European Skipper Butterfly (*Thymelicus lineola*) Associated with Reduced Seed Development of Showy Lady's-slipper Orchid (*Cypripedium reginae*)

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It has been suggested that European Skipper butterflies (*Thymelicus lineola*) trapped in the lips of the Showy Lady's-slipper orchid (*Cypripedium reginae*) may interfere with pollination. This could occur through blockage of the pollinator pathway, facilitation of pollinator escape without pollination, and/or disturbance of the normal pollinators. A large population of the orchid at an Ottawa Valley site provided an opportunity to test the interference hypothesis. The number of trapped skippers was compared in 475 post-blooming flowers with regard to capsule development and thus seed development. The presence of any skippers within flowers was associated with reduced capsule development ($P = 0.0075$), and the probability of capsule development was found to decrease with increasing numbers of skippers ($P = 0.0271$). The extent of a negative effect will depend on the abundance of the butterflies and the coincidence of flowering time and other factors. Counts of skippers trapped in flowers were found to follow closely a negative binomial distribution ($P = 0.8656$).

Key Words: *Cypripedium reginae*; *Thymelicus lineola*; Showy Lady's-slipper; orchid; European Skipper; butterfly; pollination; pollinator interference; negative binomial distribution; Ottawa Valley

Introduction

The potential for pollination interference by European Skippers (*Thymelicus lineola* Ochsenheimer) caught in the lips of Showy Lady's-slippers (*Cypripedium reginae* Walter), has been alluded to by a number of authors (e.g., Catling 1974; Barrows 1983; Vogt 1990) and repeated with additional references by Argue (2012). The usual pollinators are leaf-cutter bees (*Megachile* spp.) and syrphid flies (*Syrphus* spp.), which enter the slipper-shaped lip but cannot exit the same way as a result of its inflexed margin (Argue 2012). They first have to pass the stigma, where they deposit any pollen they are carrying and then exit by one of two openings at the base of the lip where they receive a new load of pollen on the thorax.

The flowers attract insects by a combination of odour and colour (white and pink, with pink nectar guides), but there is no nectar reward, as with other species in the genus, which are also deceptive (e.g., Argue 2012). Escape of skipper butterflies entering the lip is prevented by the inflexed margin; they are also unable to escape through the basal openings, as they are too delicate and high, with their wings closed over the top of the body. Entrapment of skippers may lead to reduced pollination by disturbance of the normal pollinators; possible mechanisms include blocking the pathway and/or facilitating pollinator escape without pollination. The effect may be substantial: Vogt (1990) found skippers in about a third of the flowers at his Vermont site, Barrows (1983) and Catling (1974) reported skippers in about half of 100 flowers at several Ontario sites.

The European Skipper was first introduced to North America in 1910 (Hall *et al.* 2014), so it has likely not evolved in the presence of the deceptive orchid flower, which might have led to avoidance. The European Skipper is now one of the most abundant butterflies in North America.

At Purdon Fen (44.99260°N, 76.54596°W) near Lanark in eastern Ontario, we had an unusual opportunity to evaluate the impact of the European Skipper butterfly on capsule (and seed) development of the Showy Lady's-slipper orchid. This orchid has an extensive distribution in northeastern North America (Luer 1975), but there are few, if any, locations where it is as abundant as at the Purdon site where the total population of stems within 1.2 ha has fluctuated between 15 735 in 1985 (Mosquin 1986) and 7367 in 2015 (Ross Fergusson, personal communication). In 2015, the skippers, which generally frequent open country, were much more abundant than usual in the wooded area surrounding the fen and had entered the wooded fen where the orchids occur.

This area is a semi-open EasternWhite Cedar (*Thuja occidentalis* L.) woodland with an understory dominated by Tussock Sedge (*Carex stricta* Lam.). During a visit to the site on 17 June 2015, we saw at least 300 skippers in 30 minutes, flying within the orchid colony, and some were trapped in the flowers (Figure 1). By

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10 July, the flowers had turned brown with development of ovaries into capsules or had more or less withered. The former had been pollinated but the latter had not (Figure 2). Because most flowers had not completely deteriorated, it was possible to open the brown lips and note the presence of skippers and count them (Figure 3). This we did to test the hypothesis that the presence of any skippers was deleterious, and the more skippers there were in a flower, the greater the likelihood of interference and the lower the likelihood of capsule development.

