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Pollination ecology and breeding systems of *Cypripedium kentuckiense* (Orchidaceae) in Tennessee[1]

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Abstract. The orchid *Cypripedium kentuckiense* is listed as imperiled in Tennessee and Oklahoma and critically imperiled in seven other states. We followed individuals' vegetative and floral traits over two seasons. Patterns of floral senescence were found to vary over two seasons and might be related to April temperatures when buds develop. Mechanical self-pollination was uncommon but perhaps "leaky." Frequencies of insect-mediated pollination were surprisingly high (62%) for a *Cypripedium* species with large flowers but conversion of flowers into fruit was low (7% and 14%), suggesting that cryptic factors lower reproductive success, not inadequate pollinator visits. Hand-pollinations suggest flowers are self-compatible. As in other *Cypripedium* species, floral architecture relates to pollinator dimensions but at this site, only two bee species, *Anthophora abrupta* and *A. bomboides*, were primary pollinators. Male *A. abrupta* were common visitors although male bees are uncommon pollen vectors of *Cypripedium* species. For the first time, exit patterns of bees inside *Cypripedium* flowers were recorded based on bee species and gender. This behavior varied and was somewhat atypical of previous studies on other bee-pollinated species in the clade and genus. Specifically, *Anthophora* species within labella chewed on the epidermis lingering within the interior and exit canals. Consequently, *C. kentuckiense* might not be a trap.

Key words: bees, floral dimensions, phenology, pollination, senescence

Ever since Darwin, scientists have continued and expanded research on the genus *Cypripedium* (Argue 2012). This genus has long been used as a model system for functional floral morphology and pollination ecology (Darwin 1877). Darwin used orchids to demonstrate the benefits of cross-pollination and evidence of natural selection (Tremblay *et al*. 2005). *Cypripedium* is distributed irregularly throughout the Northern Hemisphere and consists of 47–50 species (Cribb 1997), with 12 found in the United States (Sheviak 2002).

All members of the genus *Cypripedium* share common features, including a diandrous column with a shield-like staminodium and an inflated labellum. However, floral presentation varies among species, including floral architecture, color, and fragrance (Atwood 1984, Barkman *et al*. 1997, Sheviak, 2002). Although these features can serve

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to attract and manipulate pollinators (Tremblay *et al*. 2005), edible rewards (*e.g*., nectar and pollen) have never been identified in any of the *Cypripedium* species (Case *et al*. 1998). However, *Cypripedium subtropicum* S.C. Chen & C.Y. Lang might be an exception and might offer edible hairs or secretions to syrphid flies (Jiang *et al*. 2020). In addition, as in most orchid lineages studied to date, several *Cypripedium* species are self-compatible, but most do not self-pollinate (Edens-Meier *et al*. 2014).

Most of the insects known to carry the pollen of *Cypripedium* species are bees or flies (Argue 2012, Edens-Meier *et al*. 2014). Darwin (1877) described the process of pollen dispersal, pollen deposition, and cross-pollination. To disperse and deposit pollen, the insect's body size should correlate with the morphology of the flower's interior architecture (Nilsson 1979, Li *et al*. 2006, Case and Bradford 2009, Edens-Meier *et al*. 2018). Although the process of pollination is similar for all *Cypripedium* species, pollinator diversity often varies broadly. For example, in the large-flowered *Cypripedium reginae* Walter, only six medium-sized bees belonging to five species from three genera (*Anthophora*, *Apis*, and *Megachile* spp.) carried the orchid's pollen (Edens-Meier *et al*. 2010). Although bees with far larger (*e.g*., members of the bee genus *Bombus*) and far smaller dimensions (*e.g*., members of the bee genera *Augochlorella* and *Lasioglossum*) regularly entered the saccate labellum of *C. reginae*, they were unable to contact dehiscent anthers while exiting the flower.

The conservation status of *Cypripedium* species varies from state to state (NatureServe 2020). *Cypripedium kentuckiense* C.F. Reed is endemic to North America with a restricted geographic distribution from southern Virginia to eastern Texas. The populations of this species tend to be small and highly disjunctive (Sheviak 2002). *Cypripedium kentuckiense* is afforded conservation status in all 10 states where it naturally occurs. It is listed as vulnerable in Arkansas, imperiled in Oklahoma and Tennessee, and critically imperiled in Alabama, Georgia, Kentucky, Louisiana, Mississippi, Texas, and Virginia (NatureServe 2020).

Invasive woody and herbaceous species are suspected of causing declines in populations of *C. kentuckiense* (M. J. Tackett, personal communication). Additional threats to *C. kentuckiense* populations include over-collection (Yarian 1939), climate change (Kaye *et al*. 2019, Kolanowska and Jakubska-Busse 2020), poaching, herbivory, damage caused by feral hogs (*Sus scrofa*; Linnaeus 1758), and habitat destruction (NatureServe 2020). Furthermore, many species face multiple threats simultaneously, making recovery more difficult to address.

Molecular analyses by Li *et al*. (2011) place *C. kentuckiense* within the North American *parviflorum* clade [*Cypripedium parviflorum* var. *pubescens* (Willd.) O.W. Knight, *Cypripedium parviflorum* var. *parviflorum* Salisb., *Cypripedium montanum* Douglas ex Lindl., and *Cypripedium candidum* Muhl. ex Willd.]. The three other species in this clade all have labella smaller than *C. kentuckiense*. Consequently, they have smaller pollen vectors ranging from 3 mm to 7 mm in length (Edens-Meier *et al*. 2018, Grantham *et al*. 2019).

Little is known about the pollination biology of *C. kentuckiense* (Argue 2012). Covell and Medley (1986) examined flowers of *C. kentuckiense* in four states. In Scott County, Tennessee, they examined 200 flowers and found only two male moths of *Polychrysia morigera* H. Edwards (Noctuidae) in the labellum of one flower. In addition to the two moths, they found a large bee in a labellum in Jefferson County, Arkansas. It is unknown if these insects carried orchid pollen.

As populations of *C. kentuckiense* continue to decline (IUCN Red List of Threatened Species 2002), it is necessary, as stressed by Case and Bradford (2009), to identify pollinators in order to understand pollinator-selection pressures. Considering the current conservation status of this species, additional field studies are necessary in order to understand the ecology of this species and to make informed future conservation decisions. Therefore, we investigated floral phenology/longevity, floral characteristics, attractants, fruit production and embryonic development within seeds, pollinator diversity/behavior, and insect-pollen delivery mechanisms. Second, experimentation was required to determine the role of self-pollination and self-compatibility. Third, we asked if the large flowers of *C. kentuckiense* served as floral traps or offered floral rewards.

Material and Methods. STUDY SPECIES. The flowers of *C. kentuckiense* are described by Sheviak (2002) as green/yellow in color with reddish brown/madder spots, stripes, and reticulated markings. One or two flowers are produced per scape along with 3–6 leaves. Plant height is found to vary considerably from 35 to 97 cm. It usually grows in deciduous forests on well-drained slopes or muddy seeps (Sheviak 2002). Labellum measurements of *C. kentuckiense* are among the largest (41–65 mm) of the 12 *Cypripedium* species in the USA (Sheviak 2002) and are the largest within its clade (Li *et al*. 2011).

STUDY AREAS. Research was conducted from May 1 to May 30 in 2018 and from May 6 to May 28 in 2019. Study sites, referred to as sites 1, 2, and 3, were all located along horse trails in Scott County, Tennessee, within the Big South Fork National River and Recreation Area. Specific locations of these three sites remain confidential because of conservation concerns. Research was conducted at all three sites in 2018 and 2019. Site 1 was easily accessible whereas access to sites 2 and 3 required the use of a utility task vehicle.

Populations of *C. kentuckiense* ranged between 250 and 270 m in elevation and individual flowers were found between 50 m and 170 m from the main river channel. Although sites were often flooded for brief periods, especially early in the growing season, water tables lie well below the soil surface for most of the year.

Hardwood floodplain vegetation dominated our sites including *Liquidambar styraciflua* L., *Liriodendron tulipifera* L., *Platanus occidentalis* L., *Acer rubrum* L., *Fagus grandifolia* Ehrend, *Betula nigra* L., *Aesculus flava* Aiton, and *Juglans cinerea* L. Understory trees, shrubs, and vines included *Lindera benzoin* (L.) Blume, *Carpinus caroliniana* Walt., *Asimina triloba* (L.) Dunal, *Ulmus alata* Michx., *Toxicodendron radicans* (L.) Kuntze, and *Bignonia capreolata* L.

Voucher specimens for *C. kentuckiense* (*RMM*, *2*, MO; *RMM*, *3*, MO; *RMM*, *4*, MO; *RMM*, *5*, MO; *RMM*, *37*, MO) from sites 1, 2, and 3 were deposited in the herbarium of the Missouri Botanical Garden, St. Louis, MO. Insect vouchers will be deposited in the Division of Entomology at The University of Kansas, Lawrence, KS, USA.

PHENOLOGY AND RATES OF FLORAL SENESCENCE. Phenology and floral senescence were documented to determine the effects of climate on floral aging (see Edens-Meier *et al*. 2018). The total number of flowers was documented for all three sites during both research seasons. All phenology, floral measurements, and pollination events were recorded at site 1. Climatic conditions were recorded for both research seasons. Flowering plants were numbered and mapped. We documented floral life span by monitoring from bud stage through senescence in 2018 and 2019. The flower was considered open when the dorsal sepal separated from the labellum, exposing the dorsal opening. Flowers at site 1 were observed daily for the presence or absence of brown areas on labella. Floral stages of senescence were documented using the following code: zero indicated no brown areas; plus one indicated one to three small, brown areas (3 mm or less in diameter); plus two indicated four or more small, brown areas (3 mm or less in diameter); and plus three indicated large brown areas (5 mm or greater in diameter). Flowers were recorded as plus four when the labellum withered and collapsed or when the labellum was mostly brown in color. To determine floral longevity, the first day the flower opened was counted as day number one and the date of total senescence was recorded when the flower received a rating of plus four. Ratings were averaged to determine floral longevity.

PLANT HEIGHT, FLORAL PRESENTATION, AND DIMENSIONS. A total of 55 flowering scapes were measured (27 in 2018 and 28 in 2019). Plant height was measured from the apex of the terminal floral bract to the scape's base. To compare floral architecture with pollinator dimensions (see below) we made floral measurements following procedures described by Edens-Meier *et al.* (2011), including labellum length, width, and depth; labellum entrance length and width; and exit length and width of the basal openings.

