

Variable nursery pollinator importance and its effect on plant reproductive success

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Abstract Nursery pollination, in which insects use as hosts the very plants they pollinate, ranges from obligate mutualism to parasitism. In the non-obligate interaction between *Greya* moths and the host *Lithophragma* sp., the relative density of nursery pollinators and copollinators, which do not use plant tissues for larval development, is a key determinant of the interaction's outcome. *Silene* (Caryophyllaceae) nursery pollination by *Hadena* moths (Lepidoptera: Noctuidae), studied primarily in Europe, is considered antagonistic because copollinators comprise a substantial proportion of the pollinator community. However, there are few studies that ascertain the direction of the *Silene*–*Hadena* interaction by taking into account both pollinator service and seed predation. Here, we report a novel comprehensive evaluation of the direction of the interaction between North American *Hadena ectypa* on *Silene stellata*, by comparing the relative contributions of

nursery and copollinators to *S. stellata* pollination and relate this to variation in fruit predation and reproductive success of *S. stellata* across multiple sites and years. *Hadena ectypa* pollinator importance (pollen deposited/visit/h) varied between years, resulting from variable visitation rate. Copollinator importance was higher than *H. ectypa* in 1 year and equivalent in another. In two of three sites, lowered *H. ectypa* activity was not correlated with a significant decrease in plant reproductive success, indicating a negative interaction. Although pollinator service by *H. ectypa* is substantial in this system, copollinators' service is at least as great, and when the cost of fruit predation is factored in, the net effect of the interaction is parasitism of host plants.

Keywords Mutualism · Antagonism · *Silene* · *Hadena* · Copollinator

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Introduction

The geographic mosaic theory of coevolution provides a framework linking species interactions within and between populations to the expected geographical distributions of traits relevant to coevolution of the interaction (Thompson 2005). Much of the conceptual impetus for the geographic mosaic theory of coevolution emanates from empirical observations and results from the *Lithophragma*–*Greya* facultative nursery pollination system. Nursery pollinators use their host plant tissues as a “nursery” for the growth and development of their larval offspring. Recent work in facultative nursery pollinator systems has focused on the variable nature of the outcome of the interaction between the plant and the nursery pollinator partners, while characterizing the ecological factors responsible for the sign

switch (e.g., +/- to +/+) in order to quantify the conditions that promote the evolution of mutualisms (e.g., Thompson and Fernandez 2006; Thompson et al. 2010). Population dynamics models suggest that density of the partners (Holland and DeAngelis 2009) and third parties can destabilize pairwise interactions and may even contribute to transitions between mutualism and parasitism (Bronstein et al. 2003). Copollinators are such third parties that, as is the case for *Lithophragma* (Thompson and Cunningham 2002), may determine the evolution of traits resulting from selection due to the antagonistic or mutualistic interaction. A first step towards understanding the relevance of copollinators to the spatial or geographic distribution of coevolutionary hotspots is by robust empirical evaluation of their effect (measured in multiple sites and years) on the direction or outcome of the interaction. In this way, the geographic and temporal structure of the interactions between the focal species can be identified to determine the importance of space and time in generating a mosaic of evolutionary outcomes or whether the populations seem to be fixed in their interaction directions (Thompson 1999).

The facultative interactions between *Silene* spp. and their nursery pollinators present an opportunity to quantify the ecological factors that may tip the balance between parasitism and mutualism. Despite the potential for the *Hadena*–*Silene* interaction to contribute to a broader understanding of mutualism evolution, we have relatively little information on the contribution of *Hadena* to pollination and subsequent fruit production of *Silene* compared to their co-occurring mutualistic copollinators (reviewed in Kephart et al. 2006). Much is known of the parasitic effects of seed predation by *Hadena* on *Silene* fruit production, mostly from Europe, but less is known about the positive effects of *Hadena* moths through pollination on plant reproduction. *Silene* produces lilac aldehyde-laced fragrances that are especially important in attracting *Hadena* (Dotterl et al. 2006, 2007). However, once pollinated, the release of floral scent quickly ceases, suggesting this is a mechanism for the plant to limit egg-laying by *Hadena* (Muhlemann et al. 2006). *Hadena* larvae have also been implicated as selective agents in the evolution of flowering time traits and plant mating system through fruit predation (Biere and Honders 1996; Collin et al. 2002; Wright and Meagher 2003). The successful invasion of the European *Silene latifolia* in North America has been attributed to the absence of their associated European *Hadena* spp. (Wolfe 2002), indicating the negative impact these seed predators have on host plant fitness in their native range.

In prior work, the relative frequency of *Hadena* nursery pollinators compared to copollinators has been documented (e.g., Jurgens et al. 1996; van Putten et al. 2007; Gimenez-Benavides et al. 2007), and pollinator effectiveness has

been quantified for *Silene vulgaris* (Pettersson 1991). These studies demonstrate that the frequency of *Hadena* relative to copollinators can be highly variable, and, within a single population of *S. vulgaris* in Sweden, the four *Hadena* spp. were a minor and relatively inefficient group of pollinators compared to the more abundant and effective moth copollinators (Pettersson 1991). Specifically, we need to know the relative contribution of *Hadena* nursery pollinators and copollinators to pollination and how reproductive success is related to presence or absence of the nursery pollinator across sites and between years. These data have not been reported for any *Hadena*–*Silene* system, and they may prove useful for generating hypotheses related to whether the population distribution of coevolutionary outcomes is likely to be a mosaic and whether traits important for the interaction outcome are predicted to be under fixed or variable selection intensity.

