Killer magnolias: assessing the effect of *Magnolia macrophylla's* stigmatic secretion on its floral visitors.

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Flowers of many plants are attractive to insects and facilitate reproduction; therefore, species with flowers lethal to visitors, such as *Magnolia macrophylla* (big leaf magnolia; Magnoliaceae), present a paradox. Previous observations suggest that a stigmatic secretion produced by *M. macrophylla* is the cause of lethargic insect behavior and death within flowers. This study documents the behavior of the floral visitor taxa *Xylocopa virginica* (eastern carpenter bee), *Apis mellifera* (European honey bee), *Bombus impatiens* (common eastern bumble bee), Formicidae (ants), Halictidae (sweat bees), and Coleoptera (beetles) and quantifies the effect of stigmatic secretion on worker *B. impatiens*. I recorded video observations of floral visitors to five *M. macrophylla* ssp. *macrophylla* and three *M. macrophylla* ssp. *ashei* trees at the Arnold Arboretum of Harvard University in Boston, MA to ascertain visitor behavior within flowers. To assess if stigmatic secretion was the cause of lethargy, I performed assays testing 66 laboratory-raised worker *B. impatiens* receiving either distilled water (diH_2O) or stigmatic secretion application. Videos were scored blind for behaviors including the total length and longest period of immobile, active behavior (as a measure of lethargy), the number of leg flails, and instances of abdomen and antennae grooming. At the Arnold Arboretum, floral visitors, mostly X. *virginica* and *A. mellifera*, were observed within flowers and either tried to escape or displayed lethargic behavior. During lab assays, stigmatic secretion application only increased *B. impatiens* abdomen grooming. These results do not directly support stigmatic secretion as the cause of lethargic behavior; however, they provide a strong basis for future research on *Magnolia macrophylla* floral visitor interactions.

Introduction

Bees are abundant and critical members of many ecosystems who pollinate nearly 80% of all flowering plants (Moisset and Buchmann 2011). Many genera, including *Apis*, *Bombus*, *Xylocopa*, and *Lasioglossum*, have developed interesting life histories. The European honey bee (Wilson and Carril 2016, p.246), *Apis mellifera*, of the family Apidae, is a moderately sized bee ranging from 1 to 1.5 cm. The species forms eusocial colonies with three castes: queens acting as reproductive females, drones acting as reproductive males, and female workers that perform a variety of jobs that include rearing young, colony defense, and nectar and pollen foraging. European Honey Bees are generalist pollinators who turn foraged nectar into honey for overwinter food storage. Because of this, humans have transported and established the European honey bee globally. Bumble bees (Wilson and Carril 2016, pp.242-243), of the family Apidae and genus *Bombus*, are moderately sized bees ranging from 1.3 to 2.5 cm. Like the European honey bee, bumble bees divide their colonies into queens, workers, and drones, however, bumble bee colonies only last a single growing season and therefore have no need for honey

production. Bumble bees are generalist pollinators capable of pollinating 8 to 10 times more effectively than European honey bees depending on the plant species due to their greater pollen carrying capacity, shorter time spent at flowers, and longer active season. Additionally, the genus is found globally, with its highest diversities in the mountainous regions of Europe and Asia. A third member of the family Apidae, Carpenter bees (Wilson and Carril 2016, pp.203-204), genus *Xylocopa*, are on average exceptionally large bees ranging from 1.3 to 2.8 cm. Carpenter bees are solitary, with a female raising one to two broods per year in gallery nests. Galleries consist of a tunnel, often with multiple compartments, made in plant tissue or wood which are carved out by the strong mandibles of carpenter bees. While out foraging, carpenter bees are generalists yet frequently return to flowers of the same species. Their large size allows them to carry considerable pollen loads but also hinders them from entering tubular flowers. In such instances, carpenter bees may chew through floral tissue and rob nectar. Carpenter bees are found mainly in the tropics and sub-tropics although ten species are found within North America. Finally, sweat bees (Wilson and Carril 2016, pp.132-133), from the family Halictidae and genus *Lasioglossum*, are a collection of small bees ranging from 0.25 to 1.3 cm. Due to high species diversity within the genus, sweat bees can be solitary, semi-social, or eusocial. The majority of species are generalist pollinators; however, exceptions exist such as the subgenus *Sphecodogastra* which only visits flowers of the evening primrose (*Oenothera*). Although small, sweat bees are distributed abundantly throughout the world with higher diversity North of the equator.

In comparison to bee pollination, beetle (Coleoptera) pollination is most common in early diverged angiosperms. Flowers with beetle pollination provide floral rewards through nectar, pollen, stigmatic secretions, and edible tepals rich in carbohydrates (Thien 1974, Dieringer *et al.* 1999). Beetles became the primary pollinators of magnoliids and basal monocots but have since developed pollination relationships with six families of petaloid monocots and fourteen families of eudicots (Bernhardt 2000). The overabundance of beetles as pollinators of primitive angiosperms is likely a result of Coleoptera species diversity during the Cretaceous when early angiosperms first diverged (Young 1986). Beetles are commonly attracted to two floral morphologies: the chamber blossom, an arrangement of petals or tepals set in several whorls and commonly found in magnolia species, and the painted bowl, a bisexual structure with a deep cup shape formed by flattened perianth whorls (Bernhardt 2000). Furthermore, pollinator entrapment is used by many protogynous magnoliid species who rely on the same visiting insect, in most cases a beetle, to both deposit pollen onto female structures and later carry pollen away to other flowers. Because these species have a period of several hours between their reproductive floral phases, they use their petals or tepals as a trap to seal pollinators within the flower as it transitions from female to male (Pijl 1960).