FLORAL TEMPERATURES. Floral temperatures were recorded to determine if internal temperatures offered warmth as a reward to poikilothermic pollinators (see Edens-Meier *et al*. 2018). In 2018 and 2019, temperatures within the interior of labella of *C. kentuckiense* at site 1 were measured using an Omega Type T Thermocouple Cu-CuNi HH-25TC Thermometer, Range –80 °C to 400 °C and an Omega TMTSS-062G-6 Probe (OMEGA Engineering Inc., Norwalk, CT). The temperature probe was placed inside the labellum. After one minute, the temperature was recorded. In addition, we documented ambient air temperature, time of day, and if the flower stood in a light gap (when sunlight breaks through the woodland canopy and shines directly onto the flower).

Ultraviolet Photography. We used ultraviolet (UV) photography as pigmentation patterns, invisible to human eyes, can direct or orient the behaviors of prospective pollinators (see Papiorek *et al*. 2016). Flowers of *C. kentuckiense* were photographed using a non-UV camera (Canon Rebel T3i; Canon, Tokyo, Japan) under normal light and a UV-converted camera (Canon Rebel T2i; Canon) to determine if flowers displayed a UV response.

Floral Fragrance. Individual flowers were smelled in order to share a personal floral description (see Edens-Meier *et al*. 2018). No flowers were sacrificed to provide this description.

Analyses of Open, Insect-Pollinated Flowers vs. Bagged (Unmanipulated) Flowers. Flowers that remained open for insect pollination (unbagged) were compared with bagged flowers to determine rates of mechanical self-pollination (autogamy). Procedures followed Edens-Meier *et al*. (2010). All three sites were used in 2018 for this part of the study. Ten buds were tagged with jeweler's tags and bagged with tulle. Eleven control buds were tagged but not bagged (open flowers) on May 4, 2018. Pistils were checked on May 16 and harvested on May 30, 2018.

In 2019, site 3 was inaccessible due to flooding. Therefore, only site 2 was used for this part of the study. The bagging protocol was repeated on May 6, checked on May 14, and pistils were harvested on May 28, 2019.

Harvested flowers were fixed in 3:1 95% ethanol:glacial acetic acid for 4 hr before preserving them in 70% ethanol. This process cleared and preserved floral organs for subsequent laboratory microscopic examinations and to prepare gynoecia to view pollen tubes under epifluorescence microscopy following Edens-Meier *et al*. (2010). Due to pistil thickness, each pistil was placed on a glass slide (75 mm by 50 mm) after softening, and split vertically with a single-edged razor blade prior to staining and spreading tissues. These specimens were examined and photographed using a Zeiss Axioscope 40 and Zeiss Axioscope Imager M2 (Zeiss, Oberkochen, Germany) after 24 hr. We recorded the presence of pollen grains/tubes on the stigmatic surface and the presence or absence of pollen tubes within each style and ovary.

Hand-Mediated Pollinations. Hand cross-pollination and hand self-pollination allowed us to test for self-compatibility (see Edens-Meier *et al*. 2010). In 2018, flowers from all three sites were used. A total of 20 buds were tagged with jeweler's tags and bagged with tulle on May 4, 2018. On May 16, 2018, when flowers opened, nine were hand-cross pollinated by transferring pollen with a clean toothpick from the anthers of nine flowers a minimum distance of 1 m away from each tagged flower.

Nine more bagged flowers were hand self-pollinated by transferring pollen from the anther to the stigma in the same flowers (intrafloral selfing). Pistils at all three sites were harvested on May 30, 2018, fixed, and processed, as above. In all cases of hand-pollination, pistil collection was delayed deliberately for 2 wk because megasporogensis often occurs slowly after pollination in many orchids (Arditti 1992, Tremblay *et al*. 2005, Sogo and Tobe 2006, Edens-Meier *et al*. 2010).

In 2019, site 3 was inaccessible and we used site 2, as above. Otherwise, field procedures, harvesting, and processing of pistils were identical.

Fruit Set. To determine the natural rate of capsule production during a growing season, mature capsules were counted as in Edens-Meier *et al*. (2018). Mature capsules were counted at site 3 on June 28, 2018, and at site 2 on July 2, 2018. On July 1, 2019, mature capsules were counted at site 3 for season two.

Seeds. Due to the rarity of this species, only one mature, dehiscent capsule (produced summer 2017) was collected in spring 2018. Because the testa is transparent, we examined embryonic development in seeds. Seeds were counted and rated according to embryo development (developed or underdeveloped/absent) (see Ren *et al*. 2014). All seeds were returned to the site from which they were collected and dispersed.

Insect Observations and Collections. For two research seasons, we observed insects entering and exiting flowers of *C. kentuckiense* at Site 1 and documented their behavior. These insects were collected in order to find out which insects were attracted to the flowers (see Edens-Meier *et al*. 2018). Insect visitors were recorded during May 2018 and May 2019 for approximately 319 total hr.

Exit Patterns. The method of exit was documented for each insect observed entering and exiting the labellum to determine if the exit route differed among bee species or gender. A potential pollinator normally enters the labellum via the large dorsal opening. In order to exit the labellum, insects either leave via the same dorsal entrance or through one of the two smaller basal openings, each adjacent to the dehiscent anther. Using a frontal orientation, these small basal openings were positioned either to the left or right of the staminode. This orientation was used to document method of exit from basal openings. Insects were captured and euthanized following procedures by Edens-Meier *et al.* (2018). In 2019, ice was used to euthanize bees because it was faster than ethanol. Each plastic bag was labeled using an indelible marker to indicate bee number. If a yellow pollen mass from the dorsum of the bee's thorax adhered to the interior surface of the plastic bag, it was circled on the outside surface of the bag. A new plastic bag was used for each bee captured (see Edens-Meier *et al.* 2018). The date and time (Central Standard Time [CST]), that each insect was captured was recorded to determine when foraging peaked.

Time Spent Inside Labella. To discover if time spent within labella differed between bee species or gender, we documented the time each bee (n = 92) spent in the labellum. Timing began when the bee entered the labellum and stopped when the insect exited via one of the three openings. We often observed flowers oscillating up and down when an *Anthophora* species had entered the flower.

Light Gap. We wondered if light gaps were required for insect attraction and entry. Therefore, we documented the presence or absence of light gaps when each insect entered a labellum as described by Bernhardt *et al.* (2014).

Night Visitations. Basal openings of flowers (n = 26 in 2018; n = 23 in 2019) within the population at site 1 were blocked with ribbon, as described in Case and Bradford (2009), to determine if night visitations occurred. Yellow ribbon (2 cm wide by 19 cm long) was placed around both basal openings and secured tightly using a small binder clip (see Case and Bradford 2009). Ribbon and flower color were matched to avoid unintentional attraction to flowers. The ribbon was left in place for 15 hr overnight for one night during both seasons.

Insect Measurements and Identifications. Insects were measured to compare with floral dimensions (see above). Freshly euthanized specimens were pinned, labeled, and measured while their bodies were still pliable (see Edens-Meier *et al.* 2018). Corresponding pollen wash slides were coreferenced with bee labels and subsequently compared with the pollen library (see below). We used digital calipers (Cat No. 1235C55, ISO 17025 Calibrated, Thomas Scientific, Swedesboro, NJ), followed measurements in Edens-Meier *et al.* (2011), and identified specimens to gender, genus, and/or species. Unlike the monotypic *Anthophora abrupta* Say, 1838 which is restricted to the eastern half of North America, *Anthophora bomboides* Kirby 1837 is a polytypic species occurring across the continent with a number of forms or variants sometimes given taxonomic rank (Brooks 1983). All *A. bomboides* we collected fit the description of *Anthophora raui* Rohwer 1923 (in Mitchell 1962), which was synonymized under *Anthophora bomboides sodalis* Cresson 1879, in Brooks (1983) and Ascher and Pickering (2017). All bee nomenclature followed Ascher and Pickering (2017).

Pollen Load Analyses. To determine the extent of floral foraging on *C. kentuckiense* and coblooming taxa, we constructed a pollen library from flowering species at site 1 (see Edens-Meier *et al.* 2018). An Olympus SZ30 (Microscope Central, Feasterville, PA) was used to estimate (0–100%) pollen carried in the hind tibial and basitarsal scopa. Removal, staining, and mounting insect-borne pollen grains followed Bernhardt *et al.* (2003). Visible pollen masses in plastic bags (see above) were also removed and processed. Counting, identification, and photography of grains under white light with a Zeis Axioscope 40 and Zeiss Axioscope Imager M2 followed Bernhardt *et al.* (2014).

Floral Traps. We examined each labellum on a daily basis for dead/dormant insects (see Bernhardt *et al.* 2014). We also observed and documented insect behavior while within labella.

Floral Rewards. Insect behaviors were observed and recorded to determine if they appeared to be consuming or collecting floral substances. Each bee was examined for the presence of floral tissues.

Statistical Analyses. *Weather.* Temperature and precipitation data were downloaded from the US Climate Data website (US Climate Data 2020). In the absence of an *in situ* weather station near the location of our study, we downloaded data from the nearest stations in Jamestown and Oneida, TN, from April and May for 2018 and 2019. Temperatures for the specific location were interpolated with the package 'meteolan' (De Cáceres *et al.* 2018) for the R computational environment (R Core Team 2018). We then compared degree-warming days and daily maximum average between years via a *t*-test.

Floral Senescence. In order to assess the relationship between time and the accumulation of senesced labella over 2018 and 2019, we used quantile regression (Cade and Noon 2003). First, we regressed the number of flowers in each senescence category against the number of days since the flower opened. We then performed a second quantile regression. By this time, the independent variable was the sequential day of the year, where days are counted sequentially, one through 365. Thus, February 1 is the 32nd day of the year. For both sets of regressions we modeled the 90th quantile (tau).

Bee Visitation Times. To quantify the relationship between time remaining within the flower and flower age, the amount of time that a bee spent in the flower was regressed against the age of the flower in days. We did this only for the two common pollinators, *A. abrupta* and *A. bomboides*. In the case of *A. abrupta* we also divided the catch on the basis of gender. We performed an analysis of covariance (ANCOVA) to test if the lines have similar slopes and intercepts. The response variable (*i.e.*, the time that individual bees spent in a flower during a visitation) was log-transformed in order to meet the assumptions of the test. In this analysis we combined both years to improve statistical resolution.

In order to quantify the relationship between visitation time and age status of flowers, we performed another ANCOVA but with flower status as the independent variable. As above, the dependent variable was the time spent in the flower by visiting bees with the data log-transformed in order to meet test's assumptions, and combined years (2018 and 2019) in order to have enough statistical power.

