the same number of flowers during the 40 min of observation with GLM with quasi-Poisson errors with moth sex as explanatory variable. To test for randomness in the sequence of visitation, we divided the sequence of visitation into runs, whereby one run is a sequence of flowers of the same sex visited successively (Sokal & Rohlf 1995). We used runs tests to compare the observed number of runs of each individual moth to the expectation under the null hypothesis of randomness (Sokal & Rohlf 1995).

We estimated pollination service at two different scales: (i) a single visit to a female flower and (ii) the entire visitation sequence (all the female flowers visited during the 40 min). We tested whether male and female moths differed in their pollination efficiency (fruit initiation and, for a subset of fruits, the proportion of ovules fertilized after a single visit to a female flower). First, we assessed the fate of the visited female flowers (fruit initiated or not, $n = 291$) using generalized linear mixed models (GLMM) with binomial error distribution. For this analysis, we included all visited female flowers that were visited after at least one male flower. As fixed effects, we entered moth sex, the distance to the last male (number of female flowers visited between the last visited male flower and the focus female flower, encoded as a factor on three levels: none, one and two or more intermediate female flowers), the total number of male and female flowers visited previously in the sequence and the time spent on the flower, and the two-way interaction between moth sex and time spent on flower. The moth identity was entered as random effect. Second, we assessed the predictors of the proportion of fertilized ovules using linear mixed model (LMM) on the arcsine-transformed proportions; this analysis and transformation performed better compared to GLMM with binomial errors (which resulted in strong overdispersion). We entered the same fixed main effects as for fruit initiation, and all two-way interactions with moth sex. Both models (for fruit initiation and proportion of fertilized ovules) did not include feeding behaviour, because moths of both sexes fed on most of the visited flowers (see Results), nor oviposition behaviour by female moths, which was also highly frequent (see Results). Finally, we compared whether the absolute number of fertilized ovules (instead of the proportion of fertilized ovules) differed with the sex of the moth, using a GLMM with quasi-Poisson errors distribution with moth sex as fixed factor and moth identity as random effect. We calculated separately for male and female moths the average absolute number of ovules fertilized over the entire visitation sequence, obtained by multiplying the mean number of fertilized ovules with the number of female flowers visited by each moth. This estimates gross and not net benefits because the loss of developing fruits due to egg laying is not accounted for.

We define *feeding behaviour* as the observed insertion of the proboscis inside the corolla tube, but did not measure actual food intake. Similarly, we define *oviposition behaviour* as the insertion of the ovipositor inside the corolla tube or the display of oviposition posture on the petals. As we removed all flowers at the end of each visitation sequence, we validated that oviposition behaviour resulted in egg laying by examining all flowers for the presence of eggs. For analysis, we examine oviposition behaviour and egg laying separately.

We measured the time spent on flowers as the duration between landing on the flower and departing from it. The time spent on flower might additionally reveal a preference for a given flower sex. Using an LMM, we tested whether the sex of the visiting moth, the sex of the flower and its position in the sequence of the visitation influenced the time spent on flowers (log-transformed); we also added to the model the interactions *moth sex* × *sex of the flower* and *moth sex* × *position of the visited flower in the sequence of visitation*, and moth identity as a