Our main objective is to determine whether the nursery pollinator *Hadena ectypa* has an overall positive or negative effect on the reproductive success of its host, *Silene stellata*, and whether the outcome varies among years or sites or within flowering seasons. These data can help qualitatively assess whether this particular *Hadena*–*Silene* interaction fits the hypotheses and framework of the geographic mosaic theory of coevolution. From a more classical perspective, in order to determine the sign of the interaction, it is necessary to remove one partner from the system and observe the fitness response in the remaining partner (Janzen 1985). For the *S. stellata*–*H. ectypa* interaction, if plant fitness declines when *H. ectypa* is removed from the system then the interaction is positive, suggesting that copollinators are not important for plant reproduction. We performed this test by taking advantage of site and year differences in *H. ectypa* density and *H. ectypa* and copollinators' pollinator importance and compared maternal plant fitness among these site/year combinations. Because it is expected that nursery and copollinator density may vary from year to year and site to site, and given the important bearing this variation may have on *H. ectypa*'s effect on *S. stellata* plant reproduction (negative or positive), an assessment of the sign of the interaction would be incomplete without a comprehensive evaluation of the spatial and temporal variation in the relative densities of copollinators and nursery pollinators. This is also the first detailed study of the *Hadena*–*Silene* interaction in North America, allowing future comparisons to European systems (e.g., Brantjes 1976a, b; Pettersson 1991; Bopp and Gottsberger 2004). Specifically we asked: (1) Is pollinator importance of *H. ectypa* and the moth copollinators, taking into account both effectiveness and frequency of pollination, variable between pollinator type and between years? (2) Does the relative density of *H. ectypa* adult moths and copollinators vary within and between flowering seasons at

one site? (3) Is spatial and temporal variation in *H. ectypa* density related to fruit and seed production in *S. stellata*?

Materials and methods

Study system

Populations of the iteroparous perennial *Silene stellata* and its pollinators were studied near the University of Virginia's Mountain Lake Biological Station (MLBS) in the Southern Appalachian Mountains in Giles County, VA, during the 2004–2008 flowering seasons using plants at three sites: Meadow (37°20'53"N, 80°32'41"W, elevation \approx 1,100–1,300 m), Woodland (37°21'20"N, 80°33'14"W, elevation \approx 1,100–1,300 m), and Wind Rock (37°24'50"N, 80°31'10"W, elevation \approx 1,300 m). All three sites were located within 10 km of one another. The flowers of *S. stellata* are protandrous and bowl-shaped, with white, fringed petals that are presented horizontally. Plants lack basal rosettes, but they produce one to many reproductive stems that emerge in early spring. The flowers form a panicle inflorescence at the terminal ends of the reproductive stems with flowering occurring from early July through early September. The following data from *S. stellata* plants at the Meadow site are representative of the distribution of plant height and flower production, which gives an upper limit to the number of fruit per plant. In 2006, using a sample of 111 plants, the minimum, median, and maximum of the average height of the several stems per plant were 28, 82.5 and 126 cm, respectively. Also in 2006, the minimum, median and maximum number of flowers produced per plant was 1, 44, and 323, respectively.

Silene stellata is specialized for nocturnal moth pollination, and the nocturnal visitors of *S. stellata* include the noctuid moths *Hadena ectypa* (Fig. S1 of Electronic Supplementary Material), *Amphipoeaea americana*, *Feltia herelis*, *Autographa precatonis*, and *Cucullia asteroids* (see Fig. S2 of Electronic Supplementary Material), the arctiid *Halysidota tessellaris*, and the notodontid, *Lochmaeus manteo* (Reynolds et al. 2009). The secondary diurnal visitors are primarily halictid bees (Hymenoptera: Halictidae), syrphid flies (Diptera: Syrphidae), and bumblebees (*Bombus* spp.), and when these diurnal visitors do pollinate, they are of minor importance relative to the nocturnal pollinators (Reynolds et al. 2009). Population level outcrossing rate was relatively high (73%), and was measured in 2006 for plants of the Meadow site (Reynolds 2008).

Adult male and female moths nectar in the flowers of *S. stellata*. The egg-laying behavior of *H. ectypa* (see Fig. S3 of Electronic Supplementary Material) follows

nectaring, as female moths position the distal end of their abdomens inside the flower and oviposit on the surface of the nectaries (at base of ovary) or ovary wall (personal observation). Larvae were collected from two sites ($n = 52$) and reared to pupation in the laboratory, and 10 adult *H. ectypa* emerged, providing proof that indeed *H. ectypa* is a nursery pollinator. In the 2006 egg census of 418 flowers at the Meadow site (see below), the number of eggs per flower ranged between 0 and 24 with a median of one egg per flower and mean (SD) of 1.3 (2.2).

