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06. Pilot Study: Limitations to Pollination and Ovary Development in the Small White Lady's-Slipper (Cypripedium candidum)

Marie Faust
University of Wisconsin-Madison

John A. Harrington
University of Wisconsin-Madison, jaharrin@wisc.edu

Pilot Study: Limitations to Pollination and Ovary Development in the Small White Lady's-Slipper (*Cypripedium candidum*)

MARIE FAUST AND JOHN A. HARRINGTON[1]

Department of Landscape Architecture, University of Wisconsin-Madison, Madison, WI 53706, USA (JAH)
Conservation Biology, Nelson Institute for Environmental Studies, University of Wisconsin-Madison, Madison, WI 53706, USA (MF).

ABSTRACT The small white lady's-slipper (*Cypripedium candidum*) is threatened or endangered in 10 or more of the approximately 20 U.S. states and Canadian provinces in which it is found. Our pilot study revealed a lack of pollination occurring in Wisconsin's wild populations of this orchid. We also speculate from observations taken during the study that animal browse contributes to the state threatened status of this orchid. The results of this 1-year study are taken from three sites of low prairie in southern Wisconsin. Orchids were counted at each site; a subset of flowering plants was hand pollinated and mapped as to location. Three weeks later these plants and populations were revisited and inventoried for ovary development. Hand-pollinated plants had a significantly higher success of ovary development than plants left to natural pollination.

KEY WORDS *Cypripedium candidum*, fen, orchid, pollination, prairie

The small white lady's-slipper (*Cypripedium candidum*) is found in 17 states and 3 Canadian provinces (NatureServ 2017; Figure 1). *Cypripedium candidum* is listed as vulnerable to extirpated in all of these locations; no state or province lists this species as secure. Although considered to be locally abundant in the mid- to late 1800s and early 1900s, in 1979 the Wisconsin Department of Natural Resources (DNR; 2005) listed this perennial orchid species as state threatened (S3), vulnerable because of a restricted range with relatively few population occurrences and undergoing widespread decline (Figure 2). Bowles (1983), on the basis of historic county records, estimated a 52% decline in *C. candidum* populations over its natural range. The status of the orchid is attributed to collecting and the decline in its primary habitats of open wet prairies and calcareous fens (Figure 3), the majority of which were drained and converted for agriculture and development (Bowles 1983, Wisconsin Department of Natural Resources 2014). Two factors inhibiting population growth within habitats are inadequate moisture and light, the former due to hydrologic changes from surrounding development and the latter due to woody encroachment (Imrie et al. 2005). A third factor may be limited pollination success (Walsh et al. 2014).

Cypripedium candidum occur as single plants, often with multiple stems arising from a single branching rhizome (Michigan Department of Natural Resources 2004; Figure 4). The orchid's average mature height is 16-40 cm tall (Wake 2007, Wisconsin State Herbarium 2017). Flowering stems are terminated with a single 1.5–2.5-cm-long white pouch that is often streaked with violet lines. Once growth begins in the spring, developing flowers can be observed before the leaves have unwrapped from the stems (Michigan Department of Natural Resources 2004). Flowers persist for approximately 10 days and flowering occurs from mid-May to mid-June (Bowles 1983, Michigan Department of Natural Resources 2004). Ovary development is apparent by mid-July (Wake 2007). Seedling development is slow, and thought to require at least 12 years for maturation after germination (Curtis 1943, Michigan Department of Natural Resources 2004). Within populations, the orchid appears to depend on vegetative reproduction from adventitious buds on 2- to 3-year-old plants (Curtis 1943). Sexual reproduction maintains genetic variation within populations with bees as the principal pollinators. More specifically, small (4–6-mm long) andrenid and haclictid bees have been observed to pollinate its flowers (Catling and Knerer 1980, Bowles 1983). In addition, Pearn (2012) noted members of the *Syrphidae* (hoverflies) as possible pollinators. Bees enter the opening of the lip and once inside are routed to pass under the anthers where the sticky pollen mass will detach onto their backs. The interior lip wall is smooth at the point of entry and the reflexed lip edge creates a barrier for exiting. Exit points at the back of the flower lead under the stigmatic surface where bees deposit pollen from previous flower visits and then under an anther where a new pollen mass is deposited on their backs (Catling and Knerer 1980; Figure 5).