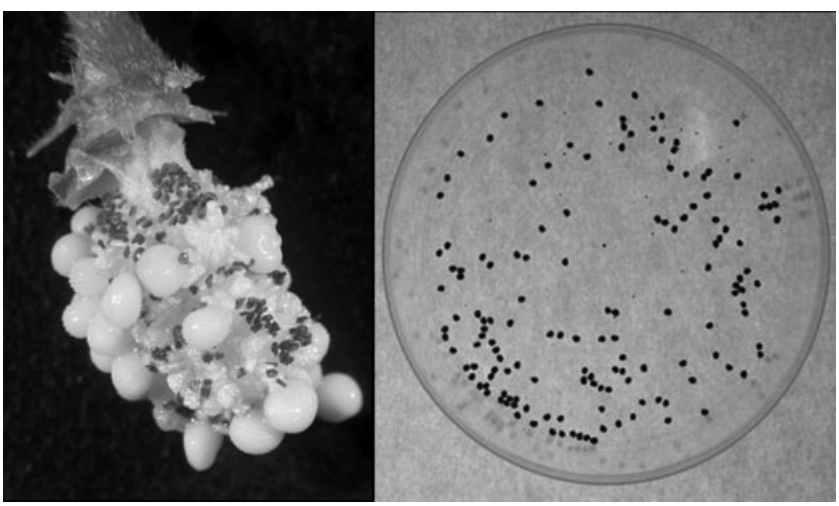

Fig. 2. Fruit of *Silene latifolia* 10 days after pollination, dissected to show developing seeds (white, large) and unfertilized ovules (dark, small). Developing seeds and unfertilized ovules were spread on a Petri dish for counts.

random factor to account for the fact that moths are the independent subjects. Again, also this model did not include feeding behaviour, because moths of both sexes fed on most of the visited flowers (see Results), or oviposition behaviour by female moths, which was also highly frequent (see Results).

We performed all statistical analyses with R 2.6.2 (2008). Model simplification was conducted through backward elimination (successively removing factors with $P > 0{\cdot}10$) until we obtained a minimal adequate model. Where applicable, we used quasi-Poisson errors in GLM to correct for overdispersion. Data are given as mean ± SE, unless specified.

Results

IS VISITATION SEQUENCE SEX SPECIFIC?

The proportion of female flowers visited by male (0·49 ± 0·03, $n = 18$, G-test, GP = 0·20, $\chi^2_{\alpha = 0{\cdot}05;1\text{d.f.}} = 3{\cdot}84$) and female moths (0·53 ± 0·02, $n = 20$, GP = 1·52, $\chi^2_{\alpha=0{\cdot}05;1\text{d.f.}} = 3{\cdot}84$) did not deviate significantly from the null expectation of 0·5, nor from each other (GLM with binomial errors, $z = 0{\cdot}832$, 36 d.f., $P = 0{\cdot}405$). The observed proportion of visited female flowers out of the total number of flowers visited was homogeneous among replicates (G-test; male moths: GH = 10·31, $\chi^2_{\alpha=0{\cdot}05;\ 17\text{d.f.}} = 27{\cdot}58$; female moths: GH = 8·74, $\chi^2_{\alpha=0{\cdot}05;\ 20\text{d.f.}} = 30{\cdot}14$). Thus, male and female moths visited both sexes of the plant, in an equivalent proportion not different from 50%. Thirty-four out of the 35 moths visited male and female flowers in a random sequence (runs tests; three male moths visited fewer than nine plants and were excluded from analysis).

DOES THE PROBABILITY OF FRUIT INITIATION DEPEND ON MOTH SEX?

On average, 83·15 ± 0·81% ($n = 20$ female moths) and 79·09 ± 0·89% ($n = 17$ male moths) of female flowers visited by female and male moths respectively developed a fruit (Table 3). Despite a slightly higher probability of fruit initiation in flowers visited by female moths, the probability for a female flower to develop a fruit after a visit by a moth did not depend significantly on the sex of the moth (GLMM, $P = 0{\cdot}103$; Table 1). Fruit development was significantly explained by pollen load on the moth. Indeed, the number of male flowers visited before (pollen uptake) and number of female flowers visited before (pollen download) significantly affected the probability of fruit initiation (GLMM, $P = 0{\cdot}039$ and $P = 0{\cdot}033$ respectively; Table 1). Accordingly, an increase in the number of intermediate female flowers visited between the focal flower and the last visited male flower reduced the probability of fruit initiation (GLMM, $P < 0{\cdot}005$; see details in Table 1). A longer time spent on the flower only marginally explained successful fruit initiation (GLMM, $P = 0{\cdot}065$; Table 1).