FIGURE 1. Fresh Showy Lady's-slipper (*Cypripedium reginae*) flower with one European Skipper (*Thymelicus lineola*) trapped inside and one trying to enter the flower, Purdon Fen, eastern Ontario, 2015. Photo: Peter W. Hall.

FIGURE 2. Shrivelled Showy Lady's-slipper (*Cypripedium reginae*) flowers, one with developed (right) and one with undeveloped capsule, Purdon Fen, eastern Ontario, 2015. Photo: Peter W. Hall.

FIGURE 3. Opened shrivelled Showy Lady's-slipper (*Cypripedium reginae*) flower with seven trapped European Skippers (*Thymelicus lineola*), Purdon Fen, eastern Ontario, 2015. Photo: Peter W. Hall.

Methods

Purdon Fen is protected and managed by the Mississippi Valley Conservation Authority (MVCA). We obtained permission to gather data; MVCA has shown much interest over two decades in accumulating information that would help to inform management (Mosquin and Brown 2006).

We sampled 475 flowers in an extensive area accessible from the boardwalk and considered to be representative of the entire colony. Within this area, we selected flower stalks approximately 1 m apart. This area included flowers that opened before the peak abundance of skippers as well as those that opened after. We avoided a small area where hand pollinations had been done to increase fecundity. We examined flowers and scored capsules as developed, partly developed, or completely undeveloped (with empty brown shrivelled capsules). For statistical purposes, we compared numbers of undeveloped capsules with the combined total of fully and partly developed capsules.

Each flower was opened and the number of skippers inside counted. We also examined 100 undamaged individual skippers to determine their sex and whether they carried any lady's-slipper pollen. Males were distinguished by a characteristic (but sometimes inconspicuous) horizontal black stigma on the forewing.

Our main study hypothesis — that there was less capsule development with skippers present and less with more skippers — was tested statistically using tests of proportions and logit modeling. Also of interest was the possibility of gender bias among counted skippers, which was tested via a one-sample test of proportions. We also considered the distribution of numbers of skippers within flowers and whether it followed either a Poisson or negative binomial model.

All statistical analyses were performed using the R statistical computing language (R Core Team 2015). The packages MASS (Venables and Ripley 2002) and binom (Dorai-Raj 2014) were used for maximum likelihood estimation of the negative binomial distribution and to calculate Bayesian credible intervals, respectively.

Results

Of 475 flowers examined, 398 (83.8%) had no capsule development and 77 (16.2%) had partly to fully

developed capsules. Members of each group with various numbers of trapped skippers are indicated in Table 1. Of the plants with no skippers trapped in flowers, 24.3% had developed capsules, compared with plants where skippers were present, of which 13.9% had developed capsules. A two-sample test of equality of proportion of developed capsules for plants both with and without skippers present was rejected in favour of the alternative that this proportion was less when skippers were present ($P = 0.0075$). A logit model of the probability of capsule development, with number of skippers as the covariate, showed a significant effect for number of skippers ($P = 0.0271$; one-sided because our alternative hypothesis was directional; we tested the null hypothesis of no effect of skipper number versus the alternative of a detrimental effect). The probability of capsule development decreased with number of skippers (estimated logit model parameters: intercept −1.37, slope −0.152). Model-predicted probabilities are plotted in Figure 4, together with sample estimates and their associated 95% Bayesian credible intervals (Jeffrey's prior) as a measure of their uncertainty.

Table 1. Number of European Skippers (*Thymelicus lineola*) in developed and non-developed capsules of Showy Lady's-slippers (*Cypripedium reginae*) at Purdon Fen near Lanark, eastern Ontario in 2015.

No. skippers	Capsule not developed	Capsule developed
0	81	26
1	104	24
2	85	9
3	57	10
4	33	1
5	23	3
6	7	2
7	4	0
8	2	1
9	1	0
10	0	1
11	0	0
12	1	0

Considering only the counts of skippers trapped in flowers, we tested the hypothesis that the total count (Table 1) follows a Poisson distribution. This would happen if the rate of entrapment was constant and did not depend on the number of skippers already trapped and if all flowers were exposed to skippers for equal amounts of time. After collapsing skipper counts of seven or larger because of small expected values, a goodness-of-fit test ($P < 0.0001$; likelihood ratio) rejected this simple model.