All statistical analyses were performed using RStudio (RStudio Team 2018).

Results. STUDY AREAS. In 2018, 34 flowering scapes were documented on 57 plants at site 1. Two plants produced two flowers per stalk, making a total of 36 flowers within that population. Damage due to horseback riders and research accidents reduced the total number of flowers available for research to 26 in 2018. At site 2, 42 flowering scapes were documented on 133 plants, and 43 flowering scapes were recorded on 140 plants at site 3.

In 2019, 28 flowering scapes were documented on 49 plants at site 1. Only one plant produced two flowers on the same stalk making a total of 29 flowers. At site 2, 50 flowering scapes were recorded on 125 plants, and 94 flowering scapes were cataloged on 224 plants at site 3.

PHENOLOGY. We arrived at site 1 on May 1 and left May 30 in 2018. Six flowers opened on May 12. When we left on May 30, 16 plants were still in flower and 10 flowers were senescent. The average life span of a flower of *C. kentuckiense* was 13 days ($n = 10$) in 2018.

No plants were flowering at site 1 on May 2, 2019. On May 6, six flowers were open. These six open flowers were not included within the longevity study. The average life span of a flower of *C. kentuckiense* was 16 days ($n = 23$) in 2019. Anthers of *C. kentuckiense* released pollen on the first day the flowers opened and continued to release pollen throughout the floral life span. In 2019, a spring flood occurred prior to flowering time and a cooler period lasted from May 12 to May 15, with an average high temperature of 17.2 °C.

There was no significant difference in the total number of degree-warming days between years ($t = -1.0325$, $P = 0.3061$). However, there was a significant difference in the average maximum high for the month of April, with 2018 averaging 14.8 ± 3.7 °C, compared to 2019 which averaged 20.6 ± 3.2 °C ($t = -8.012$, $P < 0.0001$). This average high for April 2019 was over 5 °C higher, largely due to the second half of the month, when daily high temperatures fluctuated between 19 °C and 25 °C. We did not detect a significant difference in maximum daily average in the month of May between years ($t = 0.35708$, $P = 0.7223$), with 2018 averaging a high of 24.9 ± 3.5 °C, and 2019 with an average high of 25.6 ± 4.4 °C.

RATES OF FLORAL SENESCENCE. Data collection on senescence began on the first day of floral opening and ended on May 27, 2018 when all flowers ($n = 26$) showed signs of senescence. Thirty-nine percent of the remaining flowers in the population

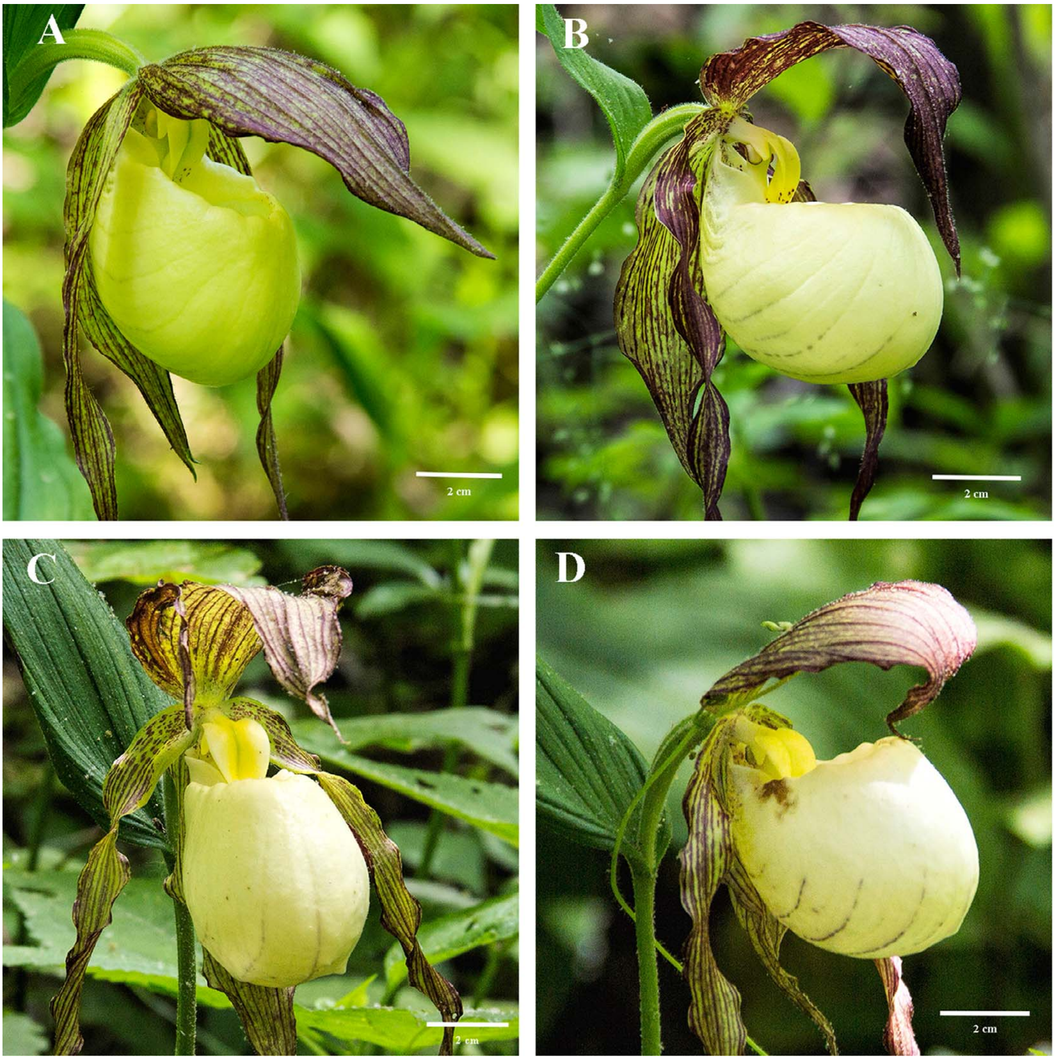

FIG. 1. Floral stages of senescence in *Cypripedium kentuckiense*: (A) Flower in state zero shows no brown spots on labellum; (B) Flower in state one shows one to 3 brown spots on labellum; (C) Flower in state two shows four or more brown spots on labellum; (D) Flower in state three shows large brown area on labellum.

were in stage 3 or stage 4 of senescence. In 2019, all remaining flowers (n = 23) were senescent by May 28.

We observed physical changes associated with senescence during the floral life span of *C. kentuckiense*. Flowers opening on the first day were glossy and turgid (stage zero; Fig. 1A). With floral aging, we observed flowers progressing through stages one through three (Fig. 1B–D), and a stage four flower turning brown and collapsing.

A quantile regression with tau = 0.9 allowed us to model the top 90% of the data over time as flowers reached the older stages (Fig. 2). In 2018, flowers remained in the first stage much longer, from 8 to 11 days, and only one flower reached the third stage of senescence, (*i.e.*, status two [intercept = –0.1 ± 0.08, and slope = 0.11 ± 0.09]). Alternatively, in 2019, aging was quicker from the first stage (*i.e.*, status = 0, Fig. 1A) to the second, with an average between 5 and 7 days (Fig. 2, Fig. 1B). Progression towards the next stages (status two and three) was even faster, taking between 3 and 5 days (Fig. 1C, D). Within 10 days, over 75% of all flowers were classified as status two or three (intercept = –0.5 ± 1.1, and slope = 0.25 ± 0.5).

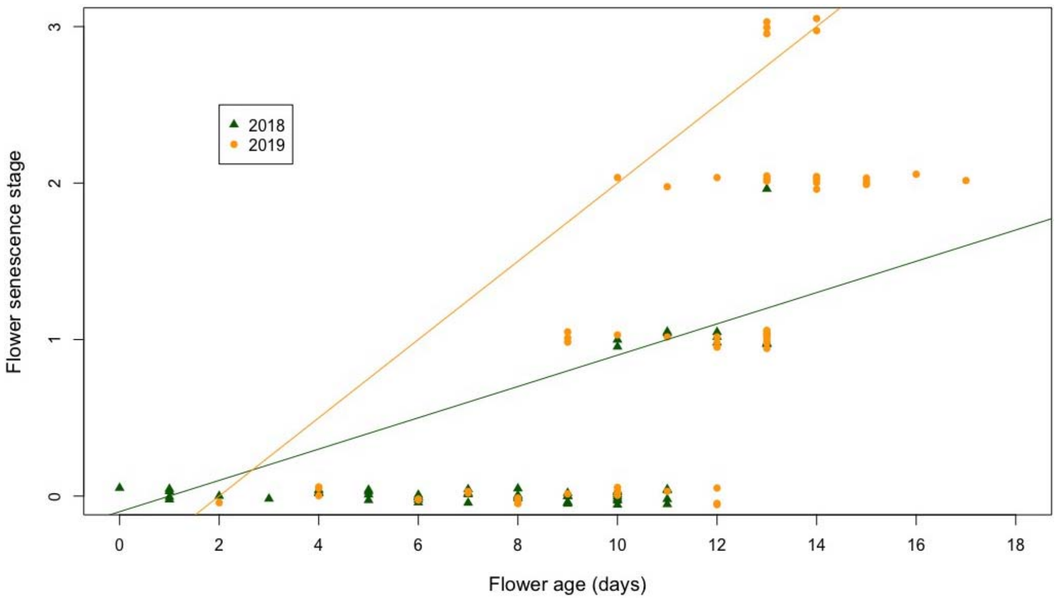

FIG. 2. Flower senescence stage as a function flower age (days) for *Cypripedium kentuckiense*. Lines represent the top 90% quantile regression (tau = 0.9), with flowers in 2018 (green triangles) aging at a slower rate than flowers in 2019 (yellow circles). Data were "jigged" to show points with multiple observations.

There were significant differences in the pace of the aging between 2018 and 2019 (Fig. 2; $F = 73.2$, d.f. = 1,55, $P < 0.0001$), with flowers in 2019 aging faster and in greater numbers.