Table 1. Minimal adequate model (generalized linear mixed model with binomial error distribution and moth identity as a random factor) for the effect of predictor variables on fruit initiation (encoded as binary response variable) for 291 flowers visited by 37 moths (one moth excluded from analysis, see Materials and methods)

Explanatory variables	Effect size	SE	z	P
Intercept	1.919	0.442	4.34	< 0.001
Distance to the last male flower (one intermediate female flower)	−1.185	0.413	−2.87	0.005
Distance to the last male flower (two or more intermediate female flowers)	−1.478	0.447	−3.31	0.001
Total number of male flowers visited before	0.210	0.102	2.07	0.039
Total number of female flowers visited before	−0.206	0.097	−2.13	0.033
Time spent on the flower	0.009	0.005	1.85	0.065

Distance to the last male flower represents the number of intermediate female flowers visited between the focal female flower and the last visited male flowers (as in Table 2).

DO POLLINATION EFFICIENCY AND SEED SET DEPEND ON MOTH SEX?

At the level of one single fruit and visit, the proportion of fertilized ovules (pollination efficiency) showed very similar effects (Table 2) as fruit initiation (Table 1). Pollination efficiency was not significantly affected by moth sex, indicating that male and female moths had similar pollination efficiencies (male moth: 0·453 ± 0·043; female moth: 0·446 ± 0·042 ovules fertilized out of all available ovules; LMM, $t = -0{\cdot}88$, 33 d.f., $P = 0{\cdot}38$). Pollination efficiency was highly variable among visits, ranging from 0 to 0·91 for male moths ($n = 18$ male moths; $n = 49$ fruits) and from 0 to 0·94 for female moths ($n = 20$ female moths; $n = 54$ fruits). Most of this variation was explained by pollen load on the moth. The distance to the last male flower visited before (i.e. the number of female flowers visited between the last male flower and the focus female flower) had a significant negative effect on the pollination efficiency (LMM, Table 2). Moreover, there was

Table 2. Minimal adequate model (LMM with moth identity as a random factor) for the effect of predictor variables on the proportion of fertilized ovules (arcsine transformed to correct for overdispersion) for 103 fruits among 35 months (three moths excluded from analysis, see Methods). Effect sizes are not back-transformed. d.f. = 63

Explanatory variables	Effect size	s.e.	t	P
Intercept	0.711	0.077	9.24	< 0.0001
Distance to the last male flower (one intermediate female flower)	−0.103	0.080	−1.29	0.21
Distance to the last male flower (two or more intermediate female flowers)	−0.462	0.091	−5.08	< 0.0001
Total number of male flowers visited before	0.037	0.018	2.00	0.05
Total number of female flowers visited before	−0.030	0.016	−1.92	0.06
Time spent on flower	0.001	0.001	1.71	0.09

Table 3. Observed occurrences of floral visits, fruit initiation and feeding behaviour (defined as insertion of proboscis into floral tube) and, for female moths, oviposition behaviour (insertion of abdomen into floral tube) by *Hadena bicruris* moths visiting *Silene latifolia* flowers