In the field, soon after the egg is laid, the *H. ectypa* larva hatches, bores into the ovary and begins consuming immature seed or ovules. Larvae are often found feeding on immature seed with half their bodies inside the fruit and the other half outside the fruit tucked between the fruit wall and the outer calyx. It is not uncommon to observe larvae on the stems of plants, presumably moving between fruit (pers. obs.). We have never observed larvae consuming non-reproductive tissues such as leaves and stems, nor have we observed any evidence of larval damage on leaves or stems. Larvae collected from plants in the field and reared in the laboratory required a mean (SD) of 36 (3.8) immature fruit to reach the pupal stage ($n = 11$, range 32–46). Given that the median flower number at the Meadow in 2006 was 44 flowers per plant (a typical size across years), if multiple larvae are present on a plant they may have to move between plants to complete development. Developmental stage of the fruit is important because young larvae will not eat seed that have become hardened (personal observation). Fruit that have been consumed by *H. ectypa* larvae are noted by a conspicuous exit hole left in the hardened ovary wall, the presence of frass, and the complete absence of seed or ovules. Flowers that have been damaged can be recognized by the complete consumption of the ovary before fruit set. *Hadena ectypa* larvae have been documented feeding on the fruits of *S. vulgaris* in Massachusetts (Nelson 2011), thus it is possible that more than one alternative *Silene* host exists. Current studies are being performed to determine *H. ectypa*'s affinity to other *Silene* spp (Castillo et al. unpublished data).

Hadena ectypa and copollinator effectiveness

At the Meadow site (2004–2006), to determine whether pollinator effectiveness varies by type of pollinator, the amount of pollen deposited per visit was estimated by counting pollen grains on previously unvisited stigmas, which were collected after visitation by moths. To ensure the flowers were unvisited, cages were placed over an entire plant until mature female flowers were observed. Stigmas collected from unvisited flowers were used as controls. The total number of samples collected was 68, 57, and 141 for the copollinators (CP), *H. ectypa* (HE) and

unvisited stigmas (NV), respectively, and the number of samples per year for CP, HE, and NV were: 17, 13, and 51 for 2004; 43, 0, and 30 for 2005; and 8, 44, and 60 for 2006. The previously unvisited flowers were observed with video cameras (night shot option Sony Digital Handycams: model #TRV17) for at most 2 h and were visited by available pollinators at the site during the time interval. After the observation interval, flowers were collected and transported to the laboratory at MLBS, and stigmas were removed and fixed in fuschin glycerine jelly on microscope slides (Beattie 1971). The video camera recordings were then viewed, and the visitor type, *H. ectypa* or copollinator, or if a moth failed to visit, was noted. If multiple visits (44 of 125 observations) were recorded during the 2-h interval of a given trial, the number of grains deposited during the observation interval was divided by number of visits to obtain average pollen deposition per visit. Moths were identified by looking for key distinguishing features such as the narrow white lateral line of the forewing and egg-laying behavior for *H. ectypa* and generally larger body size for the copollinators (Fig. S1, S3, S2, respectively, Electronic Supplementary Material). Additionally, nectaring behavior differences were noted as *H. ectypa* typically held their wings at rest and the copollinators fluttered. We noted these features based on our field experience observing moths directly in the adult moth density sampling (see next section), and in non-sampling activities.

A general linear model (GLM) was used to model pollen grains per stigma as the response and type of pollinator or control as the predictor. Significant differences among treatment means were analyzed by comparing all three treatments with one another (option pdiff = all from the GLM).

Adult moth density variation

To investigate within- and between-year temporal variation in relative densities of adult *H. ectypa* and copollinators, adult moth densities were estimated in 2005 and 2006 across the flowering period of *S. stellata* at the Meadow population. To calculate adult moth densities, the number of moths observed contacting flowers were counted in patches of 10 plants on each night of sampling. Censusing was conducted when enough plants were flowering to ensure that we could find upwards of 20 patches each with 10 flowering plants (about 7 days after initial flowering in the population). Each patch represented an area where the observers (no more than two) could see all moths on *S. stellata* flowers during each observation period, which lasted for roughly 10 s per plant. This method precluded observations when *S. stellata* flowering density was low, at both the beginning and end of the seasons, but does allow us to estimate relative frequencies when most flowers were

pollinated. Patches of plants were sampled after dusk while walking along predefined transects for a distance of up to 180 m, and then returning to the starting point along a second, but parallel, transect. At each patch, 1 of the 10 plants was randomly chosen, and the number of open flowers was counted. Thus, we obtained the average number of flowers open on a given night, by averaging across patches. Patches were haphazardly chosen along the transects, but the same patches were never sampled in consecutive nights. Sampling was not done in inclement weather (rain, drizzle) when moths do not fly. Headlamps with a red light were worn, which increased our visual acuity over white light and did not disturb moth behavior (personal observation). In 2005, on average (SE) 19.5 (1.4) patches were observed per night on 11 sample dates spanning the flowering period from 19 July to 11 August. In 2006, 19 (1.2) patches were observed per night on 14 sampling dates from 17 July to 13 August.

Because scatter plots of within season change in the densities of copollinator and *H. ectypa* moths indicated complex relationships between density and date of sampling, non-parametric regression was used to fit the model, $\text{density} = f(x) + \varepsilon$, where x is number of days since 1 January, and $f(x)$ is some unknown function that interpolates the values of x . The interpolation function is estimated by penalized least squares (Green and Silverman 1994) and the analyses were implemented with the TPSPLINE procedure (all statistical models were run with SAS, v.9.1.2; SAS Institute 2004). Four separate models were estimated: one for each pollinator type (nursery and copollinators) and year (2005, 2006).