To compare the actual timing of senescence accumulation, we performed a quantile regression (tau = 0.9), but this time against the relative Julian day (Fig. 3). In 2018, the earliest-opening flowers were observed on day 133 (May 13), whereas in 2019 it was 5 days earlier, on day 128 (May 8). In 2018, we did not observe any flower in the second stage (status one, Fig. 1B) until day 142 (May 22), almost 2 wk into the flowering season (intercept = –14.8 ± 3.2, and slope = 0.11 ± 0.08). In contrast, in 2019 we observed flowers in status one (Fig. 1B) by day 137 (May 17). Two days later, day 139 (May 19), we observed flowers in status two (Fig. 1C); and 3 days later, day 142 (May 22), flowers were in the last stage, status three (intercept = –22.5 ± 2.9, and slope = 0.18 ± 0.4; Fig. 1D). The years were significantly different from each other for the slope ($F = 116$, d.f. = 1,54, $P < 0.0001$), as well as the intercept ($F = 294$, d.f. = 1,54, $P < 0.0001$).

PLANT HEIGHT, FLORAL PRESENTATION, AND DIMENSIONS. Some staminodia had red spots whereas others did not. The staminode covered the stigma and associated anthers (Fig. 4A, B). Burgundy-colored striped and dotted patterns were apparent on the interior surface of the bottom and sides of the labellum (Fig. 4C, D). Floral hairs were present within the interior of the constricted exit channel of the flower approaching the two basal openings and extended to the floor of the inflated interior surface of the labellum where they appeared to be emerging from the red-pigmented epidermis (Fig. 4C, D). A transparent area (window) was also observed at the posterior region of the labellum within the exit channel (Fig. 4 A, D).

A significant difference in scape height of 5 cm was found between years. The average scape height (n = 55) for both years was 69.9 cm (SD = 8.5) for 2018 and 64.8 cm (SD = 9.2) for 2019 (Table 1). Plants in 2018 were 5 cm taller than those in 2019 ($t = 2.1549$, d.f. = 55, $P = 0.0355$). Floral measurements remained consistent for both years and no significant differences were found between years. The number of leaves per plant varied between four and five in both years.

FLORAL TEMPERATURES. We failed to detect any significant difference between ambient air temperature and the temperature of the interior of the labella in either year of the study (2018, $t = 0.898$, $P = 0.7161$; 2019, $t = 0.4821$, $P = 0.9224$).

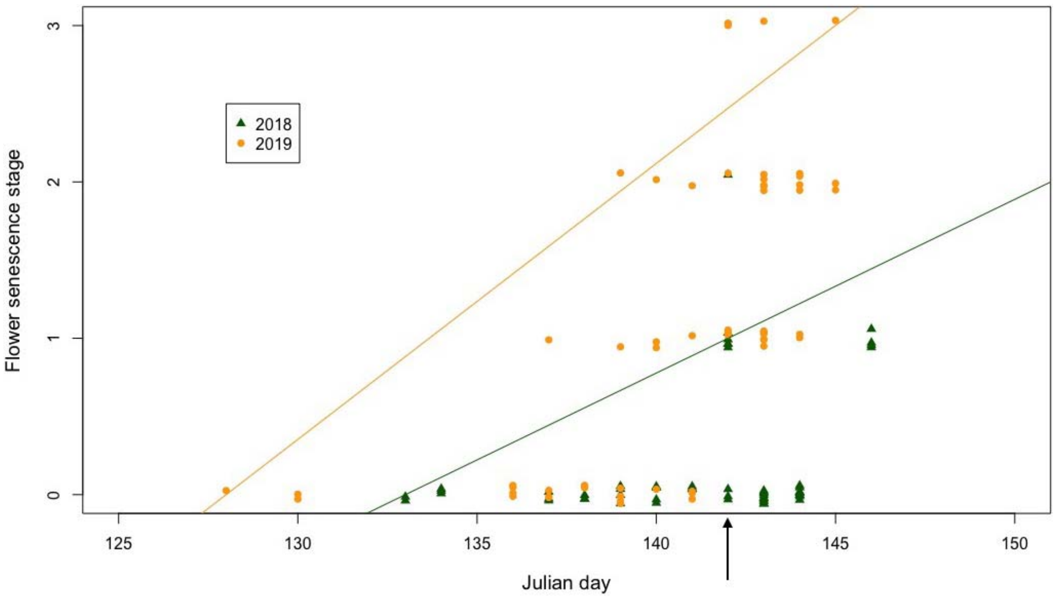

FIG. 3. Flower senescence stage as a function of relative Julian day for *Cypripedium kentuckiense*. Lines represent the top 90% quantile regression (tau = 0.9), with flowers in 2018 (green triangles) emerging later and at a slower rate than flowers in 2019 (yellow circles). Data were "jigged" to show points with multiple observations. The greatest number of bees were captured on May 23 in both years when many flowers displayed signs of senescence (see arrow).

FLORAL ATTRACTANTS. The labellum "slipper" shows a positive UV response. When highlighted by bright sunlight, two "windows" appear at the "toe" (Figs. 5A, B). A faint floral scent similar to that of *Narcissus* cultivars was detected.

ANALYSES OF OPEN, INSECT-POLLINATED FLOWERS VS. BAGGED (UNMANIPULATED) FLOWERS. Pollen tubes were found in only 1 of the 15 bagged flowers of *C. kentuckiense* (Table 2). In this specimen, one pollen tube was observed on the stigma and three pollen tubes reached the apex of the ovary. In contrast, 10 pistils of the 16 open (unbagged) flowers had pollen grains and/or pollen tubes on their stigmas (Table 2). Five of these pistils contained thousands of pollen tubes in thick skeins penetrating style tissue. Four of these pistils contained pollen tubes that penetrated the ovary.

HAND-MEDIATED POLLINATIONS. We observed pollen tube production and penetration in stigmas, styles, and ovaries of all 15 hand-self-pollinated pistils (Table 2; Fig. 5C). Seven self-pollinated pistils had pollen tubes that reached the bases of the ovaries. All of the 14 hand cross-pollinated pistils of *C. kentuckiense* showed pollen tube production on all stigmas with penetration of the style and the ovary (Table 2; Fig. 5D). Pollen tubes in three cross-pollinated pistils reached the base of the ovary.

FRUIT SET AND SEEDS. In 2018, open-pollinated fruit set was 14% (n = 7/50), whereas in 2019 fruit set was 7% (n = 7/94). The mature pod collected on March 12, 2018 measured 3 cm in length. Three hundred seeds were examined from this pod. Most of the seeds (n = 282) observed under microscopy contained a normal-sized embryo (Fig. 6), with 18 showing underdeveloped or absent embryos (see Ren *et al*. 2014).

INSECT OBSERVATIONS AND COLLECTIONS. We collected a total of 116 insects (112 bees, two unidentified beetles in the Curculionidae, and two moths in the Noctuidae) over two seasons (Table 3). Although plants flowered earlier in 2019, overall bee activity remained fairly consistent over 2 yr (n = 54 in 2018; n = 58 in 2019). The first bee carrying the orchid's pollen was caught mid-May (May 13, 2018; May 10, 2019).

Most of the bees collected belonged to the Apidae (n = 97), represented by two *Anthophora* species, *A. abrupta* and *A. bomboides*. The

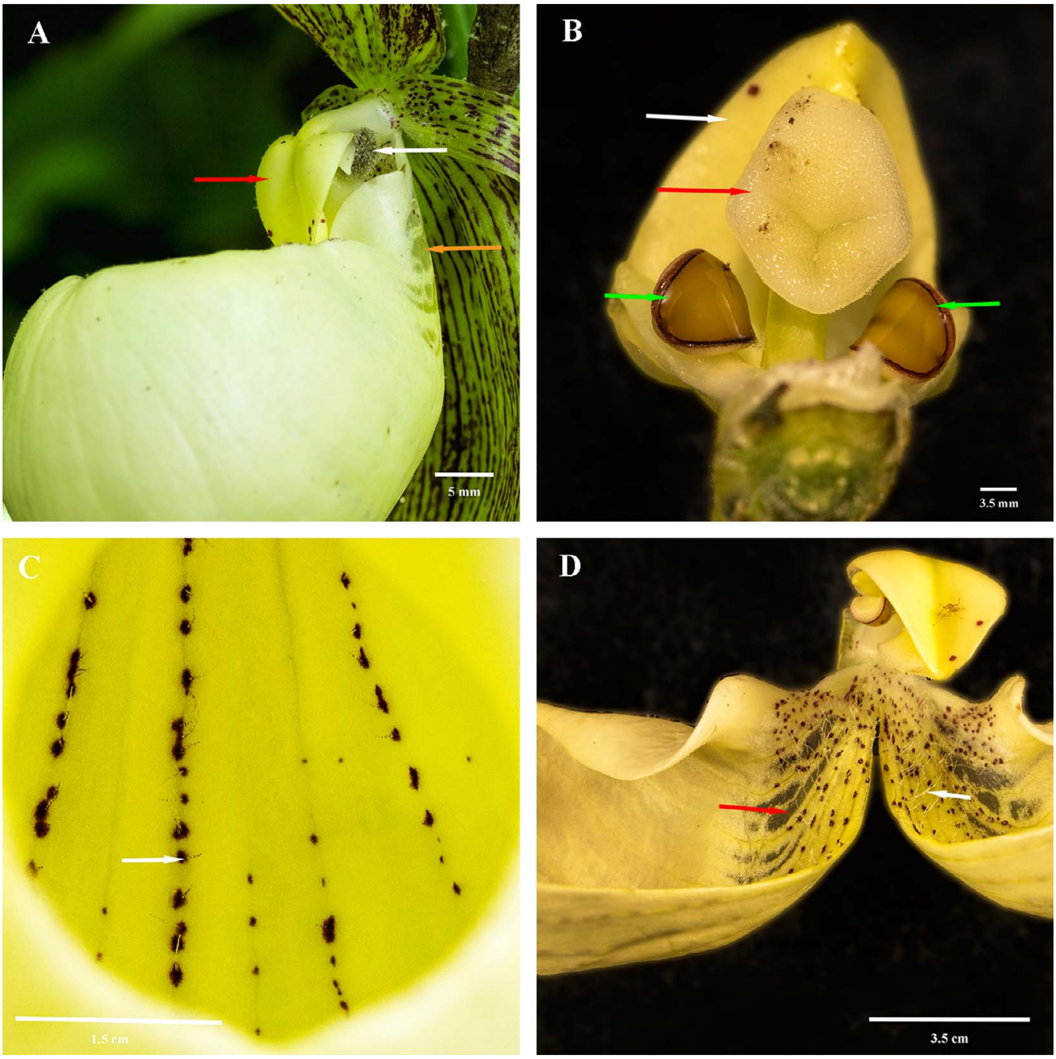

FIG. 4. (A) *Cypripedium kentuckiense* flower showing staminode (red arrow), suspected fungal growth in right basal opening (white arrow), and "windows" (orange arrow); (B) Floral structures of *C. kentuckiense*: dissected staminode (white arrow) covers stigma (red arrow) and anthers (green arrows); (C) Labellum interior of *C. kentuckiense* shows floral hair (white arrow) emerging from red, pigmented area; (D) Dissected flower of *C. kentuckiense* shows floral hairs (white arrow) and "windows" (red arrow).

remaining bees (*Lasioglossum* spp.) belonged to the Halictidae (n = 15; Table 3).