	All visits	Visits with fruit initiation*	Visits with feeding	Visits with oviposition	Visits with both feeding and oviposition	Visits with only feeding	Visits with only oviposition	Just landing	Behaviour not observed
Female moths (n = 19) on female flowers									
Number of visited flowers	9.79 ± 0.63	8.15 ± 0.16	8.26 ± 0.73	8.58 ± 0.65	7.47 ± 0.66	0.79 ± 0.22	1.11 ± 0.26	0.11 ± 0.07	0.32 ± 0.13
Percentage of visits	100	83.15 ± 0.81	82.77 ± 3.04	87.23 ± 2.79	75.07 ± 2.89	7.70 ± 2.31	12.16 ± 2.68	1.24 ± 0.86	3.82 ± 1.52
Female moths (n = 19) on male flowers									
Number of visited flowers	8.32 ± 0.65		7.74 ± 0.58	2.00 ± 0.44	1.95 ± 0.43	5.79 ± 0.72	0.05 ± 0.05	0.11 ± 0.11	0.42 ± 0.18
Percentage of visits	100		93.6 ± 2.55	27.51 ± 6.65	26.45 ± 6.26	67.15 ± 7.49	1.05 ± 1.05	1.32 ± 1.32	4.03 ± 1.59
Male moths (n = 18) on female flowers									
Number of visited flowers	6.89 ± 0.77	5.24 ± 0.14	6.22 ± 0.73					0.44 ± 0.17	0.22 ± 0.13
Percentage of visits	100	79.09 ± 0.89	89.75 ± 3.07					7.51 ± 2.81	2.74 ± 1.53
Male moths (n = 18) on male flowers									
Number of visited flowers	6.83 ± 0.65		6.33 ± 0.62					0.17 ± 0.12	0.33 ± 0.14
Percentage of visits	100		93.22 ± 2.59					2.31 ± 1.62	4.47 ± 1.8
All moths (n = 37)									
Number of visited flowers	15.97 ± 0.91		14.32 ± 0.87					0.41 ± 0.11	0.65 ± 0.16
Percentage of visits	100		89.49 ± 1.53					3.06 ± 0.86	3.91 ± 0.83

Data are given as mean ± SE. The observed occurrences of feeding and oviposition are then detailed (feeding only, both feeding and oviposition, only oviposition). In some cases, we neither observed feeding nor oviposition (*just landing*), or the behaviour could not be observed.

*Descriptive statistics for fruit initiation is shown for n = 20 female moths and n = 17 male moths (see Materials and methods for inclusion criteria).

a positive effect for the total number of male flowers visited before in the sequence, reflecting pollen upload (LMM, $t = 2{\cdot}00$, 63 d.f., $P = 0{\cdot}05$; Table 2), and a negative marginal effect for the total number of female flowers visited before in the sequence, reflecting pollen deposition (LMM, $t = -1{\cdot}92$, 63 d.f., $P = 0{\cdot}06$; Table 2). Finally, the proportion of fertilized ovules tended to increase, the longer the time spent on the focus flower (LMM, $t = 1{\cdot}71$, 63 d.f., $P = 0{\cdot}09$; Table 2).

Similarly to the proportion of fertilized ovules, the absolute number of ovules fertilized by a male moth in a single visit (152·0 ± 18·95, min = 0, max = 390) was not significantly different from the number of ovules fertilized by a female moth in a single visit (153·8 ± 18·95, min = 0, max = 397; GLMM, $t = -0{\cdot}002$, $P = 0{\cdot}88$).

DOES TOTAL NUMBER OF VISITS DEPEND ON MOTH SEX?

Over the 40 min of observation, female moths visited significantly more flowers (total number of flowers: 17·25 ± 1·14; female flowers: 9·79 ± 0·63; male flowers: 8·32 ± 0·65; Table 3) than male moths (total number of flowers: 13·72 ± 1·25; female flowers: 6·89 ± 0·77; male flowers: 6·83 ± 0·65; Table 3; GLM, 36 d.f., $t = -2{\cdot}62$, $P = 0{\cdot}01$), and were active for a significantly longer time (36 ± 1·41 min) than male moths (30 ± 1·98 min; Wilcoxon Mann–Whitney test, $W = 258$, $P = 0{\cdot}023$). Consistent with the larger number of visited flowers, over the entire visitation sequence, female moths fertilized more ovules (1530·31 ± 101·94 ovules, $n = 19$ moths) than male moths (1050·96 ± 118·12 ovules fertilized, $n = 18$ moths).

BEHAVIOUR ON THE VISITED FLOWER

On average, male moths ($n = 18$) displayed feeding behaviour in 89·75 ± 3·07% of the visited female flowers and 93·22 ± 2·59% of the visited male flowers ($n = 18$ male moths; Table 3). On average, female moths ($n = 19$; Table 3) displayed feeding behaviour in 82·77 ± 3·04% of the visited female flowers and in 93·60 ± 2·55% of the visited male flowers. Female moths displayed oviposition behaviour, in 87·23 ± 2·79% of visits to female flowers but also in 27·51 ± 6·65% of visits to male flowers (Table 3), suggesting that the cues eliciting oviposition behaviour (insertion of abdomen into floral tube) differ, but not completely, between male and female flowers.

