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Martha A. Case
William & Mary

Zachary R. Bradford
William & Mary

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Enhancing the trap of lady's slippers: a new technique for discovering pollinators yields new data from *Cypripedium parviflorum* (Orchidaceae)

MARTHA A. CASE* and ZACHARY R. BRADFORD

Biology Department, College of William & Mary, Williamsburg, VA 23187, USA

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Approximately one-third of orchid species offer no reward to their floral visitors and instead trick them into pollination. Typically, these deceptive systems have low visitation and fruiting rates because pollinators can learn to avoid non-rewarding species. Consequently, pollination ecology studies in these species often require long hours in the field to witness relatively few floral visitations relative to rewarding plants. *Cypripedium parviflorum* is a food-deceptive orchid with a pouch-like trap that temporarily imprisons pollinators. To escape, pollinators exert pressure on the stigma which facilitates pollination and widens the escape holes located near each anther. This study reports the use of a ribbon and clip to block the escape passageway of this species in order to retain and observe visiting insects. The device was tested in a large population and was shown to increase significantly the probability of observing floral visitors by nearly three-fold. Ten species of hymenopteran visitors in the families Andrenidae, Apidae, Halictidae and Megachilidae were observed, with two female *Adrena tridens* and one male *Adrena perplexa* successfully removing pollen. Insect visitation to the orchids occurred during the first half of the flowering period and was significantly associated with warm, clear days. © 2009 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2009, 160, 1–10.

ADDITIONAL KEYWORDS: *Adrena* – Cypripedioideae – food deceptive – Hymenoptera – orchid – pollination – ribbon – solitary bees.

INTRODUCTION

The bizarre and complex pollination ecology of the orchid family (Orchidaceae) has fascinated naturalists for over 100 years (Darwin, 1862; van der Pijl & Dodson, 1966; van der Cingel, 2001). Particularly intriguing is the fact that nearly one-third of orchid species offer no apparent reward to their pollinators, instead tricking them into pollination by offering a bogus reward, such as food or sex (Schiestl, 2005). Some researchers have suggested that pollination by deceit is one of the key elements in understanding the floral and species diversity of the family (Cozzolino & Widmer, 2005).

Despite the long-standing interest in the potential for pollinators to contribute to the reproductive isolation of plant populations, the evolutionary

mechanisms in operation are still poorly understood (Nilsson, 1992; Waser, 1998; Tremblay *et al.*, 2005; Peakall, 2007). One factor that is critical for understanding how plants respond to pollinator selection pressures is the ability to observe floral visitation and to identify pollinators. Deceptive orchids, however, can be particularly problematic for studies in pollination ecology because insects can learn to avoid additional encounters with non-rewarding plants (Heinrich, 1975; Dafni, 1984; Nilsson, 1992; Cozzolino *et al.*, 2005; Internicola *et al.*, 2006). This often leads to lower visitation rates and fruit production (Nilsson, 1992; Peakall & Beattie, 1996; O'Connell & Johnston, 1998; Bänziger, Sun & Luo, 2005; Tremblay *et al.*, 2005; Jersáková, Johnson & Kindlmann, 2006; Jersáková *et al.*, 2008) and reduces the likelihood of observing pollination and identifying pollinators.

With few exceptions, such as self-pollinating species, the slipper orchids (subfamily Cypripedioideae) are

*Corresponding author. E-mail: macase@wm.edu



Figure 1. A–C. *Cyripedium parviflorum* var. *pubescens* from the experimental plot showing lip morphology and trapping device. A, Side view of bloom showing large entrance orifice (right arrow) and escape orifice partially occluded by an anther (left arrow). B, *Andrena perplexa* individual inside the labellum with the ribbon covering the exit holes. C, *Andrena barbara* escaping from an exit hole into mosquito netting after the ribbon was removed.

food-deceptive orchids that attract adult insects that are seeking food for themselves or a brood-place for larvae (Pridgeon *et al.*, 1999). In this subfamily, the highly modified petal (the labellum) is modified into an inflated pouch with an entrance orifice and two basal escape orifices, each partially occluded by an anther (Fig. 1A). Visitors enter the labellum through the large entrance orifice and, if they are the right size and shape to be pollinators, they have difficulty escaping out of the entrance. Usually not more than 10 min after imprisonment (Nilsson, 1979), pollinators crawl beneath and press up against the stigma. This action transfers any pollen they may be carrying to the stigmatic surface and gives them leverage to widen the exit hole (Nilsson, 1979). Subsequently, the insect squeezes out of one of the two exit holes, picking up a new mass of pollen. For pollination to occur, a pollinator must be tricked into entering and correctly exiting at least two flowers.