The traits of Magnoliaceae flowers vary by species, and some are hypothesized to play key roles in the attraction of pollinators. For example, in *Magnolia tamaulipana*, heat production is used to attract pollinators. Larger flowers are better able to distribute vaporized scent compounds via heat currents and attract greater numbers of pollinating beetles (Dieringer *et al.* 1999). Ultraviolet reflectivity as an attractive trait has been documented in *Magnolia grandiflora* and *Magnolia macrophylla* ssp. *macrophylla* and ssp. *ashei*. Flowers of *M. macrophylla* have a purple-blue pattern at the base of their tepals which is assumed to be adaptive and play a role in the attraction of specialized bee and beetle

pollinator communities (Thien *et al.* 1995). Finally, scent production by magnolia flowers can aid in pollinator attraction. In *Magnolia ovata*, scent production plays a role in the attraction of the dynastid scarab beetle *Cyclocephala literata* in both the female and male flowering phases (Gottsberger *et al.* 2012).

Although floral nectar acts primarily as a source of carbohydrates for visitors, it is more than just sugar water and often contains secondary metabolites. Nectar secondary metabolites typically consist of flavonoids, alkaloids, terpenoids, or phenolics (Palmer-Young *et al.* 2018). Several proposed hypotheses exist as to how these compounds regulate floral interactions (Adler 2000). Among them are the pollinator fidelity hypothesis, which posits that secondary compounds in nectar encourage conspecific pollen deposition by increasing pollinator fidelity (Rhodes and Bergdahl 1981, Adler 2000). If a floral species produces nectar with secondary compounds, the compounds would discourage generalist pollinators from visiting. However, a specialist pollinator may have resistance to the toxins and therefore, the flower would act as a source of low competition rewards encouraging specialist pollination. This hypothesis relies on the assumption that specialist pollinators are more effective than generalist pollinators, but the literature does not strongly support this idea (Schemske and Horvitz 1984). A second idea is the nectar robber hypothesis. Hypothetically, plants subjected to nectar robbery could use secondary compounds to deter robbers (Barlow *et al.* 2017). This hypothesis relies on the assumption that nectar robbery reduces plant fitness, which has inconsistent results including neutral and positive benefits for some flowering species (Zimmerman and Cook 1985, Arizmendi et al. 1996, Morris 1996). The antimicrobial hypothesis posits that the sugar and nutrient-rich composition of nectar makes it a target for microbial consumption and degradation. Therefore, secondary compounds could deter or kill microbes within nectar (Baker 1978, Guerrant and Fiedler 1981, Hagler and Buchmann 1993). While the above hypotheses rely on the assumption that secondary compounds in nectar are adaptive, the pleiotropy hypothesis proposes that secondary compounds are found in nectar for non-adaptive reasons. For example, secondary compounds may be the result of past adaptations that are no longer needed and have not yet been lost. Alternatively, secondary compounds may be produced in vegetative tissue connected to the phloem, which allows the compounds to travel to the nectaries of the plant and be excreted (Adler 2000). Secondary compounds appear in the nectar of countless flowers (Palmer-Young *et al.* 2018) and are potentially responsible for bee mortality on several occasions.

Bee death during floral visits is well documented and often tied to secondary compounds in nectar. For instance, in 1932, numerous bees were found dead or dying within *V. caliornicum* flowers and high rates of bee death upon returning to an apiary occurred only during *V. caliornicum* flowering. The cause was suspected to be a toxic substance produced by *V. caliornicum* flowers (Vansell 1993). In another instance, the nectar of *Sophora microphylla* (yellow kowhai) caused a narcotic effect and was lethal to adult honeybees. The cause of mortality appeared to be alkaloids toxic to bees which reach the nectaries of *S. microphylla* flowers and are excreted with its nectar (Clinch *et al.* 1972). The nectar of *Corynocarpus laevigata* (Karaka tree) is also hypothesized to be toxic to honeybees. Apiaries suffered bee mortality during *C. laevigata* flowering, with mortality rates dropping to normal when the apiary was moved out of range of these flowers. While the cause of bee mortality was the nectar of *C. laevigata* flowers, the nectar compound responsible was not found (Palmer-Jones 1962). Finally, the

flowers of *Tilia tomentosa* (silver linden) have caused massive bee death, with the cause assumed to be the nectar carbohydrate mannose. However, recent literature analysis has shown there is no experimental evidence for the toxicity of mannose to bees (Koch and Stevenson 2017). While secondary compounds in nectar may cause bee death during floral visits, this does not explain observations of bee death on flowers without nectar.

Magnolia macrophylla (big leaf magnolia; Magnoliaceae) has a flowering cycle typical of other magnolia species; however, its flowers can cause lethargic behavior and death of visiting bees. *M. macrophylla* consists of two subspecies: *macrophylla* which is native to the southeastern United States and *ashei* which is endemic to the panhandle of Florida (Azuma *et al.* 1999, Thien 1974). Both subspecies produce exceptionally large, protogynous flowers with a chamber blossom morphology and are active for two days (Thien 1975). During their first day, flowers of both subspecies are in their female phase and form a cup shape consisting of tightly wrapped tepals with a small, 6 to 10 cm in diameter opening at the top of the cup. Between the hours of 9:00 am and 1:00 pm, receptive stigmas emit a fragrant smell and a liquid secretion which is later reabsorbed. As flowers progress into their male phase on the second day of flowering, the tepal cup opens, tepals lay perpendicular to the receptacle and the flowers reach their fully extended diameter. Additionally, the stigmas brown and are no longer receptive while stamens begin to dehisce and release pollen for foraging visitors. Unlike other Magnoliaceae species, the flowers of *M. macrophylla* remain open while transitioning from female to male. After the end of the second day, the white tepals begin to brown and shrivel, leaving only the carpels remaining (Figure 2) (Thien 1974). Interestingly, female flowers of both subspecies trap and kill certain taxa of bees, moths, and butterflies while leaving beetle pollinators unharmed (Thien 1974, Thien 1975, Thien *et al.* 1995). Furthermore, the lethal nature of female *M. macrophylla* flowers may be selective, as sweat bees of the genus *Lasioglossum* are reported to be unaffected by the flowers (Thien *et al.* 1995).