Approximately 35% (n = 39) of all the bees collected were males of *A. abrupta*, whereas all other bees were females (65%; n = 73). All bee species collected are considered pollen generalists (Brooks 1983, McGinley 1986, Gibbs 2011).

More *A. abrupta* (n = 21 males; n = 24 females) were captured in 2018 than in 2019 (n = 18 males; n = 13 females). In contrast, although only four *A. bomboides* were captured in 2018, more than four times that number (n = 17) were caught in 2019. In both years, females of the two species were in flight during the same time period and exhibited no differences in wing wear or worn body hairs, indicating they emerged at approximately the same time.

Both *A. bomboides* and *A. abrupta* showed unique behaviors. Bees were often observed flying around the labella's exterior prior to entering the labellum (see video, Edens-Meier 2018). In 2019, one flower had what appeared to be a fungal

Table 1. Floral and scape measurements of *Cypripedium kentuckiense* (n = 55) in 2018 and 2019 at Big South Fork National River and Recreation Area, TN, at site 1. Scape height is given in cm and the seven floral measurements are in mm.

	2018		2019	
Trait	Mean	SD	Mean	SD
Scape height	69.9	8.5	64.8	9.2
Number of leaves	4.5	0.5	4.5	0.5
Labellum length	46.9	4.4	45.0	5.3
Labellum width	34.0	3.3	31.9	3.2
Entrance length	20.4	2.7	20.7	3.2
Entrance width	15.1	2.3	14.1	1.8
Entrance depth	35.4	2.1	34.2	2.2
Exit length	7.5	0.8	7.2	0.7
Exit width	5.3	0.7	5.3	0.7

growth in the right basal opening (Fig. 4A). On two separate occasions, two *A. abrupta* (one male and one female) were captured exiting this flower prior to the appearance of the fungal growth. However, no bees were observed entering the flower after the fungal growth appeared.

Other visitors to the flowers included *Bombus* species both years. We observed *Bombus* surveying orchid flowers, landing on five flowers, but never entering labella.

METHOD OF EXIT. The method of exiting labella differed by gender and bee species (Table 4). Most male *A. abrupta* exited via the larger, dorsal opening (n = 21), whereas most female bees of this species (n = 15) used the right basal opening to leave the flower. In contrast, *A. bomboides* failed to show any preference in exit choice to exit via the right (n = 9) or left (n = 10) basal openings. Only one *A. bomboides* left the labellum using the larger dorsal opening. This same lack of preference for left or right basal openings was observed in halictids.

BEE VISITATION TIMES. We contrasted the time bees spent in the flower as a function of flower age, using *A. abrupta* and *A. bomboides*. The ANCOVA was significant for all terms (slopes $F = 3.7$, d.f. = 1,91, $P = 0.045$; covariates $F = 7.7$, d.f. = 2,91, $P = 0.0007$; interaction $F = 4.6$, d.f. = 2,91, $P = 0.0157$; Fig. 7), demonstrating that their behavior differed significantly. The differences in the ANCOVA are due to *A. bomboides* behaving differently from both *A. abrupta* males and females (Fig. 7); *A. bomboides* spent more time in younger flowers. As flowers aged, the time spent decreased (intercept = 3.5 ± 0.3, slope = –0.07 ± 0.02; $t = 10.8$, $P < 0.0001$; $R^2 = 22.2$). However, there was no effect of flower aging on either males or females of *A. abrupta*, with the observed slopes not differing significantly from zero (females' slope = 0.01 ± 0.02, $P = 0.5510$; males' slope = –0.02 ± 0.02; $P = 0.5641$).

We then examined the effect of flower senescence status on bee visitation time, contrasting by species and gender as above (Fig. 8). There was a significant effect of the type of bee ($F = 7.1$, d.f. = 2,91, $P = 0.0012$), as well as an interaction effect ($F = 5.1$, d.f.= 1,91, $P = 0.0131$), but not an overall effect of the flower state ($F = 0.4$, d.f. = 1,91, $P = 0.8350$). To explore the interaction effect, we ran separate regressions (Fig. 8), and as before, the differences are driven by *A. bomboides* spending greater amounts of time in flowers in the earliest stage (status zero) decreasing from there (intercept = 2.9 ± 0.32038, $t = 20.3$, $P < 0.0001$; slope = –0.29 ± 0.14, $t = -9.3$, $P = 0.0172$, $R^2 = 26.1$). For males and females of *A. abrupta*, the slopes were not significantly different from zero (females' slope = 0.07 ± 0.06, $P = 0.3007$; males' slope = –0.06 ± 0.12; $P = 0.6670$). One notable difference between the sexes of *A. abrupta* was that only the females visited flowers in the last senescence stage, status three (Fig. 8).

LIGHT GAP. A light gap was not required for bees to be attracted to and enter the flowers. For both years, 24.1% of bees entered the flowers while the flowers stood in a light gap, whereas 71.4% entered flowers standing in shade. We lacked light gap information on the remaining 4.5% of observations.

INSECT CAPTURES. The greatest number of bees caught in a single day in each year was on May 23, 2018 (n = 10) and on May 23 in 2019 (n = 12). Most bees (n = 29) were captured between noon and 1:00 pm. We captured 95% of bees exiting *C. kentuckiense* between 10:00 am and 3:00 pm.

NIGHT VISITATIONS. After carefully removing ribbon that blocked basal openings, labella interiors were checked for the presence of insects. No insects were collected from flowers that had been secured with ribbon overnight for either research season.

INSECT MEASUREMENTS. Bee size varied among species in length, thoracic width, and thoracic depth (Table 3). The average thoracic width for

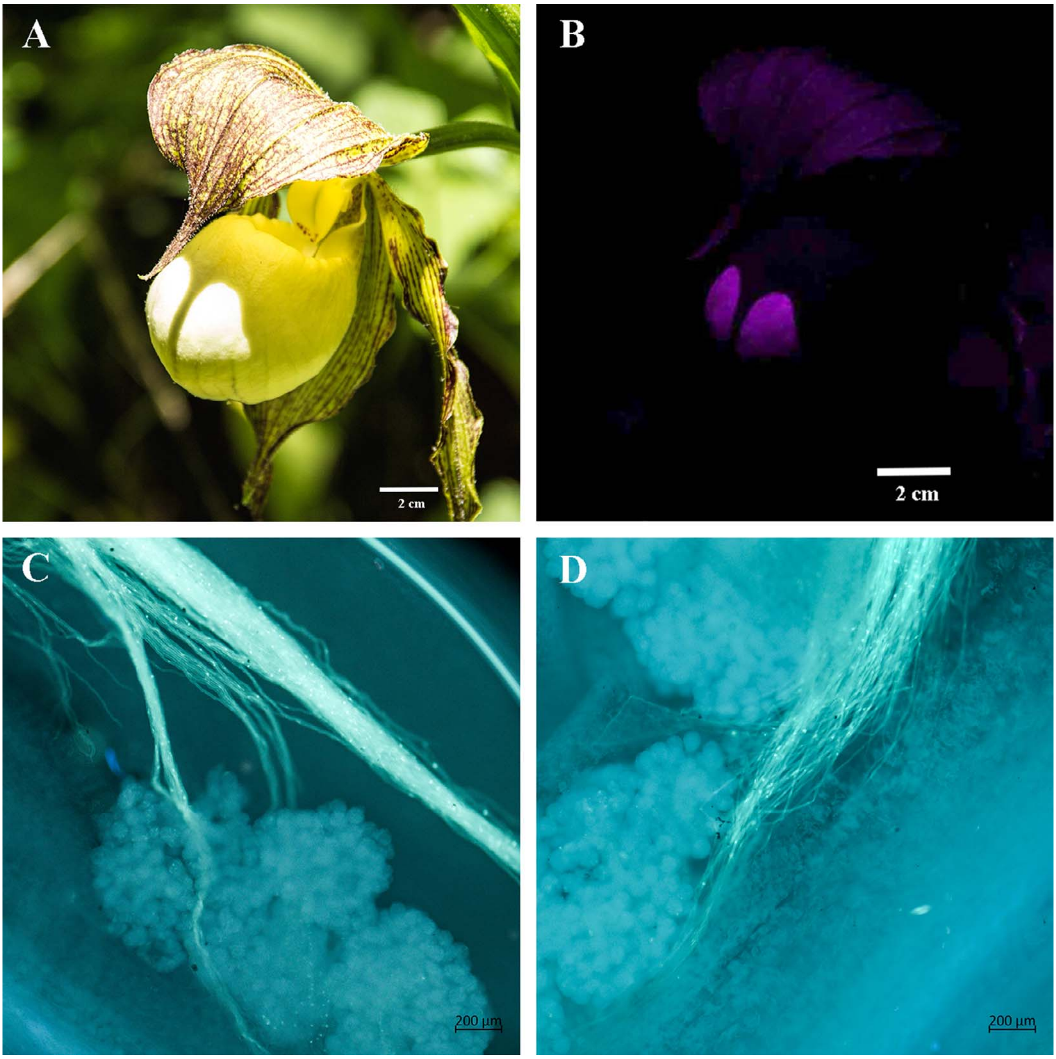

FIG. 5. (A) *Cypripedium kentuckiense* flowers stands in light gap. Notice two brightly lit areas on labellum; (B) *C. kentuckiense* flowers stands in light gap and photographed using an ultraviolet-converted camera. Notice two bright areas on labellum; (C) Pistil of hand self-pollination depicts pollen tubes in ovary; (D) Pistil of hand-cross pollination demonstrates pollen tubes in ovary.

Table 2. Pollen grains per pollen tube in *Cypripedium kentuckiense* pistils for four treatments: Bagged, Open, Hand-self, and Hand-cross.