Like other deceptive orchids (for example, Peakall & Beattie, 1996), pollination studies in the genus *Cyripedium* L. frequently require many hours in the field to witness pollination or even to find insects trapped in the labellum. For example, Li *et al.* (2006) required 47.5 h of observation and two field seasons to observe five pollinators enter the labella of *C. tibeticum* King ex Rolfe. The problem of infrequent observations is compounded by the common occurrences of sparsely populated or relatively small *Cyripedium* populations (as in many North American sites) and dense but often inaccessible populations in China (where two-thirds of known *Cyripedium* species occur; Bänziger *et al.*, 2005). Together, these factors have undoubtedly contributed to a dearth of data on *Cyripedium* pollination. For only nine of approximately 45 *Cyripedium* species have pollinators been studied (Bänziger, Sun & Luo, 2008). Virtually all studies in the genus have required extensive observation times, dense clumps of flowers to monitor or large sample sizes of flowers to obtain pollinator

data (for example, Stoutamire, 1967; Nilsson, 1979; Catling & Knerer, 1980; Sugiura *et al.*, 2001; Bänziger *et al.*, 2005, 2008; Herring, 2007; Li *et al.*, 2008). To help combat the difficulties associated with pollination studies in *Cyripedium*, the major goals of the present study were: (1) to develop a field method that would increase the chances of observing visitors and pollinators of lady's slipper orchids; (2) to apply the method to *C. parviflorum* Salisb. var. *pubescens* (Willd.) Knight; and (3) to analyse the capture data for insight into climatic conditions that could lead to a better prediction of pollinator activity. These objectives were met by designing a system that modifies the trapping device of the flower. This system retains potential pollinators in the labellum until the experimenter observes the insect and allows it to complete the pollination process. With the increase in captured visitors caused by the trapping device, sample sizes were large enough to permit a statistical analysis that correlated climatological variables with insect visitation.

MATERIAL AND METHODS

FIELD AND LABORATORY METHODS

The experimental site was located in the College Woods, a mixed hardwood forest on the campus of the College of William & Mary in Williamsburg, Virginia (USA). The site was situated near the top of a gently sloping ravine and was not shaded by anything other than the co-occurring vegetation. The canopy at the site was dominated by *Fagus grandifolia* Ehrh. and *Liriodendron tulipifera* L., and the most common understorey tree was *Ilex opaca* Aiton. *Arisaema triphyllum* (L.) Schott was blooming in the field site and *Podophyllum peltatum* L. was blooming a short distance away.

Over 70 putative genets of *C. parviflorum* var. *pubescens* were scattered across approximately 300 m² at

the site. Stems were assumed to be separate genets when the bases of their shoots did not appear to arise from a common point. Most genets were separated from each other by more than 1 m. The vast majority of genets contained zero to two flowering ramets, with the exception of one area in which five flowering stems were loosely scattered among many vegetative shoots, making it difficult to distinguish genets from ramets.

As shoots of the lady's slippers emerged, they were gently squeezed to determine the presence of a flowering bud. When in bud, flowering stems were numbered and the entire population was divided into nearest-neighbour pairs of flowering stems. Members of each pair were randomly assigned opposite treatments. Thus, genets containing two flowering stems were assigned both a control and a ribbon treatment. This design should have helped to randomize microsite and possible genetic variables associated with the population. Initial sample sizes were 49 in the treatment group and 50 in the control group. With the exception of a few late flowering individuals, all flowering stems in the population were assigned to either the treatment or control group.