Several studies have briefly described observing dead bees in the flowers of *Magnolia macrophylla* trees (Thien 1974, Thien 1975, Thien *et al.* 1995). Preliminary observations by Dr. William Friedman suggested that a stigmatic secretion emitted during the female phase of *M. macrophylla* flowering may be the cause of bee death. I investigated this phenomenon using five flowering *Magnolia macrophylla* ssp. *macrophylla* and three ssp. *ashei* trees located at the Arnold Arboretum of Harvard University in Boston, MA (Arnold Arboretum hereafter). The first goal of this study was to observe the behavior of floral visitors after entering the flowers of *M. macrophylla* trees. To achieve this, I conducted field observations, including written notes, photographs, and video recordings, of *M. macrophylla* ssp. *macrophylla* and ssp. *ashei* floral visitors. Furthermore, I aimed to verify previous observations that beetles do not die while within *M. macrophylla* flowers (Thien 1974, Thien 1975). The second goal was to determine whether stigmatic secretion is the cause of lethargic behavior and death of visiting bees. To assess this, I conducted laboratory assays where I applied diH_2O or stigmatic secretion to laboratory raised worker *Bombus impatiens* and video-recorded their behavior, scoring all recordings blind with respect to treatment. Understanding the behavior of visitors to *M. macrophylla* flowers and the effect of stigmatic secretion on bees will clarify the role of stigmatic secretion in *M. macrophylla* floral visitor death.

Methods

Field Observations and Assays

Fieldwork was conducted during May, June, and July of 2019 at the Arnold Arboretum in Boston, Massachusetts (42°18'02.4"N 71°07'33.5"W) using eight mature trees of *M. macrophylla*, five from ssp. *macrophylla* and 3 from ssp. *ashei* (Table 1, Figure 1). I observed visitors of *M. macrophylla* flowers to document visitor behavior within a flower. I scouted trees during weekdays without rain from June 7th - 28th, 2019 for flowers with trapped floral visitors at both standing height and at 3 meters high via the use of an orchard ladder. I scouted all of the female and male flowers that I could reach on the eight trees once every hour, between the hours of 10 am to 3 pm. If I found a flower with a visitor, I recorded an observation. Observations included written notes, photographs, and video recordings lasting up to 5 minutes of visitor activity within a flower. If I observed a bee trapped within a flower that appeared to be lethargic, I recorded a 2-minute or greater video of its behavior. I specifically checked whether the bee showed an inability to coordinate its legs, to fly, or to climb the inner walls of the tepals, along with any other abnormal behavior.

Cryogenic Scanning Electron Microscopy

I performed cryogenic scanning electron microscopy (Cryo-SEM) of *M. macrophylla* ssp. *macrophylla* and ssp. *ashei* inner whorl tepal surfaces to ascertain if a wax layer was present on tepals that may prevent bee escape from female flowers. All cryo-SEM images were captured using a JEOL JSM-6010 LV Scanning Electron Microscope at the Weld Hill Research Building at the Arnold Arboretum. Tepals used in Cryo-SEM were collected from flowers one hour before imaging. Tepal sections were cut from the axial surface of *M. macrophylla* flowers at four locations: the purple pigmented area (~3 cm from tepal base), the area directly above the purple pigment (~5 cm from tepal base), the concave curve of the tepal (~10 cm from tepal base), and the top of the tepal (~17 cm from tepal base). To prepare sections for imaging I mounted them onto an observation block using rice glue and froze them in liquid CO_2. Images were captured at 1000x magnification. After imaging, wax crystal density was assessed by counting wax crystals in the top right 50 μM^2 of both the top of the tepal and the purple pigmented area section images from each subspecies.

Cryo-SEM of *M. macrophylla* ssp. *macrophylla* and ssp. *ashei* pollen was performed to capture images of pollen grain structure and surface texture. Stamens and pollen were collected during flowering from both *M. macrophylla* ssp. *macrophylla* and *M. macrophylla* ssp. *ashei* flowers from June 10th, 2019 until June 25th, 2019. I only collected stamens that were still attached to the receptacle from flowers that did not show signs of herbivory. I stored both the stamens and pollen from a single flower in a microcentrifuge tube for a total of 22 samples with an estimated average mass of 150 mg. Samples were labeled with a collection date and abbreviations that marked the tree they were collected from and stored in a -80 °C freezer. Before mounting, I separated pollen from stamens using a mesh netting on which I rubbed the stamens, causing pollen release. I mounted pollen onto observation blocks using double-sided adhesive tape and froze samples in liquid CO_2 for imaging. All images of pollen were captured at 250x and 1000x magnification.