In our sample, the mean number of skippers per flower was 1.94 with a variance of 3.28; thus, skippers were over-dispersed relative to the Poisson distribution, which requires mean and variance to be equal. A common model for over-dispersed count data is the two-parameter negative binomial distribution. Maximum likelihood estimation of its parameters gave a mean of 1.94 and a dispersion parameter of 2.87. After collapsing skipper counts of nine or more because of small expected values, goodness-of-fit testing did not reject this model ($P = 0.8656$; likelihood ratio), but instead resulted in a very close fit to these data. A frequency distribution of the observed skipper counts together with Poisson and negative binomial fitted values is provided in Figure 5.

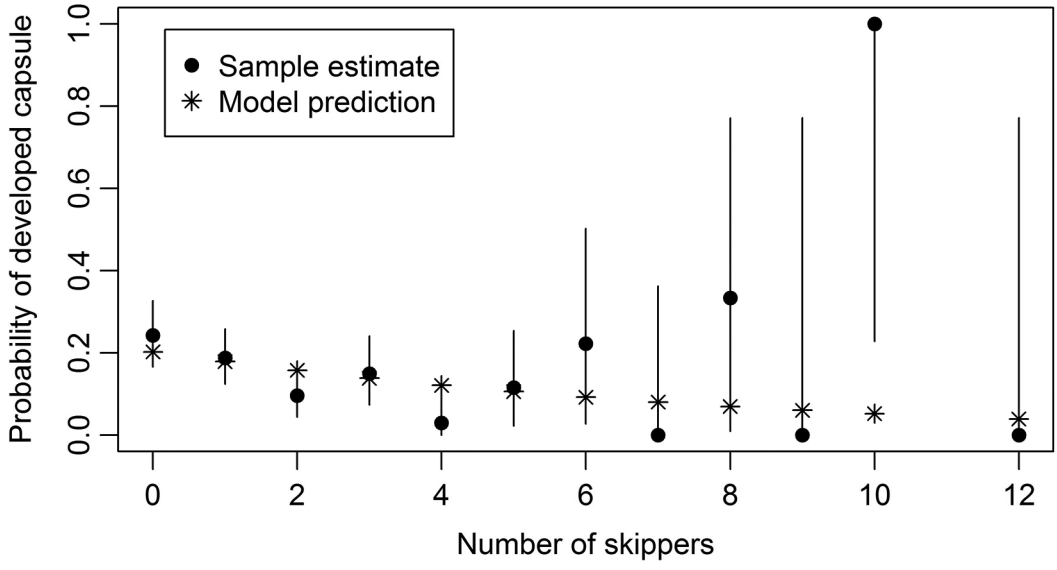

FIGURE 4. Model-predicted probability of capsule development in Showy Lady's-slipper (*Cypripedium reginae*) depending on the number of entrapped European Skippers (*Thymelicus lineola*).