For the interaction between female moths and female flowers, we can ask whether feeding and ovipositing are independent events by comparing the conditional probability (i.e. the probability that both feeding and oviposition were simultaneously observed) to that expected for statistically independent events, i.e. the product of the probability to observe feeding (all visits with feeding) times the probability to observe oviposition [all visits with oviposition; $P(A|B) = P(A) \times P(B)$ if A,B statistically independent]. For female flowers the observed conditional probability (both feeding and oviposition observed on the same flower) was of 75·07% compared to the expected probability (assuming independence of feeding and oviposition behaviours) of 72·20% (i.e. the product of 82·77% × 87·23%; Table 3). Observed (26·45%) and expected (25·75%) probabilities were also very similar for male flowers. This congruence between observed and expected conditional probabilities was also found within moths (data not shown). This is consistent with the idea that the floral cues or traits that elicit or constrain feeding and oviposition behaviours respectively, are independent of each other.

On average only 8·9% female flowers visited by female moths escaped oviposition: 7·70 ± 2·31% of female flowers were only fed upon (i.e. we observed feeding but no oviposition behaviour; Table 3) and on 1·24 ± 0·86% of the visited flowers the moth just landed. In most cases, observed oviposition behaviour was associated with the presence of an egg in the flower (i.e. there was an egg in 172 flowers out of 201 flowers in which we observed oviposition behaviour). On average, a female moth laid 8·55 ± 0·85 eggs per sequence ($n = 20$ moths). A significant majority of eggs were laid in female flowers (7·45 ± 0·74 eggs, i.e. female moths laid eggs in 79% of the visited female flowers) and only occasionally in male flowers (1·1 ± 0·37 eggs; Wilcoxon signed-rank test: $n = 20$, $V = 0$, $P < 0{\cdot}01$). Out of 172 flowers with eggs, only 10 flowers had more than one egg (up to three). Female flowers that were visited more than once by female moths (30 of 186 female flowers) did not receive significantly more eggs than flowers visited only once (GLM with Poisson error distribution, $n = 20$ moths, $z = 0{\cdot}26$, 25 d.f., $P = 0{\cdot}79$). We observed eggs both inside the corolla tube, usually on the ovary, or outside of the corolla tube, on the protruding stigma branches or on petals, whereby these different egg positions could be observed for the same individual female moth.

Both male and female moths spent significantly less time on the flowers visited late in the sequence, suggesting increasing satiation (effect of flower position in the visitation sequence; Table 4a). Moth sex and flower sex interacted significantly to determine the time spent on flowers (Table 4a). To dissect this interaction we therefore ran the minimal model separately for male and female moths (Table 4b,c). Male moths spent a significantly longer time on male flowers than on female flowers (91·08 ± 9·26 s per flower vs. 56·22 ± 5·14 s respectively; LMM with only male moths, $t = 3{\cdot}61$, 216 d.f., $P < 0{\cdot}001$; Table 4b). In contrast, for female moths the time spent on female (56·4 ± 3·13 s, $n = 20$ moths) and male flowers (65·59 ± 4·79 s, $n = 20$ moths) did not differ significantly (LMM with only female moths, factor flower sex, $t = 0{\cdot}78$, 318 d.f., $P = 0{\cdot}43$), despite the fact that they usually displayed both feeding and oviposition behaviours on female flowers (see above).