Flower Collection and Freeze Drying

I collected 22 flowers from *M. macrophylla* ssp. *macrophylla* and ssp. *ashei* trees and freeze-dried them using a Labconco Freeze Dry System to aid in the identification of compounds that cause lethargic behavior in bees. I collected 12 female flowers from *M. macrophylla* ssp. *macrophylla* trees and 10 flowers from *M. macrophylla* ssp. *ashei* trees during their peak stigmatic secretion emission period (9 am to 1 pm). I then stored collected flowers in a -80 ℃ freezer and divided them into outer whorl tepals, inner whorl tepals, and the reproductive stalk (carpels, stamens, and receptacle) before drying. Tissues were freeze-dried for 24 hours and labeled with the date they were dried and an abbreviation indicating which tree the flower came from. For long term storage, tissues were placed in airtight plastic bags and kept in a dark, dry area.

Stigmatic Secretion Collection and Preparation for Liquid Chromatography-Mass Spectrometry Analysis

Stigmatic secretion was collected during flowering from both *M. macrophylla* ssp. *macrophylla* and *M. macrophylla* ssp. *ashei* flowers from June 10th, 2019 until June 25th, 2019. I collected stigmatic secretion for later use in assays and liquid chromatography-mass spectrometry. Collection occurred from the stigmas of female flowers using a 100 µL micropipette during the hours of peak stigmatic secretion emission (9 am to 1 pm). I stored the collected stigmatic secretion from a single flower in a microcentrifuge tube for a total of 88 samples with an estimated average volume of 100 µL. All samples were labeled with a collection date and an abbreviation that marked the tree they were collected from. Samples were stored on ice while in the field and later transferred to a -80 ℃ freezer for long term storage.

Stigmatic secretion samples were sent to Dr. Phil Stevenson at the Royal Botanic Gardens, Kew, London for liquid chromatography-mass spectrometry analysis. Analysis was performed to ascertain if compounds toxic to bees, including neurotoxins, are present within the stigmatic secretion of *M. macrophylla* flowers. To provide samples for LC-MS analysis, I took ten 20 µL volumes of stigmatic secretion from previously collected samples, six from *M. macrophylla* ssp. *macrophylla* tree M-3 and four from *M. macrophylla* ssp. *ashei* tree A-1 (Table 1). I diluted half of the samples from each tree with 80 µL of 200 proof ethanol. The remaining 5 samples were kept pure. I then labeled the microcentrifuge tubes that the ten samples were stored in with the tree abbreviation the stigmatic secretion came from, the date of shipping, and if they were pure stigmatic secretion or diluted with ethanol. All samples were packaged in a styrofoam shipping container with dry ice. Samples were express shipped to Dr. Stevenson on July 1st, 2019 and arrived on July 3rd, 2019.

Stigmatic Secretion Assays

At the University of Massachusetts at Amherst, I performed stigmatic secretion assays during August and September of 2019 to assess the relationship between insect contact with stigmatic secretion and lethargic behavior. The first round of assays was performed with pairs of bees from one of the following taxa: *Apis mellifera* (n=20 individuals), *Bombus impatiens* (n=26 individuals), or *Lasioglossum* (n=20 individuals). I collected *Apis* and *Bombus* from the Franklin Dining Commons Permaculture Garden in Amherst, Massachusetts (42°23’21.1”N 72°31’23.1”W) on flowering plants of

the genera *Toona, Pycnanthemum*, and *Agastache*. I captured bees of the genus *Lasioglossum* at the UMass Crop Animal Research and Education Center in South Deerfield Massachusetts (42°28'45.53"N 72°34'46.06"W) when they became trapped in experimental field tents. Once bees were captured, I briefly placed them into a freezer where they were sedated. The time required for sedation varied by genera, with *Lasioglossum* averaging 1 min, *Apis* averaging 5 min, and *Bombus* averaging 8 min. Once a pair of bees was sedated, I placed them into separate 0.7 L deli cup observation chambers (8.25 cm tall and 11.5 cm in diameter). I randomly assigned one bee as a control, which received 20 µL of distilled water (diH_2O) placed randomly onto the underside of the bee's abdomen and legs or the top side of the bee between its wings via pipette. The second bee received 20 µL of stigmatic secretion directly onto the same areas. I selected stigmatic secretion samples at random from the samples I collected at the Arnold Arboretum. Samples were thawed before their use and were quickly refrozen in a -80 ℃ freezer after a 20 µL extraction. Video recording began before control and treatment volumes were applied and continued for 10 min after application.

After the first round of assays was completed, Dr. Lynn Adler and I determined that the quality of the collected data was not sufficient to determine if stigmatic secretion application caused behavioral changes in treated bees, and I do not report results from these assays. Numerous alterations were made to the experimental protocol to enable accurate data collection and analysis. The first notable change was the use of Biobest laboratory raised *Bombus impatiens* and the selection of only worker bees for assays. This change standardized the environmental conditions that the bees were kept in before testing. Additionally, by limiting testing to only *Bombus impatiens,* a larger sample size (n=66) could be used. The second issue was the method of application of diH_2O or stigmatic secretion to bees. Chilling bees allowed for easy application of control or treatment liquids but created problems in identifying if slow or limited behavior was due to stigmatic secretion application or bees recovering from cold temperatures. By applying diH_2O or stigmatic secretion to bees held within a bee squeezer for exactly 30 seconds I ensured that altered behavior was not a result of varied application methods. Furthermore, diH_2O or stigmatic secretion application was localized to the underside of the thorax and abdomen of a bee instead of to both the top side and underside of its body. Video recordings of bees were standardized to 10 minutes and videos were taken of a single bee at a time to ensure the video quality was sufficient for analysis. Bees were placed into smaller, 0.3 L, deli cups which better enabled clear video focus and recording by decreasing the space within which the bee could move. The final notable change was how videos were scored. I scored videos from round one while knowing the control or treatment status of the bee. In round two, I scored without prior knowledge of the bee's status ensuring that my preconceived notions of lethargic activity did not influence my analysis of each bee's behavior. Additionally, I scored videos in round 2 using the Behavioral Observation Research Interactive Software (BORIS) (Friard and Gamba 2016).