Treatment	n	Stigma	Style	Ovary upper	Ovary middle	Ovary base
Bagged	15	1	1	1	0	0
Open	16	10	5	4	4	0
Self	15	15	15	15	15	7
Cross	14	14	14	14	14	3

both female *Anthophora* species (5.6 mm) and male *A. abrupta* (5.1 mm) is similar to the average floral basal opening width (5.3 mm; Table 1). However, the average width of *Lasioglossum* species ranged from 1.6 to 3.0 mm. A female *A. abrupta* (17.43 mm length) was the largest bee that carried orchid pollen, whereas the smallest pollen-carrying bee was *Lasioglossum obscurum* Robertson, 1892 (5.32 mm length). For the *Anthophora* species we detected significant differences in body length ($F = 18.3$, d.f. $= 2{,}94$; $P < 0.0001$), with the

FIG. 6. Seeds of *Cypripedium kentuckiense* from pod collected on March 12, 2018. The red arrow is pointing to a seed containing a normal-sized embryo. The green arrow is pointing to a seed containing a small-sized embryo.

males of *A. abrupta* significantly shorter than females of *A. abrupta* (Tukey's HSD = –0.04; $P < 0.001$) and *A. bomboides* (HSD = –0.05; $P < 0.0001$). There was no significant difference in body size between the females of the two *Anthophora* species (HSD = 0.015; $P = 0.309$).

POLLEN LOAD ANALYSES. *Anthophora bomboides* and *A. abrupta* carried the pollen of *C. kentuckiense* more frequently than any other insect visitor (Fig. 9; Table 5). All *A. bomboides* (n = 21) captured exiting *C. kentuckiense* flowers carried the orchid's pollen. Only three of the smaller *Lasioglossum* species carried *Cypripedium* pollen (Table 3).

Eighty-six percent of females of *A. abrupta* carried orchid pollen vs. 69% of the males. Two males of *A. abrupta* carried pure orchid pollen loads. Of the bees observed exiting via the dorsal labellum entrance (n = 33), about 45% (n = 15) also carried orchid pollen. Of the *A. abrupta* males that exited by the dorsal labellum entrance, 27% (n = 9) also carried orchid pollen. Eleven *A. abrupta* females exited via the dorsal labellum entrance and six carried orchid pollen. One female *A. bomboides* exiting via the dorsal labellum entrance still carried orchid pollen.

Cypripedium pollen was also found on one of the two noctuid moths and one of the two weevils. One moth exited the labellum via the larger dorsal opening, but the remaining moth and two weevils were physically extracted from the labella.

Bees carrying orchid pollen also carried pollen of other coblooming plants (Table 6; Fig. 10B). Grains of *Lonicera japonica* Thunb. were frequently found on *A. abrupta* (n = 48), whereas both species of *Anthophora* carried pollen of unidentified species in the Ericaceae. *Lasioglossum coriaceum* Smith, 1853 carried the greatest number of pollen morphotypes (10 different morphotypes). One male and one female *A. abrupta* each carried eight different pollen morphotypes. Females of *A. abrupta* and *A. bomboides* carried various amounts of pollen in their hind tibial and basitarsal scopa, including full or nearly full, partial (50%, 30%, 20%), and no pollen load at all.

FLORAL TRAPS. No dead insects were observed within labella nor did any insect appear to be trapped within labella.

BEE BEHAVIOR. Bees within labella often entered the constricted exit channel, reverse their direction, reenter the labella, and continue to fly around the large chamber. This behavior was often repeated several times. On May 21, 2018, a female *A. abrupta* remained inside the labellum for 15 min displaying this repetitive, retracting behavior 53 times prior to exiting the labellum via the basal opening. *Anthophora* species were often observed chewing and clawing floral tissue within the constricted exit channel between the two basal openings. One male *A. abrupta* was observed

Table 3. Bee taxa, gender, number collected from *Cypripedium kentuckiense*, bee measurements, and number of bees that carry the orchid's pollen. All measurement units are in mm (± standard deviation).

Taxon	Gender	n	Total length	Thoracic width	Thoracic depth	Pollen
Anthophora abrupta	male	39	12.7 (0.8)	5.1 (0.4)	4.7 (0.5)	27
	female	37	14.0 (1.4)	5.6 (0.3)	5.0 (0.4)	32
Anthophora bomboides	female	21	14.4 (0.9)	5.6 (0.6)	4.8 (0.3)	21
Lasioglossum coriaceum	female	5	9.7 (1.1)	3.0 (0.2)	2.7 (0.1)	1
Lasioglossum obscurum	female	8	5.7 (0.2)	1.6 (0.1)	1.3 (0.2)	2
Lasioglossum bruneri	female	1	9.02	2.46	1.9	0
Lasioglossum sp.	female	1	6.3	1.8	1.1	0

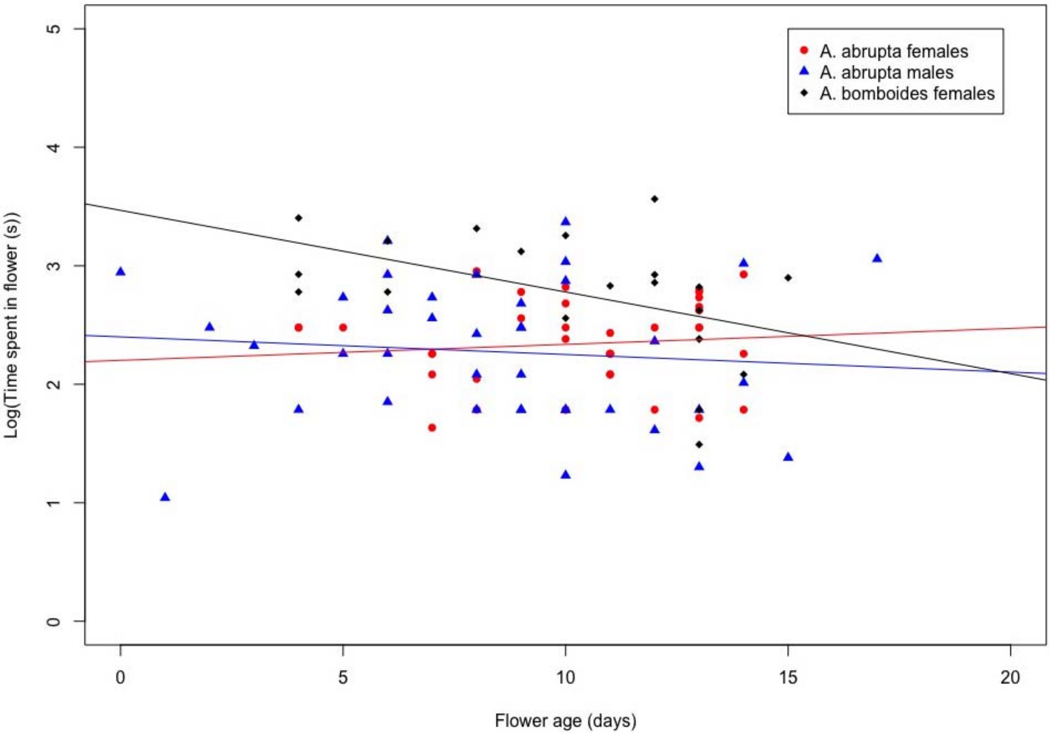

FIG. 7. Time spent inside the flowers of *Cypripedium kentuckiense* by bees as a function of flower age. Lines represent the simple linear regression for each bee species and gender (Red circles = *A. abrupta* females; blue triangles = *A. abrupta* males; black diamonds = *A. bomboides* females) showing *A. bomboides* spending significantly greater amount of time in the youngest flowers. Data were "jigged" to show points with multiple observations.

chewing one of the anthers (see video, Edens-Meier January 13, 2020), foraging on epidermal tissue within *C. kentuckiense* and contacting the anther as it continued this activity (see video, Edens-Meier February 2, 2020).

Both *Anthophora* species were observed squeezing through one of the two basal openings upon exiting. A shiny smear of pollen was often observed on the bee's dorsal thorax upon exiting (Fig. 10A). Prior to exiting, *Anthophora* species often lodged themselves under the upper edge where the labellum curved inward near the basal openings and buzz. Pollen loads from coblooming plants were often observed being carried on scopa (Fig. 10B). Bees frequently clung to the edge of labella or on dorsal sepals and groomed prior to flying away. Floral hairs (Fig. 10C) and pollen (Fig. 10D) were found in the claws of two specimens of *A. bomboides*.

Discussion. PHENOLOGY AND FLORAL SENESCENCE. Visual and olfactory floral displays attract potential

Table 4. Method of exit from labella by bees visiting *Cypripedium kentuckiensis*.

Taxon	Gender	Total	Larger dorsal opening	Right basal opening	Left basal opening	Unknown
Anthophora abrupta	male	39	21	5	6	7
Anthophora abrupta	female	37	11	15	7	4
Anthophora bomboides	female	21	1	9	10	1
Lasioglossum coriaceum	female	5	0	1	1	3
Lasioglossum obscurum	female	8	0	2	2	4
Lasioglossum bruneri	female	1	0	0	0	1
Lasioglossum sp.	female	1	0	0	1	0
Total		112	33	32	27	20

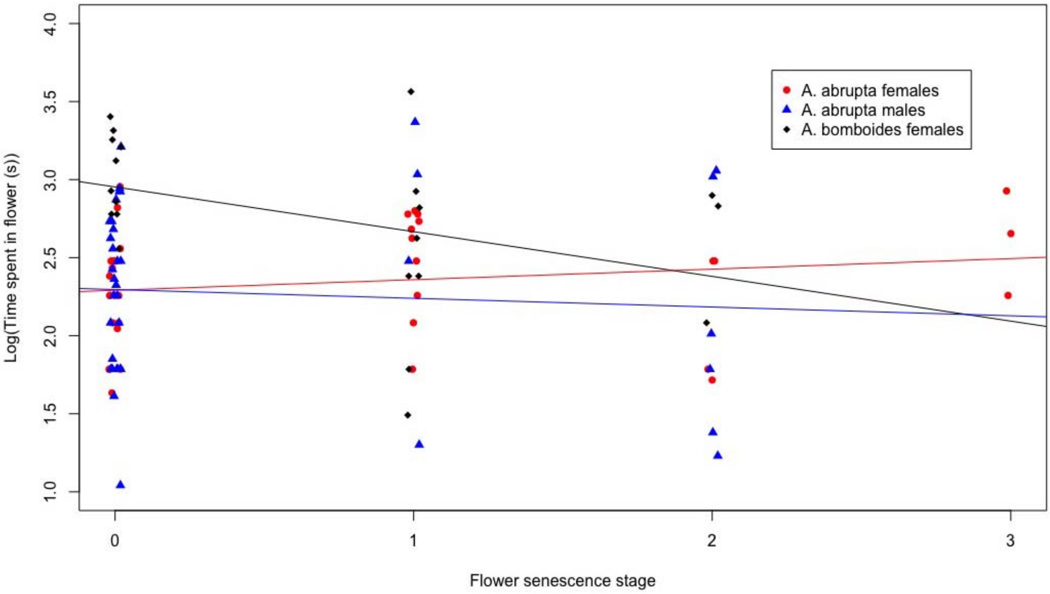

FIG. 8. Time spent inside the flowers of *Cypripedium kentuckiense* by bees as a function of flower senescence stage. Lines represent the simple linear regression for each bee species and gender (Red circles = *Anthophora. abrupta* females; blue triangles = *A. abrupta* males; black diamonds = *Anthophora bomboides* females) showing *A. bomboides* spending significantly greater amount of time in the flowers' first stage of senescence. Data were "jigged" to show points with multiple observations.