Discussion

We studied nursery pollination in *H. bicruris–S. latifolia* and specifically asked whether the male insects provide pollination service, affecting the net mutualistic benefits of this inter-

Table 4. Minimal adequate model (linear mixed model with moth identity as random factor) for the effect of predictor variables on the time spent on flowers. (a) The initial model was first run for all moths (n = 38 moths; n = 576 observed visits), then for the subset of (b) male moths (n = 18 moths; n = 236 observed visits) and (c) female moths (n = 20 moths; n = 340 observed visits)

Variable	Effect size	SE	d.f.	t	P
(a) All moths					
Moth sex	−0.19	0.13	36	−1.5	0.144
Flower sex	0.07	0.09	535	0.7	0.475
Flower position in the visitation sequence	−0.02	0.01	535	−3.2	0.001
Moth sex : Flower sex	0.39	0.15	535	2.6	0.009
(b) Male moths					
Flower sex	0.45	0.13	216	3.6	4E–04
Flower position in the visitation sequence	−0.03	0.01	216	−2.7	0.008
(c) Female moths					
Flower position in the visitation sequence	−0.015	0.01	319	−2.2	0.028

Effect sizes shown are for male moths, male flowers and male moths : male flowers.

action. Our results indicate that both male and female moths are efficient pollinators of *S. latifolia* (Fig. 3). Under our experimental conditions, male and female moths both contributed to pollination, with approximately 80% of visits to a female flower leading to fruit initiation, with on average half (45%) of the available ovules being fertilized after a single visit. Although our data were gathered under controlled experimental conditions, the observed pollination efficiency is within the range of that observed in the field: in a total of 122 developing fruits collected in nine field populations, the average per population ranged between 37% and 79% of ovules fertilized (A.M. Labouche, L. Faucqueur, B. Parra Sanchez & G. Bernasconi, unpublished data).

Pollination efficiency and fruit initiation were affected by pollen load on the moth (significant effect of distance to last male flower and trend or significant effects respectively, for the total number of male and female flowers visited before the focus flower), indicating pollen carry over. Due to random alternation between male and female flowers (every one to two visits), this suggests that pollen loads often contains the pollen of several males, consistent with multiple paternity in naturally pollinated fruits (Teixeira & Bernasconi 2007). Pollen carry over and the resulting multiple paternity may be beneficial for the plant, if more pollen donors increase offspring fitness (Marshall & Ellstrand 1986; Paschke, Abs & Schmid 2002; Bernasconi, Paschke & Schmid 2003; Bernasconi *et al.* 2004; Teixeira, Foerster & Bernasconi 2009).

Ripe seeds developed from flowers in which only feeding behaviour was observed (notably, male-visited flowers). Although in some rare cases we only observed oviposition behaviour and seed development also followed, our results indicate that feeding alone is sufficient to promote pollen transfer in this system. As female moths did not fertilize a higher proportion of ovules in a single visit than male moths, despite usually displaying both behaviours, our results also suggest that oviposition does not increase pollination efficiency. Mechanistically, that feeding behaviour alone is suffi-