Catling and Knerer (1980) found that bee pollinators of *C. candidum* are dependent on the availability of nectar from a variety of other species with overlapping blooming times. The limited pollination of *C. candidum* by bees and the consequent overall low fruit production may be due to the species' reliance on a deceptive pollination strategy, in which a flower displays cues that it holds a food reward

[1] Corresponding author email address: jaharrin@wisc.edu

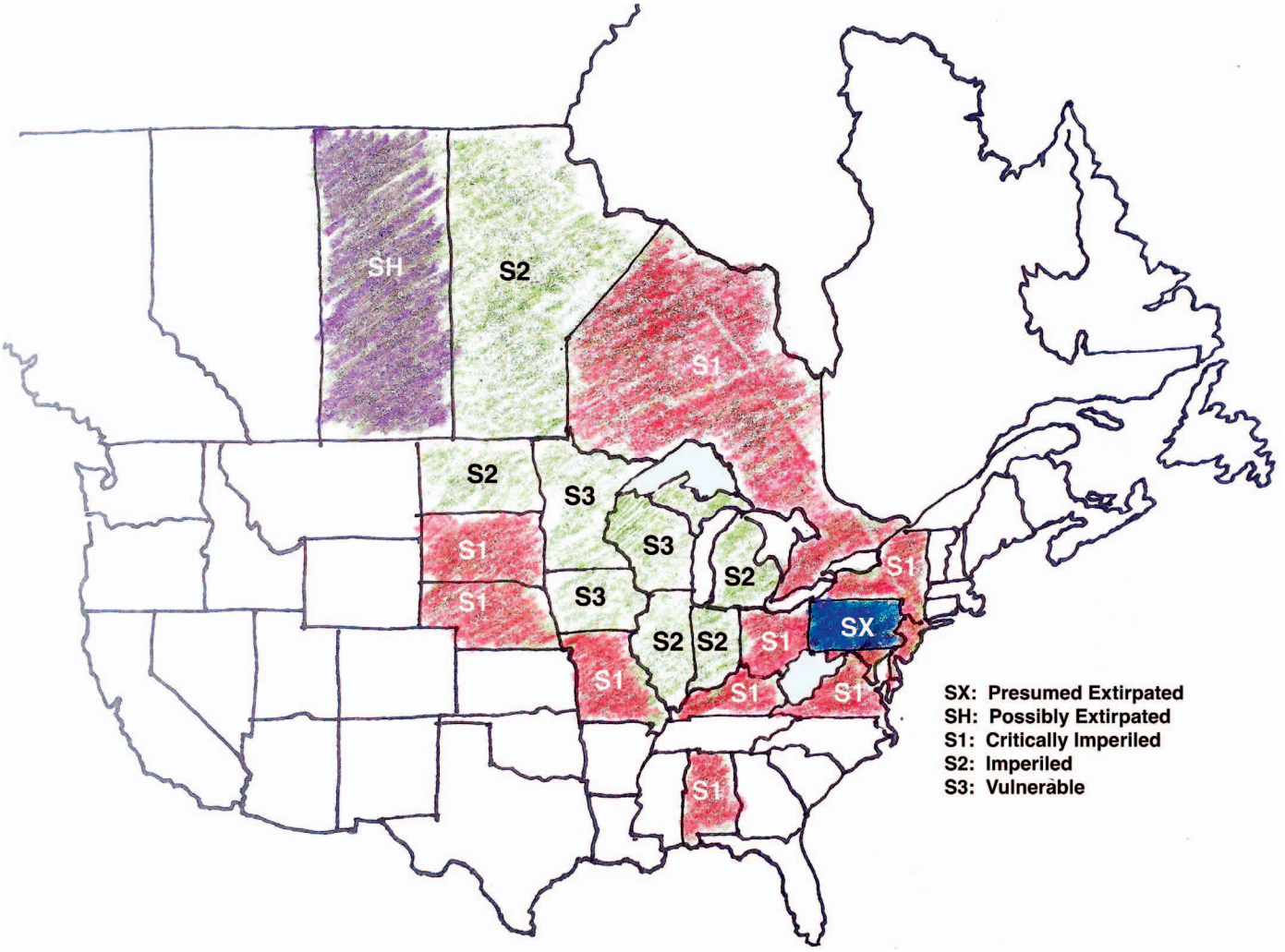

Figure 1. North American range of *Cypripedium candidum*. Modified from NatureServe Explorer: an online encyclopedia of life. Version 7.1. http://explorer.natureserve.org. Accessed 13 February 2017.

when it actually does not provide that reward. Plants that utilize deceptive pollination strategies typically receive lower pollinator visitation (Walsh et al. 2014). Combining this strategy with a short flowering period further limits the possibility of pollination and, therefore, sexual reproduction (Walsh et.al. 2014). However, *C. candidum*'s limited pollination may also be due to a decreased number of pollinators or an inability of pollinators to reach or find it among taller-growing forbs and grasses. An expanded discussion on *C. candidum* structure and pollination is presented in Catling and Knerer (1980), Bowles (1983), and Walsh et al. (2014).