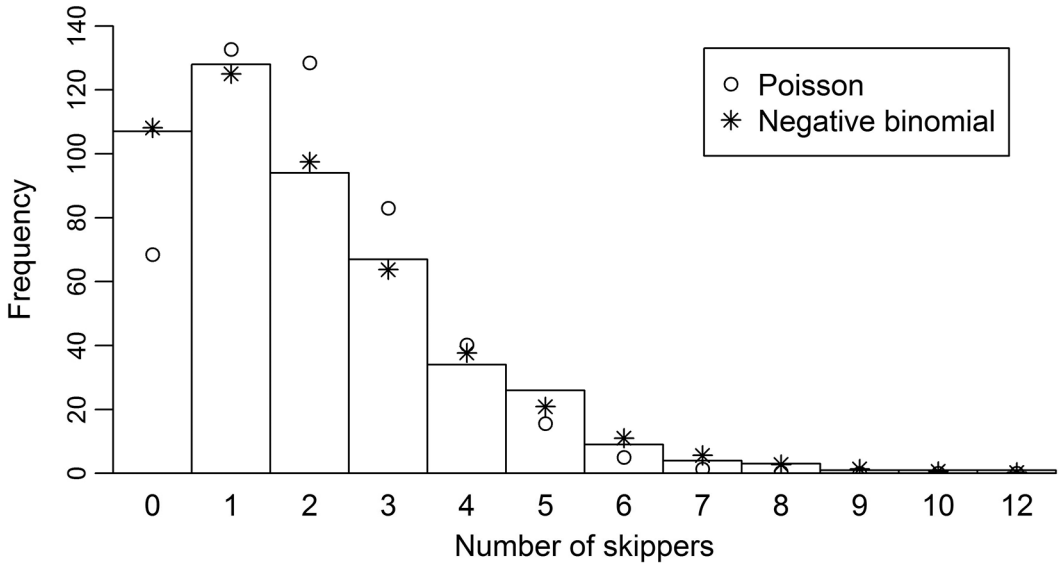

FIGURE 5. Distribution of the observed number of European Skippers (*Thymelicus lineola*) trapped in Showy Lady's-slippers (*Cypripedium reginae*), showing both fitted-model Poisson and negative binomial model counts.

Of the sample of 100 trapped skippers with an undamaged upper forewing, 92 were males and the difference in proportion of sexes was highly significant ($P < 0.0001$). Only three of 100 butterflies carried anything that looked like a pollen smear. In each case, it was on the smooth surface (by complete loss of scales) of the dorsal thorax, and microscopic examination revealed it to be stigmatic tissue. Only one monad characteristic of *Cypripedium* pollen was seen.

Discussion

In this paper, we have shown that capsule development in Showy Lady's-slipper orchids at Purdon Fen in spring 2015 was decreased when European Skipper butterflies were trapped inside the flowers and, also, that the probability of development decreased with the number of skippers trapped. We have also shown that the distribution of trapped skippers closely followed a negative binomial distribution and that there was strong gender bias in entrapment, males being predominant.

The 16.2% of orchids with capsule development in 2015 is smaller than the 22.7% with capsule development recorded at the study site by Mosquin in 1985 (Mosquin 1986: 42, Table 3). However, this decline should not necessarily be attributed to interference by skippers, because many factors can account for differences in fecundity from year to year, such as late frosts killing pollinators and/or flowers and population sizes of pollinators. We note, however, that the percentage of orchids with developed capsules, which had no entrapped skippers, was 24%; thus, the 1985 percentage, being slightly smaller than this, might be achievable in a year with very few skippers. In 2015, there was an unusually high population of European Skippers in the area, as determined by qualitative observations, so, they might have had a higher than normal impact on capsule development. Comparisons with other years could be an interesting follow-up study.

The idea that productivity in 2015 might be compared with that of past years when skippers were likely less abundant is of interest, but constrained by a lack of historical data and by a lack of information on many factors that might affect seed development from year to year. If additional risk factors are proposed, then the logit modeling in this paper could be extended.

We considered the possibility that an entrapped skipper could encounter an anther in its attempts to escape and then transfer pollen to the stigmatic surface of the same flower. This could lead to an expanded ovary with seeds. However, we have no evidence of this happening based on examination of the surfaces of trapped skippers for a pollen smear. The single monad found may have been left on the stigma by an earlier pollinating bee and then rubbed by the skipper onto its thorax. The results of the data analysis also argue against regular within-flower pollen transfer because the deleterious effect on pollination increases with the number of trapped skippers, whereas it would likely decrease with increased trapped skippers available to transfer pollen if there was within-flower pollen transfer.

Because skipper counts were negatively associated with capsule development, we examined their distribution and found the number of trapped skippers to follow a negative binomial distribution very closely. A well-

known property of this distribution is that it is equivalent to the counts being Poisson distributed with rate parameter randomly distributed according to a gamma distribution. Thus, if this decomposition is valid for the Showy Lady's-slipper, skipper counts are Poisson distributed at a flower-varying rate. Future analyses of counts may be amenable to Poisson or negative binomial regression analysis, if potential explanatory factors can be identified.

We found an extreme gender bias toward males among entrapped skippers. The likely explanation, although not sustainable by evidence in this case, is that males of most butterflies emerge before females and more likely overlap with the peak lady's-slipper flowering period.

This work is the first to associate European Skipper butterflies with a negative impact on capsule and, therefore, seed production in the Showy Lady's-slipper orchid. It is also the first time that the potential importance of this phenomenon has been suggested because of the widespread occurrence of trapped skippers.

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