Treatments and observations on each flower began as soon as the bud had opened fully (i.e. when the lateral petals and dorsal sepal were fully extended). For flowers in the treatment group, a 10 × 1 cm yellow ribbon was placed over the column and around the basal portion of the labellum, covering the exit holes associated with both anthers (Fig. 1B). The two ends of the ribbon were gently cinched up under the labellum with a small metal binder clip. At each visit to the field site, which occurred during daylight hours from approximately 07.00 h to 19.00 h Eastern Standard Time (EST), every flowering stem was systematically visited and examined for the presence of insects as well as the condition of the flower. On average, each labellum was checked once every 140 min (5.4 checks per day) from 17 April 2007 (when the first plant bloomed) to 4 May 2007. On the last date (4 May 2007), all buds had bloomed for the season and 83% of the flowers at the site had withered or were otherwise dysfunctional. With the exception of the few flowers still in bloom at the end of the experiment, insect capture data on each flower ceased when its flower was destroyed by insect or other damage, or when the tissue of the labellum became dry and collapsed. The population was revisited several times post-bloom to record the fruiting status of each bloom.

When a living insect was observed inside a labellum, a bag made of mosquito netting was placed over the entire flower and tied beneath the inflorescence with a twist-tie. In some cases, the flower needed extra support with the weight of the bag, and so a

bamboo support stake was placed in the ground next to the stem to support the bag, and the bag was tied around both the stake and the stem. In the treatment plants, the ribbon and clip were removed after the bag was in place, thereby freeing the exit holes. Anthers were checked for the presence of pollen, which could be seen with the unaided eye just below the rim of the anther near the exit hole, and the insects were then observed as they exited the flower. Exiting insects were classified into one of four categories: (1) squeezing out of the exit hole and removing pollen; (2) slipping beneath the anther without removing pollen; (3) exiting back through the entrance hole; or (4) dead in the labellum. When an insect escaped from the exit hole into the bag (Fig. 1C), the entire bag and insect were placed in a kill jar containing ethyl acetate. After several hours in the kill jar, insects were pinned promptly. In the laboratory, the insects were examined using a ×30 dissecting scope for the presence of *Cypripedium* and non-*Cypripedium* pollen (hereafter called foreign pollen). Insect measurements were also taken and included the length (from the insertion of the antennae to the tip of the abdomen), width of the widest point (either abdomen or thorax) and height of the thorax. Insect identifications were made by Sam Droege of the United States Geologic Survey, Patuxent Wildlife Research Center, Beltsville, MD, USA.

STATISTICAL METHODS

Contingency table *G*- and chi-squared tests (Sokal & Rohlf, 1995) were used to evaluate the association of experimental group with fruit set and the number of insects trapped in labella (hereafter referred to as captures). The association of trapped insects with experimental group was analysed in two ways. One analysis examined the number of plants in each experimental group with zero, one or two captures, and used a 2 × 3 chi-squared contingency table with two degrees of freedom. However, the probability of observing captures within an experimental group could be influenced by the total number of observations made on the group. Furthermore, as a result of differences in the number of days individual plants were in bloom, some plants had more observations than others. Therefore, a second method of analysis was conducted which examined the number of observations yielding captures, and employed the *G*-test to compare experimental groups. For purposes of testing the effectiveness of the ribbon barrier, all instances in which an insect was observed in the labellum but escaped during manipulations of the mosquito netting were counted as captures. These were treated in this manner because it was human error that resulted in the lost insect and not the inability of the plant to

trap an insect. Although the experiment ran from 17 April to 4 May 2007, capture data were truncated to cover only the range of dates when insects were found in the labella: 17–25 April 2007.

Climatological data were obtained for Williamsburg, VA, USA for 17 April to 4 May 2007 from Weather Underground (accessed in July 2008; <http://www.wunderground.com/>). These data included cloud conditions, temperature and wind speed in 20-min intervals from 07.00 h to 19.00 h EST. The focus of these analyses was to identify any differences in weather conditions during the capture of insects compared with conditions when no captures occurred. For all climatic investigations, experimental and control group captures were combined to analyse weather data for the entire population. Cloud conditions were divided into two categories, clear vs. the presence of clouds (i.e. scattered clouds, partly cloudy and overcast), and were analysed for associations with captures using *G*-tests. The temperature and wind speed of capture intervals were compared with conditions for intervals without captures using *t*-tests for unequal variances (SPSS, 2006).