We tested the hypothesis that a larger proportion of hand-pollinated flowers would produce developed ovaries than would flowers only exposed to natural pollinators. If the hypothesis is positive then the pollination potential for these orchids is not being maximized. More important is the difference in the proportions of developed ovaries, as we cannot assume that all plants available for natural pollination would be pollinated, even under the best of circumstances.

METHODS

Selection of Populations

Multiple prairie sites with previously recorded *C. candidum* occurrences were identified through records archived at the University of Wisconsin Herbarium and Wisconsin DNR and through discussions with DNR personnel. We visited these sites during mid-May to locate and inventory populations. Three of these sites, located in Dane, Walworth, and Waukesha counties, were selected for this study on the basis of their relatively high orchid numbers distributed across several populations, occurrence

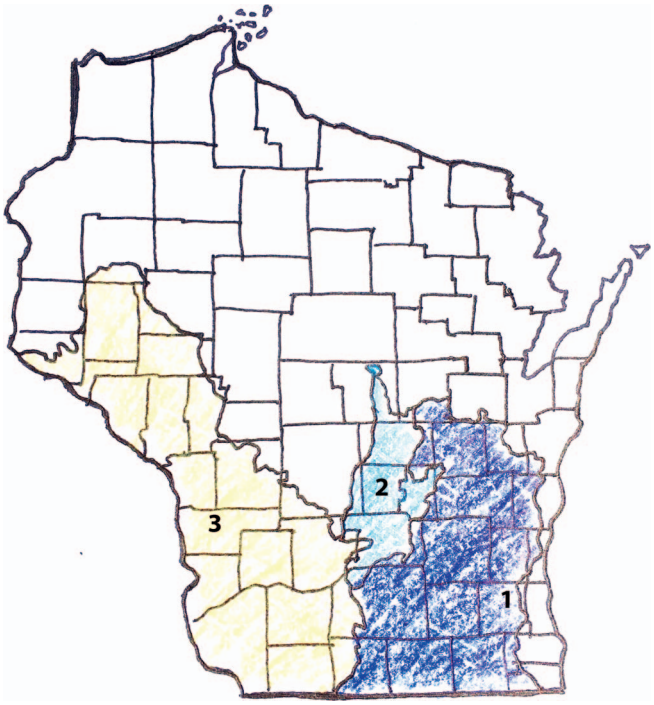

Figure 2. This map shows the ecological landscape association scores for the *Cypripedium candidum* (1 = high, 2 = moderate, 3 = low). The orchid is most prevalent in the Southeast Glacial Plains and the Central Sand Hills of Wisconsin. The shaded areas also indicate documented occurrences of *C. candidum* in the Wisconsin Natural Heritage Inventory. Modified from Wisconsin Natural Heritage Inventory, Wisconsin Department of Natural Resources, 2005. http://dnr.wi.gov/topic/EndangeredResources/Plants.asp?mode=detail&SpecCode=PMORC0Q050. Accessed 29 March 2017.

within 60 miles of Madison, and accessibility. Populations were defined as discrete groupings of orchids separated by a minimum of 100 m.

Study Sites

On site A, the orchids were loosely scattered along both sides of a creek that meandered throughout a wet prairie/fen landscape. Two locations with *C. candidum* were recorded at site B. Site B1 was in an open low prairie and site B2 occurred along and within a colony of redosier dogwood (*Cornus sericea*). Site C consists of marsh, low wet prairie, sedge meadow, and shrub carr, and had one large population along the edge of a gray dogwood (*Cornus racemosa*) colony.

Locating Plants for Pollination

Sites were visited in early June to hand pollinate a sample of the orchids in each of the populations found. We

Figure 3. *Cypripedium candidum* is found in wet prairie habitats with limited shrub cover.

Figure 4. *Cypripedium candidum* often occurs in tight clusters of multiple stems.