pollinators, whereas floral signals expressed during senescence inform pollinators that the attractive display is finished (Reid and Chen 2007). In our study, the pattern of floral senescence was significantly different between years, with flowers in 2018 spending 3 to 5 days more in the earliest stage than in 2019. This provides bees that prefer the earliest floral stages longer periods for floral visitations, and thus ensures a greater probability of pollen removal and deposition. In fact, 62% of the orchid flowers in 2018 were still in early stages of senescence by the end of May, allowing both species of *Anthophora* ample visitation opportunities. That was not the case in 2019. Flowers not only started opening earlier than bee emergence, but they also had shorter floral lifespans. By the end of the second week of the 2019 season, most flowers had entered the third or fourth stage. *Anthophora bomboides* appeared capable of detecting floral senescence, spending less time in the second stage, and avoiding later stages. Therefore, a potential consequence is a decreased likelihood of pollination by *A. bomboides* in years when the higher daily maximum that the developing flowers experience is significantly greater than the long-term average. This observation must be corroborated by future studies.

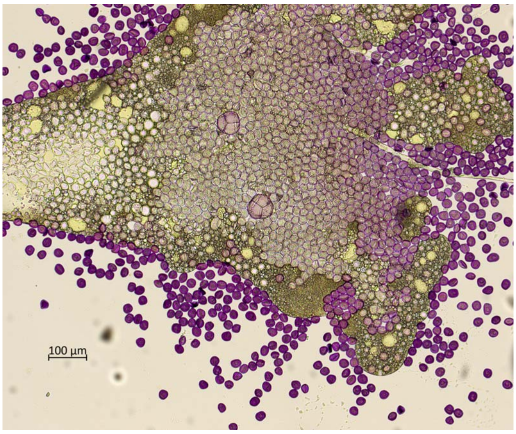

FIG. 9. Pollen sample derived from female *Anthophora bomboides* stained with Calberla's fluid. Incomplete staining due to lipophilous pollen coat retaining grains in a matrix. Tetrads identified from resident Ericaceae.

PLANT HEIGHT, FLORAL PRESENTATION, AND DIMENSIONS. Floral measurements remained consistent between the two research seasons except for taller scapes in 2018. Changes in scape height might influence reproductive success. Fruit set in

Table 5. Bee species and gender carrying pure loads of *Cypripedium kentuckiense* pollen only (Cyp pollen only), a mixed load of *C. kentuckiense* pollen and other species of pollen (Cyp pollen + other spp. pollen), only other species of pollen, and no pollen.

Bee species and gender	Cyp only	Cyp + other spp.	Other spp.	No pollen
Anthophora abrupta (f)		32	5	
Anthophora abrupta (m)	2	25	12	
Anthophora bomboides (f)		21		
Lasioglossum bruneri (f)			1	
Lasioglossum coriaceum (f)		1	4	
Lasioglossum obscurum (f)		2	4	2
Lasioglossum sp. (f)			1	
Total	2	81	27	2

C. kentuckiense was higher in 2018 (14%) than in 2019 (7%). A significant correlation was also found between scape height and reproductive success in *Cypripedium acaule* Aiton (O'Connell and Johnston 1998). We wonder if a combination of extra rain and higher temperatures in April 2019 channeled resources favoring earlier flowering at the expense of extending scape elongation.

Floral Temperatures. As ambient air temperatures increase, so does the temperature within the labellum's interior. Similar results were found in *C. parviflorum* (see Edens-Meier *et al.* 2018).

Floral Attractants. Webster (1886, p. 359) referred to "transparent lines at the upper end of the labellum" of *Cypripedium calceolus* L. and suggested that insects were drawn to light coming through this translucent tissue. Faegri and van der Pijl (1979), refer to these translucent areas a "light windows." Approximately 25% of rewarding flowers reflect UV (Chittka *et al.* 1994), and UV patterns are believed to function as floral guides for insects (Lunau 2006). Ultraviolet photography of *C. kentuckiense* indicates that parts of the labellum show a "window" response when it stands in bright light. The discernible scent of the flower is unsurprising, because chemical components of the fragrance were analyzed by Barkman *et al.* (1997) and found to be rich in fatty alcohol acetates.

Analyses of Open, Insect-Pollinated Flowers vs. Bagged (Unmanipulated) Flowers. Mechanical autogamy occurs when flowers pollinate themselves and is estimated to occur in 15% of species of North American orchids (Ackerman 1984). Because only a minute number of pollen tubes were observed in the ovary apex in only one bagged flower in this study, mechanical self-pollination appears to be unlikely in our populations. However, the system appears "leaky," allowing a few pollen grains to contaminate a stigma. This might have occurred if the tulle bag was not secured properly, allowing an insect to enter, and/or if an insect was bagged initially with the bud.

Hand-Mediated Pollinations. Although mechanical self-pollination was uncommon in this population, hand-pollination experiments showed that pistils lacked a late-acting, prezygotic, self-incompatibility mechanism. This is the typical response in hand-pollination experiments in other *Cypripedium* species (Edens-Meier *et al.* 2010, 2014). Furthermore, we observed both *Anthophora* species partially exiting via the basal openings adjacent to dehiscent anthers and then retreating back into the orchid's labellum. Therefore, insect-mediated intrafloral selfing remains possible.

Fruit Set and Seeds. Producing one of the largest *Cypripedium* flowers in the Northern Hemisphere on tall scapes does not guarantee reproductive success. Although 62% of the open (unbagged) pistils contained orchid pollen, the flower-to-fruit ratio was low in this species. Similar results of low fruit production in large-flowered *Cypripedium* species were reported in North America and Asia, including *C. acaule*, *C. reginae*, and *Cypripedium tibeticum* King ex Rolfe

Table 6. Pollen morphotypes of bees carrying mixed loads. AST = Asteraceae; Bc = *Bignonia capreolata* L.; CAR = Caryophyllaceae; Ck = *Cypripedium kentuckiense*; ERI = Ericaceae; LON = *Lonicera*; PIN = *Pinus*; Sa = *Sisyrinchium angustifolium* Mill; UE = Unidentified eudicot; UM = Unidentified monocot.

Species (gender)	AST	Bc	CAR	Ck	ERI	LON	PIN	Sa	UE	UM
Anthophora abrupta (f)		2		32	8	28		3	9	8
Anthophora. abrupta (m)	1			27	6	20	2		5	
Anthophora bomboides (f)		2		21	11	9		5	8	
Lasioglossum coreacum (f)	1	4	1	1					5	
Lasioglossum obscurum (f)				2					2	1
Total	2	8	1	83	25	57	2	8	29	9

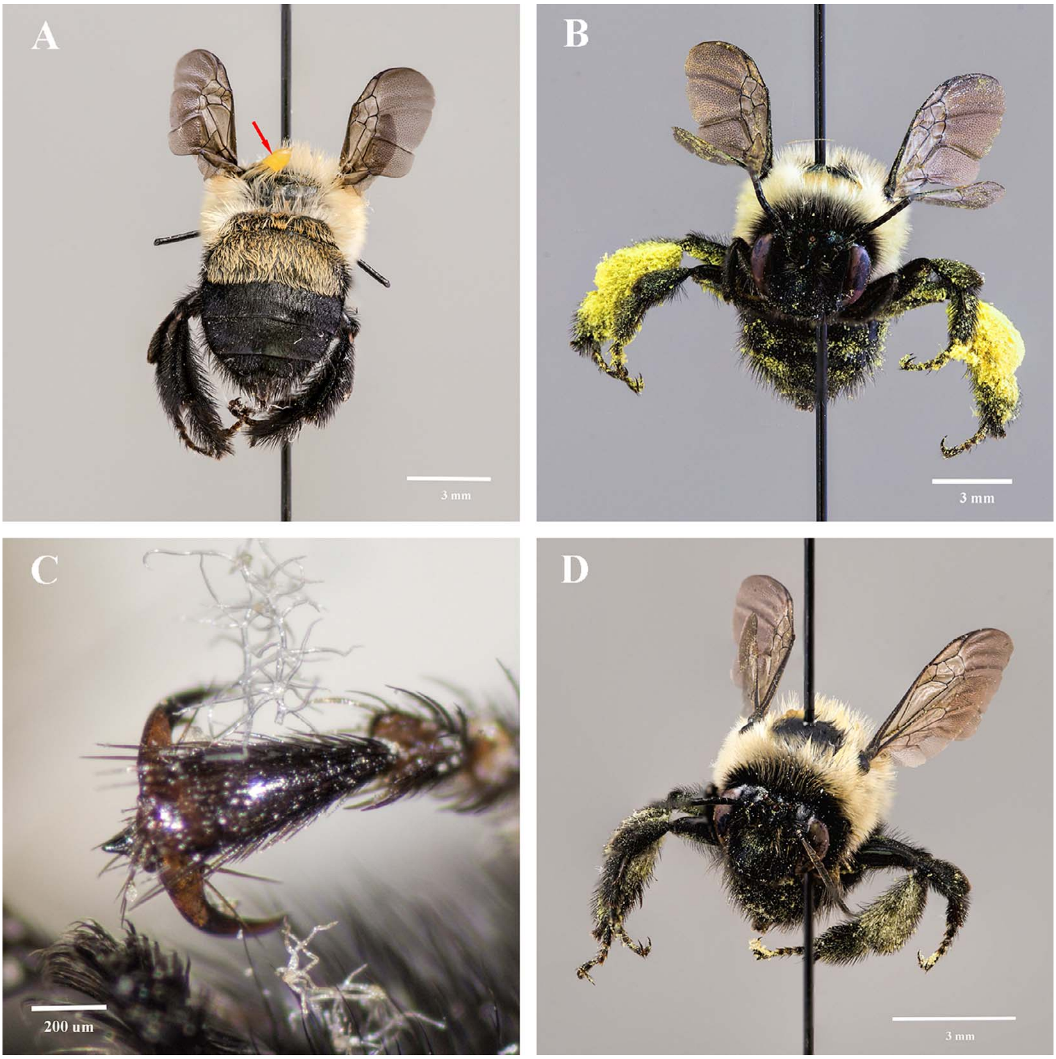

FIG. 10. *Anthophora bomboides* carrying pollen and floral hairs. (A) Mass of orchid pollen (red arrow) adheres to dorsal thorax of *A. bomboides* female; (B) A heavy load of yellow pollen carried on legs of female *A. bomboides*; (C) Floral hairs adhere to foot claw of *A. bomboides* female; (D) Pollen clings within claws of *A. bomboides* female.