Climatological associations with capture data were conducted for two major time periods: (1) within the first half of the flowering period (17–25 April 2007, when all insect captures were made); and (2) across the entire period of the experiment (17 April to 4 May 2007). For the second analysis, the number of intervals with ideal conditions for capture in the first half of the flowering period was compared with the number in the second half. In this *post-hoc* analysis, ideal conditions were defined as those associated with most captures in the first half of the flowering period (i.e. clear skies and in the range of temperatures previously associated with captures during clear sky intervals).

RESULTS

CAPTURE AND FRUIT SET DATA

Sample sizes in each group were slightly reduced as a result of the exclusion of stems that started to flower after all insect captures had ceased. In addition, three buds were destroyed before they flowered. This left 47 flowering stems in the control group and 44 in the treatment group. Twenty-five insect captures were made across treatment and control groups. Insect captures began when the first flowers initiated bloom (17 April 2007) and continued steadily through 25 April 2007, when all insect activity in the orchids stopped abruptly at peak flowering of the orchids (Fig. 2). The probability of observing 25 insects in the first half of the flowering period (2993 observations) and zero in the second half (3023 observations),

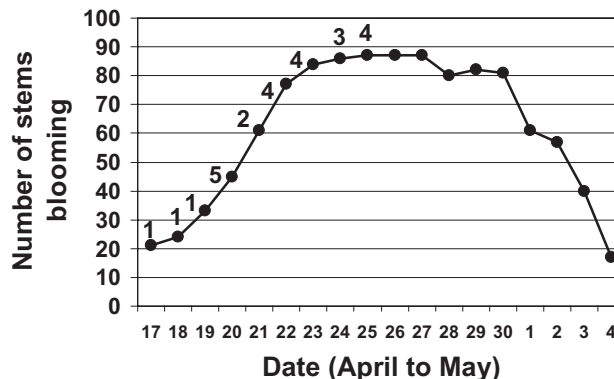


Figure 2. The number of stems blooming in relation to date and number of captures. The numbers of insects observed in labella are given above the plotted points on the curve.

assuming a random association of time interval and capture, was extremely low (2.7×10^{-8} ; Fisher's exact test).

The probability of finding a trapped insect for all observations in the treatment group (0.0129; 18 insects among 1394 observations; Table 1) was significantly higher ($G = 6.69$, $P < 0.01$) than that of finding a trapped insect for all observations in the control group (0.0044; seven insects among 1599 observations). In addition, the capture rate per plant for the treatment group (0.41; Table 1) was significantly higher ($\chi^2 = 6.13$, $P < 0.05$) than the capture rate for the control group (0.15). Fruit set was not significantly different between the control (14.8%) and treatment (18.2%) groups ($G = 0.18$, $P > 0.5$; Table 1).

Of the 25 insects captured in the labella, 18 were successfully transferred to kill jars, mounted and identified. Under a $\times 30$ dissecting scope, *Cypridium* pollen was seen as an orange–yellow mass immersed in a thick viscid secretion on the dorsal side of the thorax. Pollen grains were clumped together, even if the mass itself was spread out over the thorax. The pollen mass also severely matted dorsal thoracic setae on the insect, and this matting was never seen on an insect without *Cypridium* pollen. In contrast, foreign pollen was evident as a lighter coloured powdery dusting with non-sticky grains that could be present anywhere on the insect, but especially on the legs. All captured insects contained foreign pollen, but the amount varied from only a few perceptible grains on one *Adrena tridens* pollinator (captured on 22 April 2007 at 16.29 h) to copious amounts on another (captured on 21 April 2007 at 14.12 h).

The mounted insects represented four hymenopteran families and ten species. Fourteen of the 18 identified insects were female (Table 2). Nine individuals were observed to exit through an exit hole and

Table 1. Number and frequency of insects trapped in the labellum (i.e. captures) and fruit set data for control and treatment groups

Type of comparison	Control	Treatment (traps)	Test statistic and value; significance
By observations ($N = 2993$)			
Captures	7	18	
No captures	1592	1376	
Capture frequency	0.0044	0.0129	$G = 6.69; P < 0.01$
By plants ($N = 91$)			
Plants with 0 captures	40	31	
Plants with 1 capture	7	8	
Plants with 2 captures	0	5	
Capture frequency	0.15	0.41	$\chi^2 = 6.13; P < 0.05$
Fruit set ($N = 91$)			
Number	7	8	
Frequency	0.148	0.182	$G = 0.18; P > 0.50$

For capture data, two methods of analysis are presented. Analysis by observations examines the total number of observations yielding captures in each group. Analysis by plants examines the number of plants with zero, one or two captures in each group.