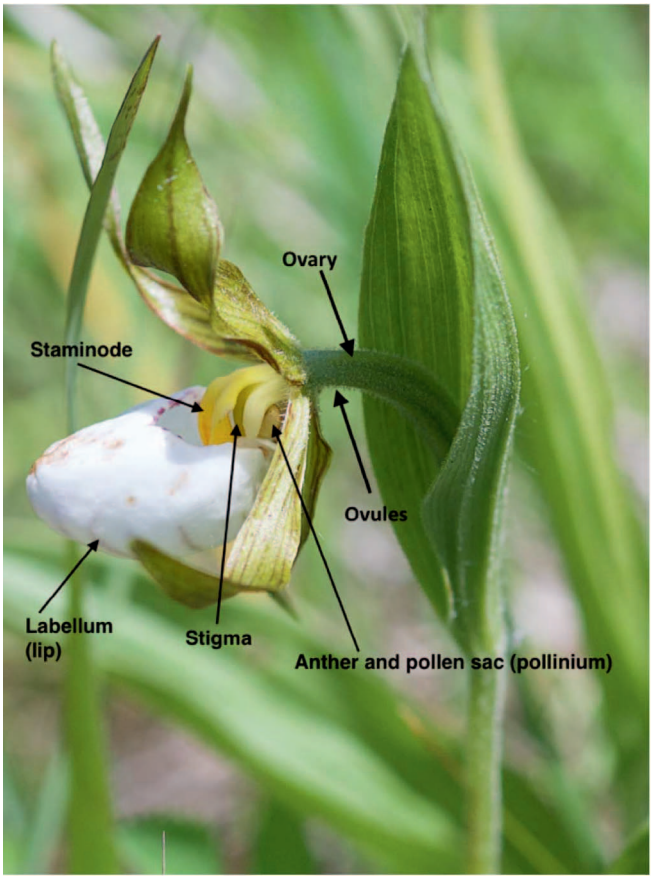

Figure 5. Flower of *Cypripedium candidum* and its structure.

Figure 6. *Cypripedium candidum* with developed ovary signifying successful pollination and partially removed protective cheesecloth mesh. This individual resides along the edge of a dense colony of redosier dogwood (*Cornus sericea*).

conducted a walk-through survey at each site where orchids were previously observed by field botanists and land managers. When an orchid population was found we recorded its stem numbers. To help us avoid missing stems and counting stems twice, we walked a series of belt transects each approximately 140 cm wide and spaced approximately 10 cm apart. We proceeded to the next belt transect once we had walked 10 m without observing another orchid. We continued adding belt transects until no more orchids were recorded. Stems within each belt transect were recorded in two separate categories: nonblooming and blooming. The presence of all other blooming species within and adjacent to the population was also recorded. Twelve to 30% of the flowering stems within a population were randomly assigned for hand pollination. Locations of orchid populations were recorded using a handheld GPS.

One of the two pollinia and anther caps was collected from a single flower and stem of one orchid and transferred to the stigma of a flower of a different plant. The sticky pollinium is easily removed with a toothpick and we immediately transferred it on the toothpick to the stigmatic surface of another plant located 30 cm or more from the source plant. All plants at a site were pollinated on the same day. Cheesecloth was wrapped and tied with decomposable hemp string around the flowers of the hand-pollinated orchids to prevent insect herbivory and natural pollination (Carlson 1940). In addition, these small orchids are difficult to locate once taller grasses and sedges mature. As the GPS unit accuracy was insufficient to pinpoint specific individuals that occurred within centimeters of each other, the cheesecloth assisted with relocating the pollinated orchids. All blooming orchids that were not hand pollinated were recorded as individuals available for natural pollination.

Data Collection: July

All three sites were revisited in early July to inventory the orchids for ovary development. The numbers of total individuals and individuals with and without developed ovaries were recorded for each population. Flowers with a green or brown enlarged fruit were scored as having successful pollination and ovary development (Figure 6),

Table 1. Ovary development in hand-pollinated and naturally pollinated orchids.

Pollination Group	Number	%
Hand-pollinated plants	33	100
Ovary development	30	91
Ovary failed to develop	3	9
Flowers available for natural pollination	137	100
Ovary development	37	27
Ovary failed to develop	100	73

and flowers with shrunken or missing fruit were scored as failed pollination (ovary abortion). Orchids that developed ovaries not enclosed with a cheesecloth bag were recorded as developing ovaries by natural pollination. Orchids exposed to natural pollination were not marked in the study as the populations at each site are concentrated and once located, flowering stems were relatively easy to count.