Using this sampling scheme allowed us to estimate the average number of *H. ectypa* moths versus copollinator moths per flower during a 10-s interval and it was four-fold useful. First, we have a standardized and near instantaneous measure of the mass of moths in a patch of plants for *H. ectypa* moths and copollinators in 2005 and 2006 that we can use to investigate the within-season change in the relative densities of copollinators and *H. ectypa*. Second, we can estimate the relationship between *H. ectypa* egg density and adult density in order to develop a proxy for adult density. If we can find a relationship between egg and adult density then sampling eggs with flowers is a more practical way to assess *H. ectypa* adult density. Third, we can transform our instantaneous density measurements into copollinator and *H. ectypa* visitation rates as visits per flower per hour. In order to perform this transformation, we multiplied the density values, calculated separately for *H. ectypa* and copollinators across all patches within each sampling date, by 10 (seconds of direct observation per plant) \times 6 (10 s intervals min^{-1}) \times 60 (min h^{-1}) = 3,600 to obtain visitation rates. This approach yielded 11 observations of visitation rate each for copollinators and *H. ectypa*

in 2005 and 14 in 2006. To test if this extrapolation was accurate, we compared the mean visitation rate in 2005 ($n = 22$) and 2006 ($n = 28$) of *H. ectypa* + copollinator (i.e., the nocturnal pollinators) to the mean and 95th percent confidence intervals of our direct camcorder observations of nocturnal moth pollination in 2005 and 2006, which is published in Reynolds et al. (2009). Finally, we were able to calculate and make statistical comparisons of pollinator importance between copollinators and *H. ectypa* within and between years according to Reynolds and Fenster (2008).

H. ectypa egg density variation

To estimate the proportion of flowers with eggs, which reflects the population and year-specific probability that a flower contains an egg, a single flower was collected from multiple plants haphazardly chosen along transects at each of the three sites (Meadow, Woodland and Wind Rock) for 4 years (2005–2008). As the flowers progress to female stage after 24 h, we collected flowers that had been in female phase for two nights in order to standardize the time available for female moth oviposition. Presence or absence of eggs was determined by examining the flowers under a dissecting scope, the morning after the flower had experienced its second night in the female phase. The proportion of flowers with eggs was estimated as the number of flowers with eggs divided by the number of flowers sampled. During 2005, on average (SE) 84 (18), 106 (3.2) and 57 (14) flowers were sampled on 4, 3, and 3 dates across the flowering season at the Meadow, Woodland and Wind Rock sites, respectively. In 2006, on average (SE) 32 (3.1), 59 (18), and 43 (7) flowers were sampled 21, 4, and 4 times at the Meadow, Woodland and Wind Rock sites, respectively. During 2007 on average (SE) 46 (2.5), 32 (8), 35 (4) flowers were sampled 20, 6 and 4 times at the Meadow, Woodland, and Wind Rock, respectively. In 2008, on average (SE) 34 (4), 30 (9), and 38 (3) flowers were sampled 8, 3, and 4 times at the Meadow, Woodland, and Wind Rock, respectively. The sampling date in 2008 was delayed due to later flowering initiation. Although fewer samples were collected at the Woodland and Wind Rock sites than at the Meadow site, nonetheless the sampling allowed us to assess *H. ectypa* egg-laying activity across the entire season at these two sites. The proportion of flowers with eggs was modeled as a binomial response (logit link function) and site, year and site \times year interaction as predictors using the GENMOD procedure in SAS. Orthogonal contrasts were used to test whether the differences between means among the treatment levels were significantly different from zero. For the year effect, with four levels (2005–2008), we first tested the hypothesis that mean egg density was significantly different between

earlier (2005–2006) or later years (2007–2008) because different investigators collected the data in the time periods. Next, differences were tested within the earlier (2005 vs. 2006) or later (2007 vs. 2008) categories. Mean differences in egg density among sites was tested by first comparing the Meadow to the Woodland and second the Meadow and Woodland together versus the Wind Rock site. As with the adult density data, a non-parametric regression function was estimated to determine the relationship between egg density and date of sampling for the combined 2005–2008 samples for each site using the statistical software package (R development core team 2009).

H. ectypa egg density as a predictor of variation in *H. ectypa* adult density

To test the hypothesis that egg density is a predictor of adult moth density, a regression model was fit with square-root transformed adult density data as the response and egg density as the predictor for the 2005 and 2006 seasons using the REG procedure in SAS. Performing the square-root transformation linearized the relationship between adult density and egg density. The data used to test the hypothesis were adult density and egg density sampled the same day or adult density data collected within 1 day of the egg density sampling date. Four observations met these criteria in 2005 and 12 in 2006.

Others have also used egg presence and absence to infer whether a pollinating seed predator is mutualistic or antagonistic (e.g., Thompson and Fernandez 2006), and in the *S. stellata* system, there is ongoing research to determine the relationship between the number of *H. ectypa* eggs laid in all flowers within a given individual and resultant plant reproductive success (Kula et al. unpublished).

Spatial and temporal variation in fruit and seed set

To determine the sign of the interaction and whether copollinators could compensate in terms of fruit and seed production in the absence of *H. ectypa*, all mature fruit, including reproductive units that did not set seed, were collected from *S. stellata* plants near the end of flowering in mid-August from three sites, Meadow, Woodland and Wind Rock, for 2 years. Individual plants were haphazardly chosen while walking transect lines at the three sites in 2005 and 2006. We sampled 122 plants in 2005 and 111 in 2006 from the Meadow site. Thirty-three plants were sampled in 2005 and 29 in 2006 from the Woodland site. Twenty-three plants were sampled in 2005 and 29 plants were sampled in 2006 from the Wind Rock site. The fruits were scored in the laboratory for successful fruit set (fruit with mature seed), number of seeds and whether a fruit had

been eaten by *H. ectypa* larvae. Flowers that had been eaten are readily determined by the presence of a calyx, and absence of corolla and pistil. The GLM procedure was used to model fruit set (number of fruits setting seed/total number flowers), seed set (number of seed per fruit), and proportion of flowers eaten as the response variables and site, year and year by site as the predictors. Pairwise differences among all treatment level means (ls means option, pdiff = all) were compared.