Table 2. Capture data for insects found in *Cypridium parviflorum* var. *pubescens* labella

Family, species count and species	Sex and exit method	Experimental group	Capture date and time (EST)
Andrenidae			
(1) <i>Andrena barbara</i>	Female, 2	Treatment	April 20; 12.50 h
<i>A. barbara</i>	Female, 3	Treatment	April 22; 14.18 h
(2) <i>Andrena miserabilis</i>	Female, 2	Treatment	April 20; 14.33 h
(3) <i>Andrena perplexa</i>	Female, 2	Treatment	April 20; 13.00 h
<i>A. perplexa</i>	Female, 3	Treatment	April 22; 14.18 h
<i>A. perplexa</i>	Female, 2	Control	April 22; 14.27 h
<i>A. perplexa</i>	Male, 4	Treatment	April 23; 18.05 h
<i>A. perplexa</i>	Male, 1	Treatment	April 24; 14.32 h
(4) <i>Andrena tridens</i>	Female, 1	Treatment	April 21; 14.12 h
<i>A. tridens</i>	Female, 4	Treatment	April 21; 14.19 h
<i>A. tridens</i>	Female, 1	Treatment	April 22; 16.29 h
(5) <i>Andrena vicina</i>	Female, 4	Treatment	April 20; 12.30 h
Apidae			
(6) <i>Ceratina calcarata</i>	Male, 3	Treatment	April 25; 13.00 h
(7) <i>Nomada sulphurata</i>	Male, 4	Control	April 24; 14.25 h
Halictidae			
(8) <i>Lasioglossum laevissimum</i>	Female, 4	Treatment	April 18; 10.17 h
<i>L. laevissimum</i>	Female, 2	Treatment	April 25; 14.24 h
(9) <i>Lasioglossum oblongum</i>	Female, 4	Control	April 23; 13.15 h
Megachilidae			
(10) <i>Osmia pumila</i>	Female, 2	Control	April 19; 12.01 h
Total individuals	18		

Manner of exiting the flowers: 1, through the exit hole and removing pollen; 2, through the exit hole without removing pollen; 3, through the entrance orifice; 4, slow or dead individuals removed from the labellum by hand. All captures were made during the spring of 2007.

either pick up pollen during escape (three) or slip beneath the anther without releasing any apparent pollen (six). The individuals identified as pollinators in the field were the only specimens observed to contain *Cypripedium* pollen under $\times 30$ magnification in the laboratory, and comprised two female *A. tridens* and one male *A. perplexa*. Of the remaining captures, three flew out of the entrance orifice and six were removed from the labellum by hand because they were sluggish or possibly dead. Seven individuals escaped from the labellum and back into the field site whilst we were removing the ribbon barrier or placing the insect into the kill jar. Two procedures were then instigated that effectively prevented any further losses of insects. These procedures were: (1) removal of the ribbon and clip after the mosquito net bag was over the flower; and (2) placement of the entire bag with the insect into the kill jar. Five of the seven escapees had a morphology consistent with Hymenoptera (specifically Halictidae and Andrenidae), and two were tentatively identified as Diptera.

The three insects that had *Cypripedium* pollen on their thorax were 11–14 mm long, 2.5–3.0 mm wide and had a thoracic height of 2.5–3.0 mm. Insects not carrying *Cypripedium* pollen varied widely in dimensions (6–15 mm in length, 1.5–4 mm in width and 1–3.5 mm in thoracic height), but most had at least one dimension within the range of values reported above for *Cypripedium* pollinators. Only *Ceratina calcarata* and *Lasioglossum* spp. were outside of these values and smaller than pollinators for all dimensions (6–7 mm in length, 1.5–2.0 mm in width and 1–1.5 mm in thoracic height).