Site A had eight populations of *Cypripedium candidum* loosely scattered along both sides of a creek. Three of the larger and most distinct populations were chosen for sampling with a total count of 209 orchids. Of these, 76 were blooming and 24 were hand pollinated. Site B1 contained 130 orchids; 75 were blooming, and 12 of these were hand pollinated. Site B2 had a population of 63 orchids; 35 were blooming and 20 were hand pollinated. These orchids were located adjacent to a colony of *Cornus sericea* (redosier dogwood). Site C had one large *Cypripedium candidum* population estimated at 320 individuals and was also along the edge of a *Cornus racemosa* colony. Of these orchids, 132 were blooming and 35 were hand pollinated.

For the analysis, the data for all three sites were compiled. To determine whether the proportions of hand pollination and natural pollination were equal we used Fisher's exact test, where $P \leq 0.05$ would suggest that the proportions were not equal. Fisher's exact test was used because of our small sample size (McDonald 2014).

RESULTS

Thirteen orchids developed ovaries at site B1 and a similar number had missing flowers and torn stems, similar to that of deer herbivory. We hand pollinated 12 orchids but no orchids with or signs of cheesecloth bags were present. As we could not determine the pollination treatment of the 13 orchids with developed ovaries or the browsed orchids, site B1 was excluded from the analysis. For sites A, B2, and C, we recorded 592 individual orchid stems. Of these stems, 243 (41%) had flowers, and therefore were included in the study. We hand pollinated and bagged 79 (33%) stems and left 164 blooming stems available for natural pollination.

Table 2. Fisher's exact test (two-tailed) for pollination type and ovary development. $P = 1.10 \times 10^{-11}$, odds ratio = 0.037, confidence interval = 0.011–0.129.

Pollination Type	Ovary Developed	Pollination Failed	Totals
Hand pollination	30	3	33
Open pollination	37	100	137
Totals	67	103	170

The latter number likely fluctuated throughout the reproductive season, since orchids do not all bloom at the same time.

We counted 463 individuals in July at sites A, B, and C; this count was 129 fewer individuals than the 592 recorded during the May visits. Of these individuals, 170 bloomed during the study and 67 developed an ovary or seed capsule. Although 79 orchids were hand pollinated, only 33 were still bagged and relocated when sampling postpollination in early July, and 30 (91%) had developed ovaries. Of the 137 flowering plants available for natural pollination, 37 (27%) developed ovaries (Table 1). The proportion of ovaries that developed between hand pollinated and natural pollination was not equal ($P = 1.09 \times 10^{-11}$, odds ratio = 0.037, confidence interval = 0.011–0.129, Table 2).

DISCUSSION

We designed our study to determine if the pollination potential of *Cypripedium candidum* populations in Wisconsin is being met. If it is, we would expect that the proportion of orchids with successful pollination to be similar, regardless of whether a plant undergoes natural pollination or hand pollination. Similar to studies by Wake (2007) in eastern South Dakota and Walsh et.al. (2014) in Ohio, the results of this study found that, proportionately, hand-pollinated plants had much greater ovary development than plants relying on natural pollination.

Natural pollination is limited in *C. candidum* and may be one of several contributing factors to population declines in *C. candidum*, but we know of no studies that have explored this possibility. Pollination and successful ovary development do not in themselves result in an orchid that reaches reproductive maturity and contributes to a population. Seed predation, disease, herbivory, and weather are only some of the factors that influence orchid success during the lengthy period to maturity.

Limited pollination can be related to many factors, including a lack of pollinators, possibly due to fragmented and fewer habitats and an inability of pollinators to access *C. candidum* flowers hidden or covered by tall vegetation (Wake 2007). In addition, *C. candidum* relies on other plant

species in proximity to attract pollinators and the potential pollinators of *C. candidum* are dependent on the availability of nectar from many other flowering species blooming at the same time (Catling and Knerer 1980). Our study sites were of relatively high-quality wet prairies. Although a large number of wet prairie and fen plant species surrounded the orchid populations found in this study, the majority of these species were not blooming at the same time as the orchids. Species that were blooming were sparse, but included *Galium boreale*, *Hypoxis hirsuta, Phlox pilosa*, *Saxifraga pensylvanica*, and *Cypripedium parviflorum*.