The second round of assays was performed between December 2019 and February 2020 using one laboratory raised female *Bombus impatiens* per assay. Bees (n=66) were taken from 6 commercial colonies acquired from Biobest USA Inc. (Leamington, Ontario). In December, I performed 18 assays using 3 colonies: 12 assays on December 11th, 2019 and 6 assays on December 13th, 2019. On January 30th, 2020, I performed 16 assays using the same 3 colonies. In February, I performed 32 assays using 3 new colonies. I performed 12 assays on February 6th, 2020 using 2 of the 3 new colonies, and 6 assays on

February 11th, 2020 using the same colonies as February 6th, and 14 assays on February 13th, 2020 using all 3 new colonies. When performing an assay, I pulled a worker bee from a colony in a dark room under a red light, and as a bee was pulled, I randomly assigned it as control or treatment via a coin flip. The bee's status was recorded on a datasheet along with the colony the bee was pulled from, date and time of application, the bee's unique ID consisting of a number between 1 and 66, and the stigmatic secretion sample information if it was a treatment assay. I then placed the bee into a bee squeezer and held it against a cloth mesh using a foam plunger. If a bee was assigned control, I applied 20 µL of diH_2O directly onto the underside of the bee's thorax and abdomen. If a bee was assigned treatment, I applied 20 µL of stigmatic secretion to the same areas. The same stigmatic secretion selection and storage protocols were used as in the first round of assays. Once either diH_2O or stigmatic secretion was applied to the bee it was left in the squeezer, contacting the liquid for 30 seconds. I also started a hand timer at the same time liquid was applied. Once the thirty seconds were over, I placed the bee into a 0.3 L deli cup (4.5 cm tall and 11 cm in diameter). I then quickly moved the hand timer and deli cup containing the bee onto a lab bench under natural light and video recorded the movements of the bee for 10 minutes. After recording assays, I placed the bees into individual collection tubes. I labeled the tubes with the date of the assay, the bee's unique ID number, and a C or T corresponding to the bee's assigned control or treatment status. Bees were frozen, later pinned, and are stored at the Adler Laboratory at the University of Massachusetts at Amherst.

Video Scoring and Data Analysis

I scored video recordings from the second round of assays using BORIS for both time-dependent and time-independent behaviors. Recordings were scored blind to the control or treatment status of the assay to avoid human biases. Time-dependent behaviors were scored by the number of seconds they occurred for and included mobile, active behavior, immobile, active behavior, and immobile, inactive behavior. Mobile, active behavior was displayed when a bee constantly moved around the observation chamber. A bee displayed immobile, active behavior when it remained in the same location for 5 seconds or greater and groomed its antennae or abdomen, flailed its legs, or moved its wings. Immobile, inactive behavior was displayed when a bee remained in the same location for five seconds or greater and did not move its body at all. Time-independent behaviors were chosen based on the most frequently observed lethargic bee behaviors recorded during field observations and were scored for the number of times they occurred. These included antennae grooming, abdomen grooming, and leg flailing. Antenna grooming was when a bee used its front set of legs to groom its antenna and abdomen grooming was when a bee used its rear set of legs to groom its abdomen. Leg flailing occurred when a bee moved either of its thorax legs up and down rapidly in a 180-degree arc.

Behavior data were aggregated using Microsoft Excel and analyzed using box plots and one-tailed, heteroscedastic t-tests to determine if treatment bees behaved differently than control bees. Due to experimental error, assay 19 was replaced with assay 63 and assay 38 was replaced with assay 65. The remaining assays numbered 61, 62, 64, and 66 were discarded to keep the total number of scored assays at 60. I then analyzed and graphed the total duration and longest interval of a bee's immobile, active behavior in seconds. Immobile, non-moving behavior did not occur frequently enough to analyze. Additionally, I analyzed and graphed the number of leg flailing, antenna grooming, and

abdomen grooming events. Duration values were rounded to the nearest whole number to allow for t-test analysis in Excel. Time-independent behaviors that did not occur for a certain bee were recorded as a zero value. I designated numbers outside three standard deviations above or below the mean as outliers. Because outliers were not a result of improper measurements or faulty equipment but instead accurate data values from scored assays, all t-test data sets included outliers. Excluding these outliers did not qualitatively change results.

Table 1. Mature *Magnolia macrophylla* individual abbreviations, subspecies, and accession numbers. Accession numbers were assigned by curatorial staff at the Arnold Arboretum.