CLIMATOLOGICAL ASSOCIATIONS

Sixty-eight per cent of the captures (17/25) occurred between 13.00 h and 15.00 h EST (Fig. 3) and included most of the identified species (Table 2). Generally, captures occurred at or close to the onset of peak daily high temperatures. In the analysis of climate for 17–25 April 2007, clear conditions were associated with 84% of the captures (21/25), but clear conditions comprised only 65.4% (195/298) of the non-capture intervals ($G = 4.03$, $P < 0.05$; Table 3). The mean temperature of capture intervals (25.3 °C) was significantly higher than that of non-capture intervals (19.9 °C; $t = 4.66$, $P < 0.001$), but no significant difference was found for average wind speed (12 km h⁻¹ for capture vs. 10.4 km h⁻¹ for non-capture intervals; $t = 1.47$, $P > 0.10$; Table 3). The period from 26 April to 4 May 2007 showed a significant reduction in favourable conditions for insect capture compared with the first half of the flowering period (45% ideal conditions before peak flowering compared with 30% after; $G = 15.4$, $P < 0.001$).

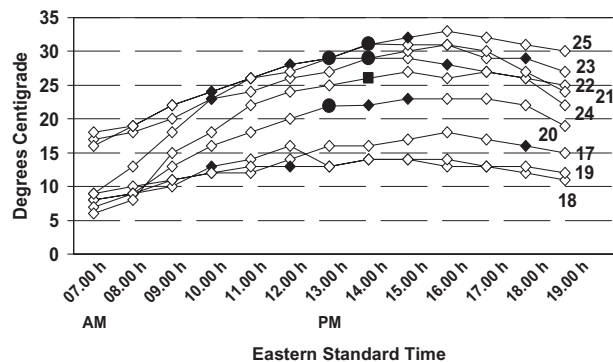


Figure 3. Insect captures in relation to temperature, time of day and date in April 2007 (dates are given at the right end of each data plot): filled diamonds, one capture; filled squares, two captures; filled circles, three captures; open diamonds, no captures. Capture data were rounded to the nearest hour. Climate data were obtained for Williamsburg at <http://www.wunderground.com/>

DISCUSSION

The ribbon barrier increased the likelihood of observing an insect trapped in the labellum by nearly threefold. It also permitted an evaluation of insect exit strategy, pollen receipt and identification. All insects receiving pollen in this study were in the treatment group, which is most probably a result of the increased ability to observe visitors in this group. In sparse or large populations, in which it is difficult to monitor each plant continuously for the duration of flowering, the protocols developed here should help to increase the sample sizes for floral visitors and pollinators. This is especially important when working with non-rewarding pollination systems. Although the protocols were tested and developed with *C. parviflorum* var. *pubescens* individuals, it is likely that these methods can be applied successfully to other members of Cypridioideae because of the highly similar morphologies and pollination mechanisms (Pridgeon *et al.*, 1999).

The observed increase in insect captures in the treatment group is most probably a result of the increased difficulty of insects escaping when the exit holes are blocked with ribbon. Although it is also possible that features of the ribbon itself increased visitations to the plant, fruit set data suggest that this is not the case. As insects were allowed to exit normally after the ribbon was removed, and labella were checked frequently, increased visitation rates would most probably increase successful pollination and fruit production. Although fruit production was low in this study, and consistent with other reports for this species (Newhouse, 1976; Tremblay, 1994; Herring, 2007), it was not significantly different between the control and treatment groups. This

Table 3. Climatic variables associated with insect captures from 17 to 25 April 2007

Variable	Captures (<i>N</i> = 25)	No captures (<i>N</i> = 298)	Test statistic and value; significance
Cloud conditions			
Clear	21 (84%)	195 (65.4%)	<i>G</i> = 4.03; <i>P</i> < 0.05
Other	4 (16%)	103 (34.6%)	
Temperature (°C)			
Mean	25.3	19.9	<i>t</i> = 4.66; <i>P</i> < 0.001
Standard error	1.1	0.43	
Wind speed (km h ⁻¹)			
Mean	12.0	10.4	<i>t</i> = 1.47; <i>P</i> > 0.1
Standard error	1.0	0.45	

Climatic data were obtained for most 20-min intervals from 07.00 h to 19.00 h.

suggests that the ribbon barrier had no effect on attractiveness or pollination rate, but instead acted to retain insects temporarily in the labellum.