(Tremblay *et al*. 2005; Edens-Meier *et al*. 2010, 2014).

Numerous biotic and abiotic factors affect fruit maturation and seed production in this genus (Bernhardt and Edens-Meier 2010). Orchid species, regardless of lineage, often produce more flowers than fruits (Tremblay *et al*. 2005). In addition, flowers and immature fruits of varying plant species are naturally aborted (Stephenson 1981). Pollination of orchid flowers does not guarantee fruit maturation (Tremblay *et al*. 2005). Lipow *et al*. (2002) found that far more ovaries contained pollen tubes than the number of fruits produced in *Cypripedium fasciculatum* Kellogg ex S. Watson. Developing buds of *C. parviflorum* were aborted following flooding (Edens-Meier *et al*. 2010). Predation can also limit fruit set (Walsh *et al*. 2014), destroying up to 75% of the fruit of *C. reginae* (Edens-Meier *et al*. 2010). Finally, post-zygotic rejection following vector-mediated self-pollination might contribute to low fruit set, as in the epidendroid, *Calanthe yaoshanensis* Z.X. Ren & H. Wang (Ren *et al*. 2014).

Examination of seeds in a single, dehiscent fruit indicated successful embryonic development in the majority of ovules and, indeed, more successful than other orchids which depend upon cross-pollination (Ren *et al*. 2014, Tao *et al*. 2018). The contents of the single fruit indicated normal embryonic development.

INSECT OBSERVATIONS AND COLLECTIONS. With such infrequent visits by weevils and moths, the dominant insects observed and collected exiting *C. kenuckiense* over two seasons were the bee genera *Anthophora* and *Lasioglossum*. The two *Anthophora* species visited *C. kentuckiense* both years. Females of both *Anthophora* species emerged at approximately the same time in this study. However, in at least one other study in Maryland, *A. bomboides* emerged 2 wk later than *A. abrupta* (Norden 1984).

Both *Anthophora* species have been recorded visiting a large variety of plants in many different families (Brooks 1983), but appear to be rare visitors to orchids. *Anthophora abrupta* and *Anthophora terminalis* Cresson, 1869 were identified as pollinators in *C. reginae*, but only one specimen of each species was caught (Edens-Meier *et al*. 2011). Females of both *Anthophora* species were superior to males in transporting orchid pollen at our sites, primarily because males of *A. abrupta* preferred to exit the labellum via the large, dorsal opening. In addition, this is the first known record of *A. bomboides* visiting and carrying orchid pollen.

METHOD OF EXIT. The method of exiting labella differed between male and female *A. abrupta*. Males preferred the larger, dorsal opening whereas most female bees used the right basal opening to leave the flower. *Anthophora bomboides* and bees of *Lasioglossum* did not show a preference for exiting via the right or left basal openings.

BEE VISITATION TIMES. *Anthophora bomboides* spent more time in younger flowers than in older flowers. Only two female *A. abrupta* were observed visiting flowers in the last senescence stage. In contrast, visits of both sexes of *A. abrupta* did not correlate with specific periods of floral aging.

The floral life span for *C. kentuckiense* ranged from 13 to 16 days. This was similar to *C. parviflorum* (Edens-Meier *et al*. 2010, 2018), a member of the same North American clade. By May 23, 2018, and May 23, 2019, the date that most bees were captured, many flowers were already 15 days old and showed signs of senescence. To maximize pollination, anthesis and bee emergence should synchronize (Tremblay *et al*. 2005, Vaudo *et al*. 2015, Edens-Meier *et al*. 2018). We propose that reproductive success of this orchid depends on the peak emergence of the two *Anthophora* species within our study site. Furthermore, reproductive success in *C. kentuckiense* is also linked to the flower releasing viable pollen throughout its life span. However, floral phenology does not appear to be fixed in this species. If *C. kentuckiense* produces flowers earlier each year, the emergence of *A. abrupta* and *A. bomboides* could become out of sync, as observed in the rare and federally listed *Clematis socialis* Kral and its primary pollinator, *Anthophora ursina* Cresson, 1869 (Wall *et al*. 2003). Of course, emergence dates and flight periods for *A. abrupta* are known to vary as much as a month from year to year, presumably as a function of temperature (Rau 1926, Norden 1984). The decoupling of a plant species dependent on only a few pollinators, as found in *C. kentuckiense* populations, could result in its future extirpation from these Tennessee sites.

LIGHT GAP. Unlike in the allied species, *C. montanum*, studied by Bernhardt *et al*. (2014), a light gap was not necessary to attract bees to flowers of *C. kentuckiense*. The primary pollinators were more likely to visit the flowers when they stood in the shade.

INSECT MEASUREMENTS AND IDENTIFICATIONS. Floral architecture and pollinator dimensions are of primary importance in *Cypripedium* pollination (Li *et al*. 2008, Edens-Meier *et al*. 2014). Although flowers of *C. kentuckiense* allow insects of all sizes to enter the labellum, bee dimensions, especially thoracic width and depth, determine whether bees will be able to deposit pollen on the surface of the hanging stigma and contact dehiscent anthers upon exiting (Edens-Meier *et al*. 2011, 2018). The two *Anthophora* species in this study were significantly larger than the smaller halictids. These larger bees contacted the dehiscent anther as they squeezed through the basal openings, but this occurred infrequently in the smaller, narrower *Lasioglossum* species.

POLLEN LOAD ANALYSES. Eighty of the 97 *Anthophora* individuals captured carried the orchid's pollen. Although *A. abrupta* and *A. bomboides* carry pollen from other coblooming angiosperms, the pollination system of *C. kentuckiense* appears to be highly canalized, depending on two large bee species to transport pollen. However, our pollen load analyses showed repeatedly that both *Anthophora* species were generalist, polylectic foragers. We note that *Lonicera japonica*, an invasive but extremely fragrant plant species, serves as a pollen and nectar resource for both *Anthophora* species. *Anthophora abrupta* was also found to use *L. japonica* as a pollen source in Maryland (Norden 1984).

FLORAL TRAPS. *Cypripedium* labella are believed to serve as traps (Bänzinger *et al*. 2008, Case and Bradford 2009, Bernhardt *et al*. 2014), but this might not apply to *C. kentuckiense*. The unusual behavior displayed by both *Anthophora* species can be interpreted in a number of ways. However, in two research seasons, none of the bees appeared to be trapped within labella and we never observed bee corpses as did Bernhardt *et al*. (2014).

FLORAL REWARDS. The evidence of floral rewards offered by *C. kentuckiense* remains elusive. One hypothesis is that floral rewards are not being offered and the ancestral architecture of *Cypripedium* functions universally within all species to temporarily detain insects within an inflated labellum. Then, the insects' movements are canalized as they exit constricted openings and their dorsa are smeared with viscous pollen. However, *Cypripedium* species vary in size, height, color, pigmentation, and fragrance. With such variation, a second hypothesis for floral rewards exists (Jiang *et al*. 2020).

Tremblay *et al*. (2005) identified food, floral fragrances, and waxes/resins as three floral rewards offered by orchids. We wonder whether substances produced within the labellum of *C. kentuckiense* serve as a resource for some bees. Both *Anthophora* species spent an unusually long time flying, buzzing, clawing, and chewing on floral tissue within the labellum, especially tissue between the two basal openings, as compared to most of the smaller *Lasioglossum* species.

It is worth noting that captured females of *A. abrupta* and *A. bomboides* carried various amounts of pollen in their hind tibial and basitarsal scopa. We interpret this to mean that some females were interrupting pollen-foraging trips to spend time in the labellum of the orchid for reasons that are not clear. We suggest that *Anthophora* species entering *C. kentuckiense* might be collecting one or more substances (*e.g*., floral hairs, fragrance molecules) that might be beneficial to bees, as previously suggested by Edens-Meier *et al*. (2011). Darwin (1877) observed floral hairs within labella and recognized the possibility that insects could be attracted to trichomes secreting a viscid fluid. Although Darwin believed the fluid might be nectar, Knoll (1922, as cited in Nilsson 1979) identified the fluid as oil (unspecified). Stoutamire (1967) reported that floral hairs within the labellum were often chewed. However, Nilsson (1979) concluded that definitive evidence of bees eating floral hairs or secretions was lacking. We found floral hairs clinging to the claw of a female *A. bomboides*, which can be interpreted as either accidental (using the floral hairs to pull themselves toward the basal openings; applying thoracic vibration) or intentional collection of hairs. We also note that secretory hairs appear to offer essential rewards to syrphid flies in *C. subtropicum* (Jiang *et al*. 2020).

The labellum of *C. kentuckiense* is spacious enough for bees to fly freely within the interior. Are the primary pollinators collecting floral scents to attract mates or control bee parasites? Certain bee species are suspected of using floral compounds to control microbial pathogens within the nest (Danforth *et al*. 2019).

Half of the *A. abrupta* were males. No males of *A. bomboides* visited the flowers. Males of *A. abrupta* are known to chew tissue of *Pastinaca sativa* L. and possibly other materials (Rau 1926), collect the exudate on their labral mustaches, and mark certain objects that outline a flight path that might facilitate mating (Norden and Batra 1985). We wonder if *A. abrupta* males entered *C. kentuckiense* labella to obtain floral compounds for the same purpose. Similar behavior and labral morphology are unknown in *A. bomboides* (Brooks 1983, Norden 1984). We encourage future researchers to investigate whether *C. kentuckiense* is offering rewards.

CONSERVATION. We encourage conservation agents and the public to actively protect this imperiled species and dissuade individuals from collecting these orchids as described by Yarian (1939) and Brown (1995). Finally, seed banking is also encouraged to preserve genetic diversity within this species.

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