Abbreviation	Subspecies	Accession #
A-1	ashei	396-96*B
A-2	ashei	1067-64*A
A-3	ashei	396-96*A
M-1	macrophylla	961-89*A
M-2	macrophylla	959-89*A
M-3	macrophylla	961-89*B
M-4	macrophylla	960-89*A
M-5	macrophylla	299-2001*A

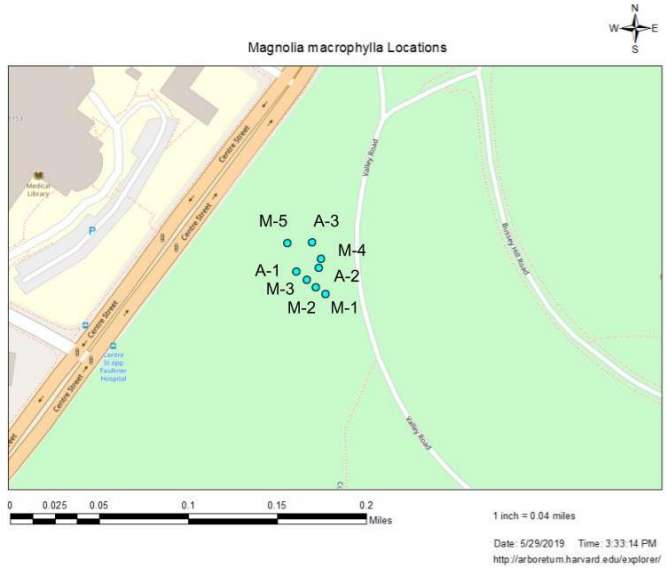

Figure 1. A map of mature *Magnolia macrophylla* trees cultivated at the Arnold Arboretum. Members of subspecies *macrophylla* are labeled M-1 through M-5. Members of subspecies *ashei* are labeled A-1 through A-3. The map was taken from the Arnold Arboretum's explorer webpage.

Results

Floral Morphology and Phases

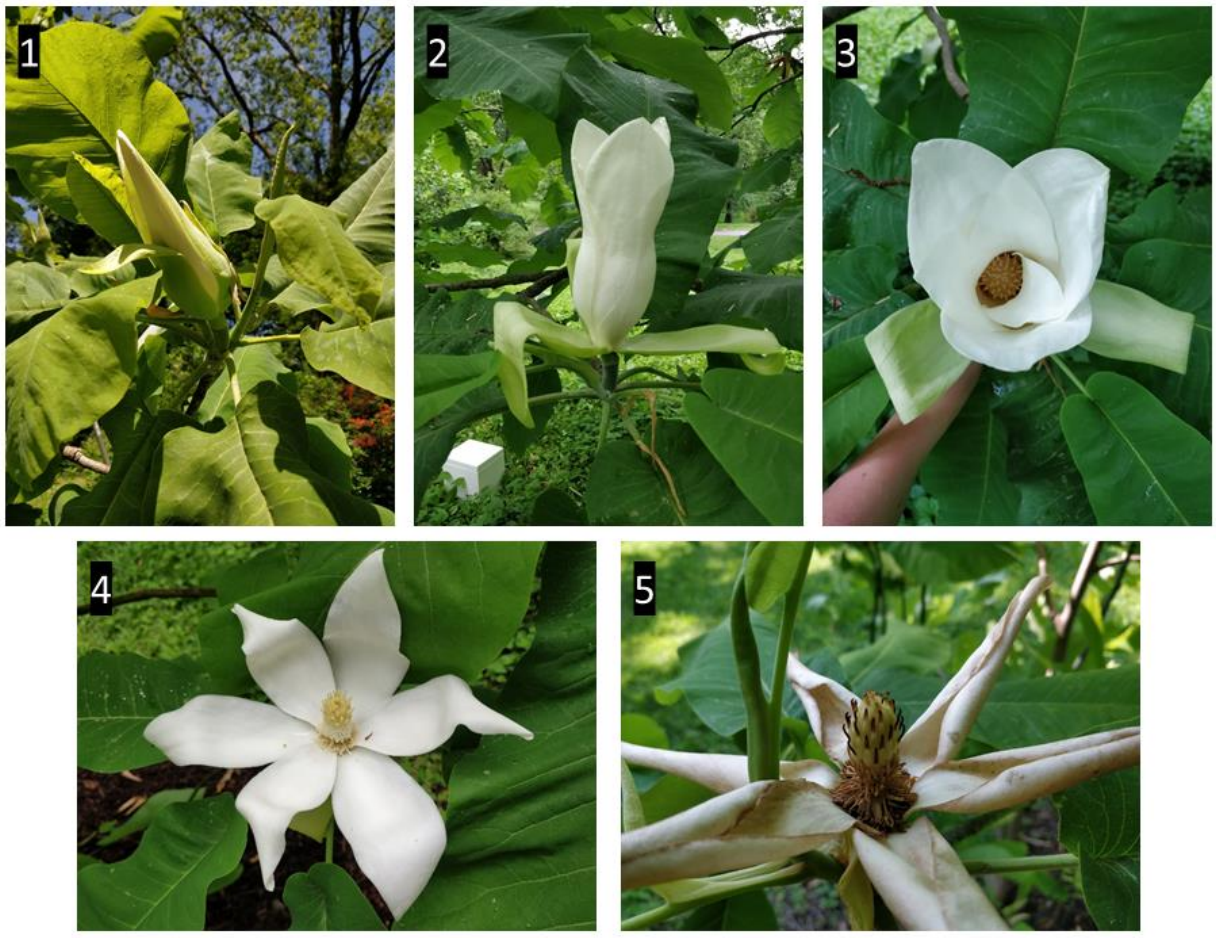

Figure 2. Phases of *Magnolia macrophylla* ssp. *macrophylla* and ssp. *ashei* flowering. Image 1 depicts a floral bud one day before opening. Images 2 and 3 are of the same, 1-day old flower in its female phase. Image 2 is a horizontal view of the flower and image 3 is a vertical view. Image 4 depicts a 2-day old flower in its male phase. Image 5 depicts a flower which has begun to degrade one day after its male phase. Photos were taken on June 3rd, 2020 at the Arnold Arboretum by Liam Cleary.