Two climatic variables were significantly associated with insect visitation during the period 17–25 April 2007. These were warmth and the presence of clear days. These variables are well known to influence pollinator activity in many insects (Kevan & Baker, 1983; Bertin & Sholes, 1993; Herrera, 1995; Høye & Forchhammer, 2008), and have also been associated with pollinator activity in *Cyripedium* species (Nilsson, 1979; Erneberg & Holm, 1999; Herring, 2007; Li *et al.*, 2008). In particular, the presence of direct sunlight on exposed orchids has often been implicated as a factor that increases insect visitation rates (Tremblay *et al.*, 2005). Solar radiation measurements were not made directly in our study population. However, data from a nearby weather station showed that the percentage of sunny intervals was higher during captures (84%) than when no captures occurred (65.4%), suggesting the importance of sun. The presence of sun would also increase ground temperature, and it is therefore not surprising that the average temperature during captures (25.3 °C) was significantly warmer than when no captures occurred (19.9 °C). However, the range of capture temperatures was considerable (13–32 °C), and this may reflect temperature tolerance differences among the ten different species in the sample (Kevan & Baker, 1983). Lastly, captures were common in the early afternoon between 13.00 h and 15.00 h EST. Again, this finding suggests an association with warm temperatures and/or light. During the dates of the experiment, solar noon corresponded to approximately 13.00 h (field-site time). The height of the sun may have minimized shadows in the heavily wooded site, maximizing the number of orchids in direct sunlight.

A highly significant outcome was the termination of all insect visitations at peak flowering of the orchid

population (Fig. 2). This is apparently not related to flower senescence, as over 20% of the stems initiated bloom within 4 days of the last insect capture, and the vast majority of other blooms were also in good shape at this time. Climatic conditions may have played a role, as there was a significant decrease in favourable conditions for capture in the second half of the flowering period. However, the number of intervals with favourable conditions was only reduced by 33% and insect visitation was reduced by 100%. Thus, these climatic variables may not be sufficient to explain the complete absence of insect captures.

There have been other reports in the literature addressing this curious decrease in pollinator activity during peak or otherwise intense blooming. This phenomenon is often associated with deceptive orchids (Ackerman, 1981; Fritz, 1990; Sabat & Ackerman, 1996; O'Connell & Johnston, 1998), but similar results have been reported in at least one non-orchid forest herb, *Geranium maculatum* L. (Bertin & Sholes, 1993). In deceptive orchids, the explanation most commonly evoked is pollinator learning (Peakall, 1990; Peakall & Beattie, 1996). If anthesis is timed with insect emergence, it is expected to lead to a flurry of visitations by naive insects, followed by decreased activity when insects learn that there is no reward. Early insect visitors during this study may have learned to avoid additional plants and left the population by peak flowering. However, it is difficult to explain why any insects emerging after peak flowering did not visit the blooms, especially given the diversity of insects attracted by *C. parviflorum* var. *pubescens* (ten species in this study alone). The learning abilities of pollinators may also depend on the prevalence, spatial distribution and aggregation of co-flowering herbs, which could either pull pollinator attention away from deceptive orchids or increase their pollination depending on specific circumstances (Internicola *et al.*, 2006; Jersáková *et al.*, 2008).

Although there were few rewarding co-flowering herbs within the field site, foreign pollen was observed on all of the captured insects, and many other flowering plants were starting to flower in the regions neighbouring the field site. Thus, there may have been competition for pollinator attraction from other nearby open flowers, but it is not easy to explain how the presence of these other species might relate to the abrupt halt in insect visitation of *Cypripedium* flowers.

One factor that corresponded to the loss of visitation in this study was forest canopy closure. Absence or closure of the canopy has been associated with a decrease in pollination or pollen removal in other studies (Bertin & Sholes, 1993; Walters & Stiles, 1996; O'Connell & Johnston, 1998). The presence of a canopy would dramatically reduce solar radiation, which could then influence many microsite variables, including visibility of flowers, temperature or a decrease in plant productivity, leading to reduced fragrance emissions. Thus, the loss of insect activity may be complex, with contributions from several potentially interacting factors.