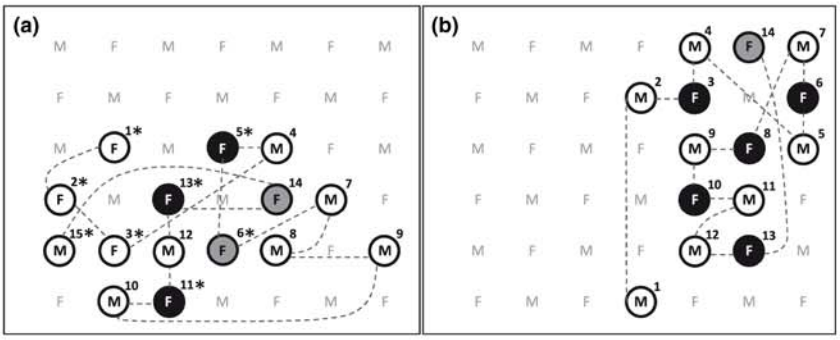

Fig. 3. Representative visitation sequences, and costs/benefits of visitation for one female (a) and one male (b) moth. In each test, we displayed 21 female (F) and 21 male (M) flowers at randomized positions. Visited flowers are highlighted (bold outline). (a) Costs of female visitation arise through nectar feeding (not shown; feeding was observed in 82·77 ± 3·04% of the visited female flowers) and through egg laying (marked by an asterisk). Benefits arise through pollination efficiency as a function of pollen load, which in turn depends on the distance between the focus female flower and the last visited male (indicated by shading). Black: female flowers visited immediately after the last-visited male flower (expected pollination efficiency 53·7%); grey: female flowers visited second after the last-visited male flower (expected pollination efficiency 35·1%); not shown in this figure: female flower visited after two or more intermediate female flowers from the last-visited male flower (expected pollination efficiency 18·6%); white: female flower visited prior to a visit to a male flower (expected pollination efficiency 0%). Costs and benefits of female moth visitation to male plants are defined by analogy, and under natural conditions will depend additionally on the siring success within each visited female flower (as a function of multiple pollinator visits (Teixeira & Bernasconi 2007) and timing of visitation (Burkhardt, Internicola & Bernasconi 2009). (b) Male moth visitation incurs benefits of pollination to both plant sexes with similar efficiency as for female moths. Black: female flowers visited immediately after the last-visited male flower (expected pollination efficiency 57·3%); grey: female flowers visited second after the last-visited male flower (expected pollination efficiency 28·8%); not shown in this figure: female flower visited after two or more intermediate female flowers from the last-visited male flower (expected pollination efficiency 7·3%); white: female flower visited prior to a visit to a male flower (expected pollination efficiency 0%). Costs arise through nectar feeding but there are here no costs of egg laying. Feeding was observed on average in 89·75 ± 3·07% of the flowers visited by male moths. Further refinements of costs estimates depend on factors such as the probability of successful egg and larval development, feeding duration, and pollination-induced floral wilting.

cient for pollen transfer is also consistent with the exserted position of both stigmas and anthers in *S. latifolia* flowers, which easily come into contact with the moth body during feeding (A.M. Labouche, personal observation).

These findings differ from those in other nursery pollination systems. In the Yucca/Yucca moth or in some of the fig/fig wasp systems, active pollination is carried out by the female insect following oviposition (Pellmyr *et al.* 1996; Pellmyr 1997; Jousselin & Kjellberg 2001; Cook *et al.* 2004). *Greya politella* passively pollinates the hermaphroditic flowers of *Lithophragma parviflorum* during oviposition and feeding rarely leads to seed production (Pellmyr & Thompson 1992). Also in *Silene dioica*, pollination by *Perizoma affinitatum* is ensured passively by the female probing with its abdomen both male and female flowers before ovipositing in female flowers only (Westerbergh 2004). However, similarly to our findings, in the interaction between *Trollius europeaus* and *Chiastocheta*, male flies also contribute to pollination: pollination is ensured passively by both sexes which enter the globeflower, where they mate and females also lay eggs (Despres 2003). This indicates that male-derived pollination benefits may occur in different, although potentially a minority of associations, and it would thus be interesting to explore the role of male insects across more systems, including other *Silene/Hadena* species pairs. Ultimately, this should help to understand why in some systems males contribute to pollination benefits, while in others pollination depends on oviposition (through active pollination for example) and males play no role.