Results

Hadena ectypa and copollinator effectiveness

Results from the general linear model demonstrate the pollinator type or control (no visits) were significant predictors of pollen grain deposition (effectiveness) on stigmas ($F = 13.94$, $df = 2, 265$, $P < 0.0001$). Data were pooled across years as a GLM demonstrated year ($F = 0.29$, $df = 2, 124$, $P = 0.7488$) was not a significant predictor of pollen grains on stigmas for the nocturnal pollinators. The mean (SE) pollen grain deposition was 69 (8) *H. ectypa*, 78 (8) for copollinators, and 34 (5) for unvisited stigmas (average ovule number per pistil is 25 ± 0.4 ; Reynolds et al. 2009). There was no significant difference between *H. ectypa* and copollinator effectiveness ($P = 0.7274$), but *H. ectypa* ($P = 0.001$) and copollinators ($P < 0.0001$) were both significantly different from the control (unvisited).

Adult moth density variation and pollinator importance

We observed a total of 7 adult *H. ectypa* and 69 moth copollinators and 75 *H. ectypa* and 57 moth copollinators in 2005 and 2006, respectively. These moths were counted and confirmed by sight by observing a total of 234 patches (10 plants per patch) across 11 sampling dates in 2005, and 265 patches across 14 sampling dates in 2006. Within the 2005 *S. stellata* flowering season, average adult *H. ectypa* and copollinator moth density ranged between 0–0.00089 and 0–0.0040 moths per flower, respectively. Within the 2006 season, average *H. ectypa* and copollinator density ranged between 0–0.013 and 0–0.0098 moths per flower, respectively. While these numbers appear low, they represent the mass of moths available at any given time for flower visitation at the Meadow site. For example in 2005, out of 10,000 open flowers, at any instant we would expect a maximum of nine *H. ectypa* moths and 40 copollinators on nights of peak activity. By contrast, in 2006, we would expect a maximum of 130 *H. ectypa* and 98 copollinators.

Non-parametric regression analysis indicated that copollinator density increased across the flowering season in

2005 and *H. ectypa* was uniformly low and unchanged across the flowering season (Fig. 1a). By contrast, in 2006, *H. ectypa* density decreased and copollinator density increased across the flowering season (Fig. 1b).

Extrapolating the adult density data for *H. ectypa* and copollinators across the 2005 and 2006 flowering periods, we found that the mean (SE) visitation rate (visits/flower/h) for both copollinators and *H. ectypa* together were 0.48 (0.11) and 0.89 (0.22) for 2005 and 2006, respectively. These means and standard errors completely overlap the means and standard errors reported in (Reynolds et al. 2009, see Fig. 2 in that paper). Thus, we are confident that our estimates from the adult density data in this study are consistent to those from direct observations using cam-corders, published in our previous study.

In order to determine whether *H. ectypa* is actually an important pollinator compared to the copollinators in terms of delivering pollen grains to the stigmatic surface, we multiplied the mean visitation rates of copollinators and *H. ectypa* with their respective pollinator effectiveness estimates to obtain mean pollinator importance and then calculated the confidence limit on the mean using the procedure explained in detail in Reynolds and Fenster (2008). In 2005, *H. ectypa* pollinator importance was significantly lower (based on non-overlapping 95% confidence limits) than *H. ectypa* in 2006. The pollinator importance of copollinators was greater than the pollinator importance of *H. ectypa* in 2005, but similar in 2006 (Fig. 2).

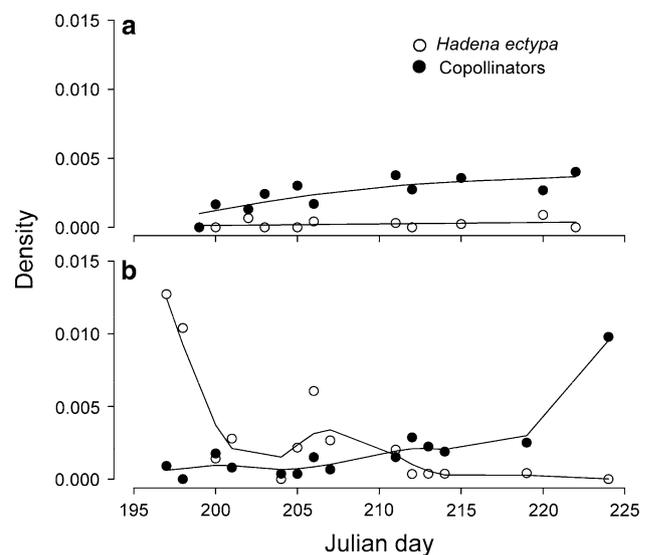


Fig. 1 Temporal changes in adult *Hadena ectypa* and copollinator densities (number of moths observed per flower) across the flowering period of *Silene stellata* at the Meadow site in **a** 2005 and **b** 2006. Lines are predicted curves fit with penalized regression splines