Magnolia macrophylla flowers morph between four stages during their 2-day flowering period (Figure 2). On day 0, floral buds consist of tepals that form a tight, conical chamber around the reproductive organs (Figure 2, Image 1). When buds burst, they open into female flowers with a vertical "cup" morphology formed by their 6 inner and outer whorl tepals. Seen within the center of the tepal "cup" opening are the stigmas, which produce stigmatic secretion between 9 am and 1 pm. The tepal whorls of both subspecies constrict to a diameter of 6 to 10 cm roughly two-thirds of the way up from the base of the flower, forming an opening through which floral visitors enter. Female *M. macrophylla* ssp. *macrophylla* flowers range from 16 to 23 cm tall and female *M. macrophylla* ssp. *ashei* flowers range from 8 to 15 cm tall (Figure 2, Images 2 & 3) (Thien 1974). On their third day, flowers go into their

male phase and both tepal whorls lie perpendicular to the receptacle. Stamens have begun to dehisce, releasing pollen, and can be seen around the base of the tepals. Stigmas no longer produce secretion and are not receptive. When fully open, *M. macrophylla* ssp. *macrophylla* flowers have a diameter of 32 to 46 cm and *M. macrophylla* ssp. *ashei* flowers have a diameter of 16 to 30 cm (Figure 2, Image 4) (Thien 1974). One day after their male phase, flowers have begun to degrade. Tepals brown and later fall off of the reproductive stalk, stamens have fully dehisced, and stigmas are inactive (Figure 2, Image 5).

Floral Visitor Observation Data and Images

During field observations of *M. macrophylla* trees, I recorded both female (n=26) and male (n=11) flowers with visitors from the taxa *Apis mellifera*, *Xylocopa virginica*, *Bombus impatiens*, Formicidae, Halictidae, and Coleoptera. *A. mellifera* (n=29) was the most abundant species found within flowers. I observed *A. mellifera* foraging on the stamens of male flowers (n=8) in groups of 1 to 3 bees who were not lethargic (Figure 3). However, on female flowers, I observed *A. mellifera* (n=21) trapped within the floral cup. Bees were unable to fly past the inwardly sloping tepals which formed the opening of the cup and were forced back onto the stigmas. They would then contact stigmatic secretion and become lethargic shortly after (Figure 4). Lethargic *A. mellifera* moved slowly or remained immobile, often had outstretched wings, rapidly lifted their thorax legs up and down in a 180° arc (leg flails), and exhibited frequent abdomen and antennal grooming. *X. virginica* (n=23) were the second most abundant species I observed and were the only taxa that I found lethargic on male flowers. Male flowers with lethargic *X. virginica* (n=9) lacked stigmatic secretion but consistently had herbivory on their reproductive organs (Figure 5). On one occasion I observed an *X. virginica* bee chewing on floral reproductive structures with its mandibles, indicating the herbivory seen in other observations may be caused by *X. virginica* bees. Within female flowers, I observed *X. virginica* (n=14), who like *A. mellifera*, were unable to overcome the inwardly sloping tepals and later became lethargic after stigmatic secretion contact (Figure 6). I observed *B. impatiens* (n=2) within female flowers who showed lethargic behavior similar to that of *A. mellifera*. Formicidae (n=3) were observed foraging on pollen from dehisced stamens that collected on the tepals of male flowers and never showed lethargy. I observed a non-lethargic Halictidae (n=1) resting on the top of the tepal cup of a female flower which flew away when disturbed by the wind. The final taxon I observed was Coleoptera (n=2) on both female and male flowers who did not show signs of lethargy.

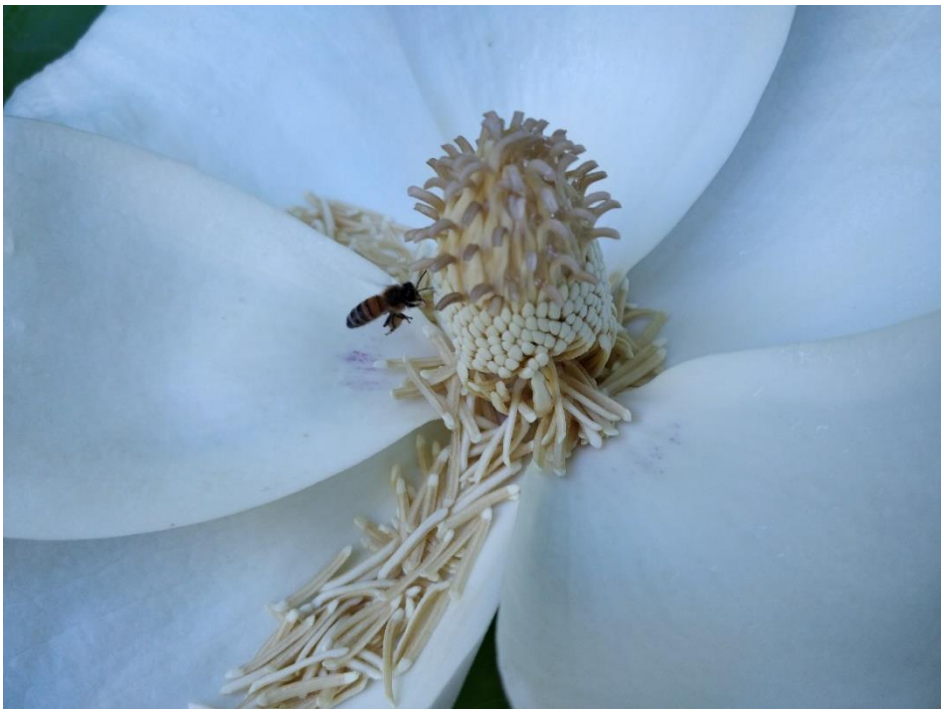

Figure 3. A worker *A. mellifera* foraging on pollen from the anthers of a male *M. macrophylla* ssp. *macrophylla* flower. Pollen was collected from anthers attached to the reproductive stalk or from dehisced anthers which aggregated at the base of the reproductive stalk where the tepals join. The image was captured on June 17th, 2019 at the Arnold Arboretum by Liam Cleary.