Prior to this study, the only observations of insects exiting *C. parviflorum* (*s.l.*) and receiving pollen were made by Stoutamire (1967), involving *Ceratina calcarata*, and Herring (2007), who identified three additional species, *Andrena krigiana*, *Augochlora pura* and *Lasioglossum rohweri*. In addition, four other hymenopterans [*Apis mellifera*, *Agapostemon splendens*, *Lasioglossum coriaceum* and *Osmia vicina* (= *O. pumila*)] and one dipteran (*Eristalis dimidiatus*) have been observed with *C. parviflorum* pollen smears (Guignard, 1886; Stoutamire, 1967). In this study, three individuals of two newly reported species were observed exiting the flowers and receiving pollen. These were two female *A. tridens* and one male *A. perplexa*. *Andrena* species are common pollinators of *C. calceolus* (Nilsson, 1979), and members of the genus have been found visiting or pollinating several other *Cypripedium* taxa (Stoutamire, 1967; Catling & Knerer, 1980; Bänziger *et al.*, 2005, 2008).

Eight species of visitors were trapped by *C. parviflorum*, but did not acquire pollen. These visitors were from the hymenopteran families of Andrenidae, Apidae, Halictidae and Megachilidae (Table 2). Bee pollination is widespread in *Cypripedium* (Cribb, 1997), and commonly includes all of these families (Pridgeon *et al.*, 1999). Two species observed to either receive or carry pollen in previous studies, a male *Ceratina calcarata* in Stoutamire (1967) and female *O. pumila* in Guignard (1886), were visitors in this study, which failed to receive pollen when they exited. The male *Ceratina calcarata* in our study flew back out through the entrance hole and the female *O. pumila* exited normally, but without obtaining pollen.

Similarly, only one of the five *A. perplexa* individuals successfully removed pollen, and the others escaped through the entrance, died in the labellum or failed to remove pollen on exit (Table 2). The observation that pollinating species do not always remove pollen is consistent with previous findings in *C. parviflorum* (Nilsson, 1979; Herring, 2007), and points to a difficulty in assessing pollinators. Effective pollen removal and pollination depend on a large number of factors, including the age and flexibility of the labellum (Nilsson, 1979), presence and condition of the pollen, condition of the insect, climatological variables (Corbet, 1990) and correspondence of pollinator size to labellum dimensions (Stoutamire, 1967; Nilsson, 1979; Li *et al.*, 2008). Variance in morphology within and among orchid populations will probably influence the effectiveness of insect species as pollinators. For example, Herring (2007) described effective pollinators in Missouri populations of *C. parviflorum* to be 6.6–7.8 mm long and 1.6–2.3 mm wide. In the present study, *Lasioglossum laevissimum* specimens were in this size range, but were observed to slip easily beneath the anther and not to acquire pollen. This suggests differences in orchid functional morphology among the two studies, and is consistent with the variation reported in *C. calceolus* populations from Europe (Erneberg & Holm, 1999).

In *C. parviflorum* and its varieties, the labellum dimensions are known to vary widely within populations, among varieties and geographically (Newhouse, 1976; Weldy *et al.*, 1996; Wallace & Case, 2000). Consistent with this variation, this species has been observed to attract approximately 16 genera of insects, including such disparate visitors as Coleoptera, Diptera and Lepidoptera, which become trapped in the labellum (Guignard, 1886; Cockerell, 1915; Robertson, 1924; Stoutamire, 1967; Newhouse, 1976; Barrows, 1983; Herring, 2007). Heinrich (1975) suggested that food-deceptive orchids should display variation in traits used to attract pollinators in order to retard the learning process. Although this hypothesis is consistent with high levels of variation in *C. parviflorum* and other *Cypripedium* taxa (for example, Sugiura *et al.*, 2001), the general attractiveness of *C. parviflorum* also ensures that a number of different insects are available as potential pollinators. For pollination ecology studies, this variation probably promotes highly idiosyncratic pollinator affinities that vary widely in time and space, and requires a large number of pollination ecology studies to derive meaningful trends.

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