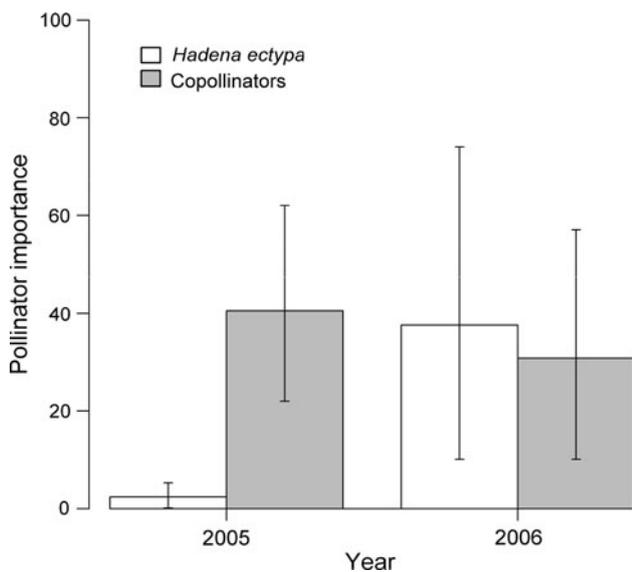


Fig. 2 Mean ($\pm 95\%$ confidence limits) pollinator importance (pollen grains deposited \times visit $^{-1} \times$ h $^{-1}$) of *H. ectypa* and copollinators in 2005 and 2006

H. ectypa egg density variation

To estimate the proportion of flowers with eggs, we collected a total of 3,680 flowers across the 4 years and three sites of study. The results of the linear model fit to the egg density data from the 4 years and three sites indicated that site ($F = 18.21$, $df = 2$, 72 , $P < 0.0001$) and year ($F = 7.71$, $df = 3$, 72 , 0.0002) were significant predictors of egg density, but the interaction between site and year ($F = 1.89$, $df = 6$, 72 , $P = 0.09$) was only marginally statistically significant (Fig. 3). Overall, egg density was lowest in 2005 and lowest at the Wind Rock site. Orthogonal contrasts between the levels of the site variable indicated that egg density was not significantly different between the Meadow and the Woodland ($F = 0.27$, $df = 1$, 72 , $P = 0.67$), and egg density was significantly higher at the Meadow and Woodland than Wind Rock ($F = 35.26$, $df = 1$, 72 , $P < 0.0001$). Orthogonal contrasts indicated that together egg densities in 2005 and 2006 were not significantly different than 2007 and 2008 ($F = 0.19$, $df = 1$, 72), although 2005 was significantly lower than 2006 ($F = 22.39$, $df = 1$, 72 , $P < 0.0001$), and 2007 and 2008 were not significantly different ($F = 0.09$, $df = 1$, 72 , $P = 0.76$).

Figure S4 (Electronic Supplementary Material) contains the scatter plots and non-parametric regression functions pooled across the 4 years of study. These plots demonstrate that egg density declined across the season at the Meadow, was uniformly moderate at the Woodland and relatively low at Wind Rock.

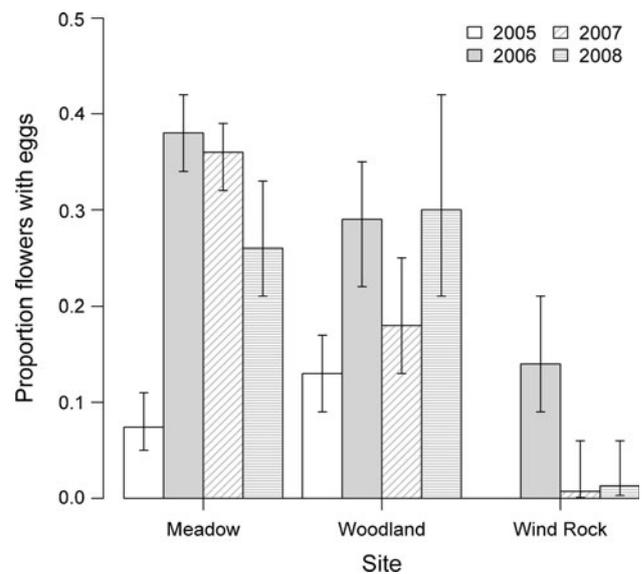


Fig. 3 Variation in the probability that flowers sampled have at least one *H. ectypa* egg across sites and years. Bars means \pm SE

H. ectypa egg density as a predictor of variation in *H. ectypa* adult density

The probability of finding eggs in flowers was a significant predictor of adult *H. ectypa* density at the Meadow site ($F = 13.64$, $df = 1$, 14 , $P = 0.002$, $R^2 = 0.49$).

Spatial and temporal variation in fruit and seed set

Site ($F = 18.85$, $df = 2$, 341 , $P < 0.0001$), year ($F = 6.89$, $df = 1$, 314 , $P = 0.009$) and the year \times site interaction ($F = 11.74$, $df = 2$, 314 , $P < 0.0001$) were significant predictors of fruit predation. Figure 4a shows the results of all pairwise contrasts between treatment levels. Notably, fruits eaten by seed predators and flowers with eggs were not sampled from Wind Rock in 2005 indicating the absence of *H. ectypa*. However, fruit predation was observed at Wind Rock in 2006. Fruit predation and the presence of eggs were also observed at the Woodland and Meadow populations in 2005 and 2006.