Theoretical developments that highlight the role of interspecific population regulation for the stability of mutualisms (Holland, Deangelis & Schultz 2004) not only do not yet include male-derived benefits, they also do not yet include male-derived benefits, and therefore most closely apply to systems without male pollinators. However, male-derived pollination benefits that are free of oviposition costs (Fig. 3) are likely to modify the benefit and cost functional responses in some of these associations such as *Silene* and *Trollius*, which may be further modified by the effects of variable insect sex ratios. Available models also assume hermaphroditic plants, and do not directly apply to dioecious host plants. This neglects the possibility that male and female plants may evolve sexually dimorphic strategies (e.g. for flower production). *Silene latifolia* is well known for sexual dimorphism (Delph & Meagher 1995; Delph, Knapczyk & Taylor 2002). However the contribution of mutualistic and antagonistic components of biotic interactions to selection on sexually dimorphic traits is still unresolved. Despite the ability to distinguish flower sex (Brantjes 1976b), in our experiment male and female moths were equally likely to visit male and female flowers and switched randomly between them. This is consistent with the fact that both moth sexes are efficient pollinators, because if they would specialize on one plant sex, this would compromise pollen transfer. However, both male and female moths fed for a significantly longer time on male flowers, which have richer nectar than female flowers (Shykoff & Bucheli 1995; Witt *et al.* 1999). While fruit initiation and pollination efficiency showed only a trend ($P < 0{\cdot}10$) to increase with the time spent on the visited female flower, we did not investigate whether the time spent on male flowers correlates with pollen export. It would thus be interesting to explore in future studies whether the increased nectar quality of male flowers represents a male strategy to increase pollen export. In our experiment, all plants had only one flower. However, under natural conditions male plants have larger floral displays (Delph & Meagher 1995) and receive more visits than female plants (Shykoff & Bucheli 1995), and future studies under field conditions should explore how this dimorphic trait influences the distribution of pollen uptake and download (Harder & Johnson 2005).

Without accounting for oviposition, over all visits, male moths ensured fertilization of an average of 1051ovules and females 1530 ovules. However, visits by female moths to female flowers resulted in egg laying in about 79% of the visits (Fig. 3). Depending on egg/larval survival, the net pollination benefits conferred by the female moth may thus decrease dramatically. To resolve whether the outcome is mutualistic or antagonistic, would require a comprehensive estimate of total costs and benefits over the entire interaction and thus on a longer time scale and under field conditions. Several factors can play a role in determining the net benefits. On one hand, not all eggs hatch successfully (Burkhardt, Delph & Bernasconi 2009) and plants respond to infestation with increased fruit abortion (Jolivet & Bernasconi 2006, Burkhardt, Internicola & Bernasconi 2009; Elzinga & Bernasconi 2009). On the other hand, larvae usually consume more than one fruit on the same plant (Biere & Honders 1996). Also, visitation patterns might differ if moths were observed throughout their entire lifetime. Future field studies should explore the effects of these different components of the interaction, as well as the role of potential co-pollinators.

Because of the presumably high costs imposed by seed predation, and as our results show that pollination is mainly achieved through feeding behaviour, it would be of particular interest to examine if and which floral traits elicit or can constrain oviposition behaviour. In the present study, fewer than 10% of the female flowers visited by female moths escaped oviposition. The question arises of whether flowers that escape oviposition differ morphologically from flowers that are used for oviposition and what determines this. The conditional probabilities to observe both feeding and oviposition behaviours were very close to the predictions for statistically independent events for both male and female flowers, consistent with the idea that these two behaviours are elicited or constrained by different (independent) floral traits. Although only very few eggs were laid in male flowers, moths also displayed oviposition behaviour (insertion of abdomen into floral tube) in about 27% of the visited male flowers, suggesting that there is some degree of overlap between male and female flowers for traits associated with oviposition behaviour.

In conclusion, our study shows that both females and males of *H. bicruris* provide pollination benefits in the interaction with *S. latifolia*. As our results also show that plants of both sexes are attractive to moths of both sexes, this raises the

question of whether pollination by male moths under field conditions compensates, at least partly, for the loss of seeds due to seed predation following visits by female moths and how this affects the net benefits of the interaction to the plant. Like male insects, co-pollinating species may also provide pollination without imposing at the same time costs due to oviposition. However, co-pollinators can reduce the relative benefit of being pollinated by the seed predator, leading to a purely antagonistic outcome of the nursery pollination system (Thompson & Cunningham 2002). Conversely, if the male of the seed predator insect increases the net benefits to the plant, this is likely to affect the strength of the interspecific population regulation between the seed predator and the plant (Holland, Deangelis & Schultz 2004). This indicates that the role of males in at least some species of pollinating, seed-eating insects needs to be taken into account when analysing the evolutionary stability of such mutualisms.

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