Population Dynamics: Flowering and Ovary Development

Of the orchids found, 37% bloomed during the time periods we sampled. This percent is lower than many reported in past studies on population dynamics of *C. candidum*. For instance, Curtis (1954) found an average flowering rate of 60% over 18 years, Carroll et.al. (1984) documented a flowering rate of 48%, and Bowles (1983) reported flowering rates among four Illinois populations to span from 39.7 to 91.5%. Low flowering rates may be attributed to a multitude of biological and environmental factors and stressors including drought and low light levels, which can lead to orchids allocating their resources to vegetative growth (Bowles et al. 1992). A small percentage of ovaries (14%) developed into fruits in our study. This percentage is near the lower range for development reported in past studies. Curtis (1954) reported an average fruiting rate of 22% over 18 years. Carroll et al. (1984) documented a fruiting rate of 62%, and Bowles (1983) found fruiting rates among four Illinois populations to span from 4.6 to 54%. Environmental conditions and management practices can affect ovary development. However, pollinators are necessary for ovary development in some species, and *C. candidum* is not known to self-pollinate (Catling and Knerer 1980). Pollination success is thought to be low for *C. candidum* (Nies 2014), yet some degree of sexual reproduction success provides the genetic diversity that enhances adaptability to disease, predation, and environmental change (Wake 2007).

Possible Herbivory

On the return visits to inventory for ovary development, we were confronted with an unexpected dilemma; a total of 46 orchids or 58% of the orchids whose flowers were pollinated and bagged with cheesecloth were not found. The GPS waypoints were sufficient to locate the sample areas and, where still present, the cheesecloth was sufficiently visible that hand-pollinated orchids were quickly identified. The cheesecloth surrounding the flowers was unlikely to have come off or deteriorated during the 3-week period since it was placed over the flowers; and we found almost no cheesecloth on the ground. However, we cannot rule out deterioration of the cheesecloth.

Although herbivory was not an objective of this study, one unanticipated study outcome was the large numbers of orchid flowers that were eaten in areas where the hand-pollinated and cheesecloth-bagged flowers could not be found. The flower stems showed tears similar to those caused by deer browsing and not a sharp cut, which would be more indicative of rabbits. Human disturbance was deemed unlikely as the cause because of the remoteness of the populations and the occurrence of this phenomenon at separate sites. Flowers of several species of orchids are known to be a preferred deer and rabbit browse (Alverson et al. 1988), although this has not been documented for *C. candidum*. We also observed that many of the orchids with removed blossoms occurred in sites with low shrub density, whereas orchids near and along shrub borders retained blossoms and the cheesecloth bags. Although we observed this occurrence at all sites, it was most pronounced at site B1. One possible explanation is that the cheesecloth itself was an attractant to deer. Several research findings suggest that deer are attracted to visual flags while foraging (Wiegman and Waller 2006, Frerker et al. 2014). Other studies have shown that several orchid species are favored by deer and other herbivores, and these species could be experiencing reduced reproductive success and population decline (Stuckey 1967, Alverson et al. 1988, Brzosko 2002, Knapp and Wiegand 2014). However, Nies (2014) reports from personal observations that deer do not seem to prefer *C. candidum* and may even bed in proximity without consuming them. An inventory of existing *C. candidum* in eastern Wisconsin along with a series of exclosure studies will be conducted in Wisconsin during 2017 and 2018.

Shrub Densities

Cypripedium candidum is highly dependent on full sun in open areas and, as with many prairie species, populations begin to decline with the invasion of woody plants (Curtis 1946). In addition to the potential shading effects of encroaching woody vegetation, increased heterospecific stem density has been shown to reduce pollination and population recruitment (Wake 2007). Even so, nearly all the larger-sized *C. candidum* populations in this study occurred near or along shrub edges. We observed only small populations of orchids growing in the opening away from shrubs.

However, our study does not suggest that these shrub edges are the orchid's preferred habitat or that their populations will remain stable in such a context. Imrie et al. (2005) conducted a study on a small nature reserve in Ontario and reported that *C. candidum* and shrubs prefer similar wetland edge environments, and that *C. candidum* declines due to woody succession are not always immediate.

If true, the environment in which these orchids grow appears to be conducive to shrub establishment, to their eventual detriment. We have not found studies that suggest how long these orchids can survive once shrub encroachment occurs.

CONCLUSIONS

Our pilot study suggests that the pollination potential of *C. candidum* is limited in Wisconsin wet prairies, but not why or whether a reduction in pollination is a factor in the orchid's decline. Future studies focused on whether a reduction of pollination is contributing to the orchid's decline, and if the lack of pollination is due to an absence of pollinators or an inability of these pollinators to find and access the orchids. Changes in the surrounding vegetation structure would aid land managers who have responsibility for lands upon which *C. candidum* grows.

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