Year ($F = 29.96$, $df = 1$, 341 , $P < 0.0001$), year \times site ($F = 3.32$, $df = 2$, 341 , $P = 0.0374$), but not site ($F = 1.26$, $df = 2$, 314 , $P = 0.2853$) were significant predictors of fruit set. Figure 4b shows the results of all pairwise contrasts between treatment levels.

Year ($F = 17.63$, $df = 1$, 314 , $P < 0.0001$), site ($F = 19.28$, $df = 2$, 314 , $P < 0.0001$), but not the site \times year interaction ($F = 1.24$, $df = 2$, 314 , $P = 0.2906$) were significant predictors of seed set. Fig. 4c shows the results of all pairwise contrasts between treatment levels.

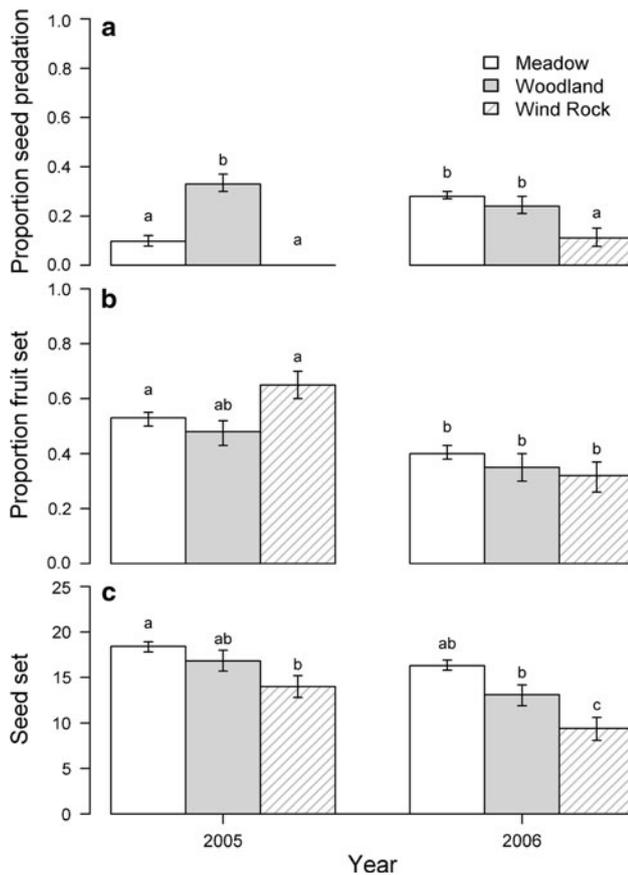


Fig. 4 Variation in **a** fruit predation, **b** fruit set and **c** seed set of *Silene stellata* across the three sites in 2005 and 2006. Bars sharing the same letters are not significantly different at the family-wise $\alpha = 0.05$ level

Discussion

We have quantified the relative importance of *Silene stellata* pollination by *Hadena ectypa* and associated copollinators, measuring plant reproductive success, fruit predation and all the while estimating the relative density of the pollinators within and between populations and among years. The results demonstrate that the interaction between *H. ectypa* and *S. stellata* is parasitic, but we have suggestive evidence that, under certain conditions (e.g., high *H. ectypa* density and early in flowering), the interaction may tend towards mutualism. Two arguably short-sighted approaches to quantifying the outcome of the interaction between *H. ectypa* and *S. stellata* are (1) to directly measure the ratio of ovules matured to seed as a result of pollination to those eaten by larval seed predators, and (2) indirectly evaluate the relative costs and benefits by measuring reproductive success in the absence of the focal partner. However, it is now widely recognized that these approaches are lacking. For one, interactions are contextual, not strictly pairwise, in that they occur among a

community of species that may affect the interaction outcome (Stanton 2003). Also, third partners can have an important impact on the spatial and temporal structure of the population dynamics of the partners (Bronstein et al. 2003). Furthermore, interaction outcomes are not constant among the distribution of populations across the species range and through time (Thompson and Cunningham 2002; Thompson and Fernandez 2006; Thompson et al. 2010). Theory and empirical work has suggested that the density of the interacting partners is tantamount to the direction of the interaction (Thompson and Pellmyr 1992; Holland and DeAngelis 2009). We detail below how our own empirical results of *S. stellata* nursery pollination by *H. ectypa* amid a community of moth copollinators relate to theory and results from other study systems on the coevolution of interactions and the conditional sign changes of the interaction's direction.

Because *H. ectypa* and the other nocturnal moth copollinators are equally effective pollinators in terms of pollen grain deposition onto stigmas, any difference in their total contribution to pollination (pollinator importance) is due to differences in their visitation rates. In both years of study, copollinators were common relative to *H. ectypa*, and clearly in 2005, when mean (LCL, UCL) copollinator importance [38 (10, 74)] was 16 times higher than *H. ectypa*'s [2.4 (0.15, 5.3)], nursery pollination is antagonistic for *S. stellata*. However, in 2006, *H. ectypa* and copollinator mean (LCL, UCL) importance were equivalent [copollinator: 31 (10, 57); *H. ectypa*: 38 (10, 74)] making it more difficult to determine the sign of nursery pollination for *S. stellata*. Given that the mean ovule load for a *S. stellata* flower is 25 (Reynolds et al. 2009), it is probably not unreasonable that (at the Meadow) a moth is able to deposit enough pollen for full seed set in one visit, and assuredly multiple visits in the course of the night could result in full seed set (Reynolds et al. 2009). Thus, our calculations of pollinator importance indicate when the interaction may turn positive. When the density of *H. ectypa* is much greater than copollinators (e.g., early in flowering 2006), most of the benefit to plant reproduction is attributable to nursery pollination. Furthermore, since both male and female *H. ectypa* visit the flowers of *S. stellata* and the sexes appear to have similar pollinator effectiveness, as in the European *H. bicruris* and *S. latifolia* (Labouche and Bernasconi 2010), one may consider the benefit of males to plant reproductive success to partially blunt the negative effects of fruit predation due to female *H. ectypa*. These ideas are the subject of current investigation for *H. ectypa*–*S. stellata* nursery pollination.

Although there may be certain restricted conditions when the nursery pollination interaction is positive, on average nursery and copollinator importance was equivalent when *H. ectypa* density was at its highest in 2006.

Thus, the cost to the plant of partnering with *H. ectypa* in terms of lost fitness due to fruit predation can be substantial in flowering seasons when *H. ectypa* is abundant. Each *H. ectypa* larvae consumes between 30–40 flowers and/or unhardened fruit, again demonstrating that the cost to *S. stellata* individuals of interacting with *H. ectypa* can be severe. This laboratory estimate may be overstated as larvae in the field are likely to require fewer seed resources to complete development and thus the actual cost to plants in nature may be smaller especially if copollinator density is low. However, we were able to directly estimate the fruit predation rates from the *S. stellata* plant reproduction study. Fruit predation rates were ~10% in 2005 and 30% of total fruits produced in 2006, which reflect the variable adult *H. ectypa* populations observed in those 2 years. In another non-obligate system, *H. bicurris* lays its eggs on female plants of dioecious *S. latifolia* where each pistil has hundreds of ovules and the larvae also eat more than one fruit (but many fewer than *H. ectypa*) to complete their development (Labouche and Bernasconi 2010). Thus, our data indicate that in years like 2006 when pollinator service between copollinators and *H. ectypa* is equivalent, heavy larval seed predation turns the nursery pollination interaction negative. We conclude from the pollinator importance study that *H. ectypa* adult activity is negatively associated with female reproductive success of *S. stellata* even when present at the highest observed average density.

Another line of evidence that the interaction is negative comes from the fruit and seed sampling study across the three sites. The regression demonstrating adult *H. ectypa* to be a significant predictor of *H. ectypa* eggs per flower allows us to use presence and absence of eggs as a proxy of *H. ectypa* adult activity. Thus, the low level of *H. ectypa* activity in 2005 compared to 2006 at the Wind Rock and Meadow sites allows us to address the question of what happens to plant reproductive success when *H. ectypa* is absent or at reduced density in the systems. At Wind Rock, seed and fruit set significantly declined when *H. ectypa* entered the system, and we would have expected the opposite if the relationship were a mutualism. Likewise at the Meadow site, fruit set significantly declined and, although not a significant difference, seed set was also reduced when *H. ectypa* density (and pollinator importance) increased. That copollinators at the Wind Rock and Meadow sites were able to compensate for the absence of *H. ectypa* yet again leads us to argue that the interaction is parasitic.

Our results discussed thus far provide comprehensive evidence across populations and years that the effect of *H. ectypa* on *S. stellata*'s female reproductive success in North America is negative and support earlier findings from investigations of species occurring in Europe (e.g., Brantjes 1976a, b; Pettersson 1991; Bopp and Gottsberger

2004). It is tempting to propose that this study system does not fall under the rubric of the geographic mosaic theory (GMT) of coevolution because the interaction direction is negative and fixed among sites and years. By contrast, the GMT suggests a more fluid dynamic of interaction sign changes and the full spectrum of outcomes among populations in a geographic setting and through time. We note that our three study sites were all located within 10 km of each other, hardly “geographic,” and therefore future studies will encompass populations from the entire species’ range. We also recognize that testing the direction of the interaction by quantifying maternal fitness components as measured by seed set is only part of the story, since pollination by nursery and copollinators may have different outcomes on seed quality (Herrera 2000; Kula et al. unpublished). We also recognize that results from the site and year plant reproduction study do not present the full description of the interaction between *S. stellata* and *H. ectypa* because *H. ectypa* pollinator density varies across the flowering season (Figs. 1 and 4; Kula et al. unpublished).

The substantial within-season, -site and -year variability in nursery pollinator and copollinator density that we document here could quite possibly translate to variation in individual plant net reproduction. This is a current topic of investigation in our laboratory (Kula et al. unpublished data). What we do know from theory of pairwise interactions is that perturbations in density of partners can have consequences for the outcomes of pairwise interactions (Holland and DeAngelis 2009). We can consider in the context of Holland and DeAngelis (2009) what may drive a possible mutualistic outcome for *Silene–Hadena* to a parasitic one. One can imagine that, in a particular flowering season, a large increase in density of *H. ectypa* moths, which exist in relative isolation from the copollinators early in flowering, may result in a shift from a potential mutualistic coexistence with *S. stellata* to overexploitation and complete reproductive failure of early flowering *S. stellata*. However, a key variable in these nursery pollinator systems is the divergent life histories: often the plants are iteroparous and the insects have short lifespans (Bronstein et al. 2003), as also seen in our study system. Therefore, it is likely that the plant partners could absorb large fluctuations in the moth’s density by virtue of the plant’s longevity. These theoretical considerations suggest that the sign of the interaction between *S. stellata* and *H. ectypa* may be a function of the within- and between-season variability in the relative density of the *H. ectypa* and associated copollinators, which we have extensively documented.

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