Figure 4. A worker *A. mellifera* showing lethargic behavior while resting on receptive *M. macrophylla* ssp. *ashei* stigmas coated with stigmatic secretion. The image was captured on June 15th, 2019 at the Arnold Arboretum by Liam Cleary.

Figure 5. A lethargic female *X. virginica* bee resting next to the chewed reproductive structures of a *M. macrophylla* ssp. *macrophylla* flower. The image was captured on June 12th, 2019 at the Arnold Arboretum by Liam Cleary.

Figure 6. Two active *X. virginica* bees attempting to escape a female *M. macrophylla* ssp. *ashei* floral cup. The image was captured on June 13th, 2019 at the Arnold Arboretum by Liam Cleary.

Tepal Wax Layer Imaging

Cryogenic scanning electron microscopy revealed an increasing wax crystal gradient from the top to the bottom of the axial surface of *M. macrophylla* ssp. *macrophylla* and ssp. *ashei* inner whorl tepals. In both figures, the top electron micrograph image corresponds with the top removed tepal section and shows a visibly lower density of white particles indicating a lower wax crystal density. Moving downwards, the density of wax molecules increases until its highest concentration at the purple pigmented area at the base of the tepal (Figure 7, Figure 8). *M. macrophylla* ssp. *macrophylla* tepals had a wax crystal density of 104 crystals per 50 μM^2 at their top and a density of 198 crystals per 50 μM^2 at their bottom. *M. macrophylla* ssp. *ashei* tepals had a wax crystal density of 87 crystals per 50 μM^2 at their top and a density of 191 crystals per 50 μM^2 at their bottom. Each wax crystal had an average length of 3 µM.

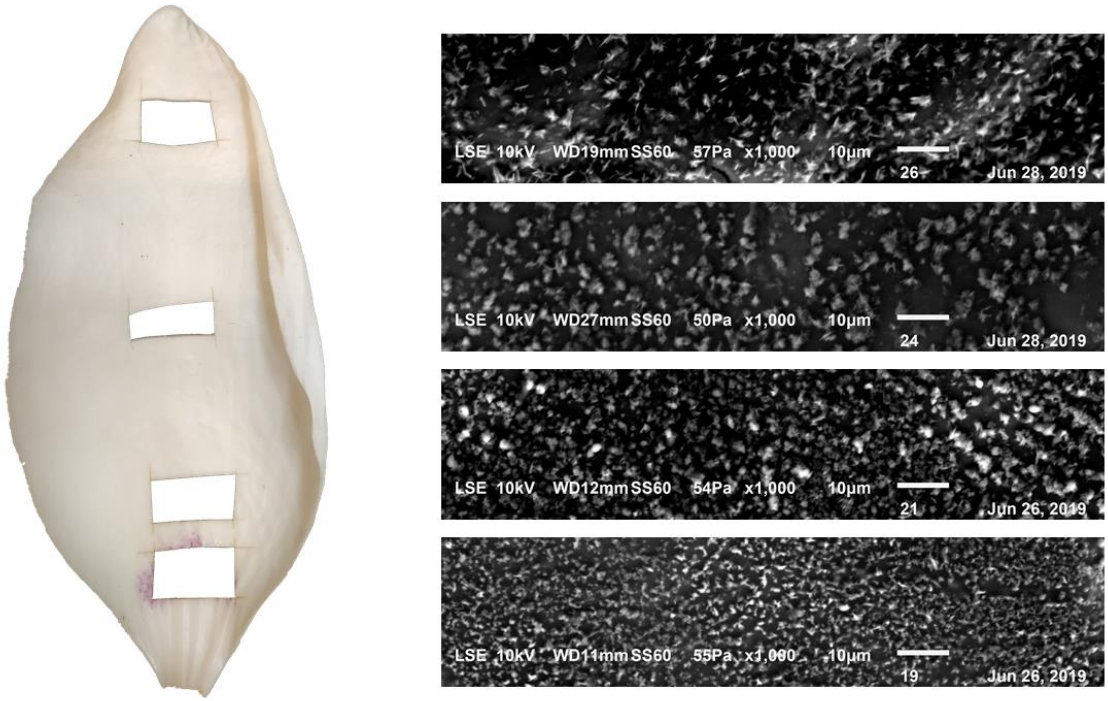

Figure 7. Shown left is a *M. macrophylla* tepal with sections removed corresponding to images on the right. Shown right are cryo-SEM images of axial surface tepal sections from a female *M. macrophylla* ssp. *macrophylla* flower. All images were captured at 1,000x magnification. The top two images were taken on June 28th, 2019, two days after the bottom two images on June 26th, 2019 using a tepal from a different female *M. macrophylla* ssp. *macrophylla* flower.

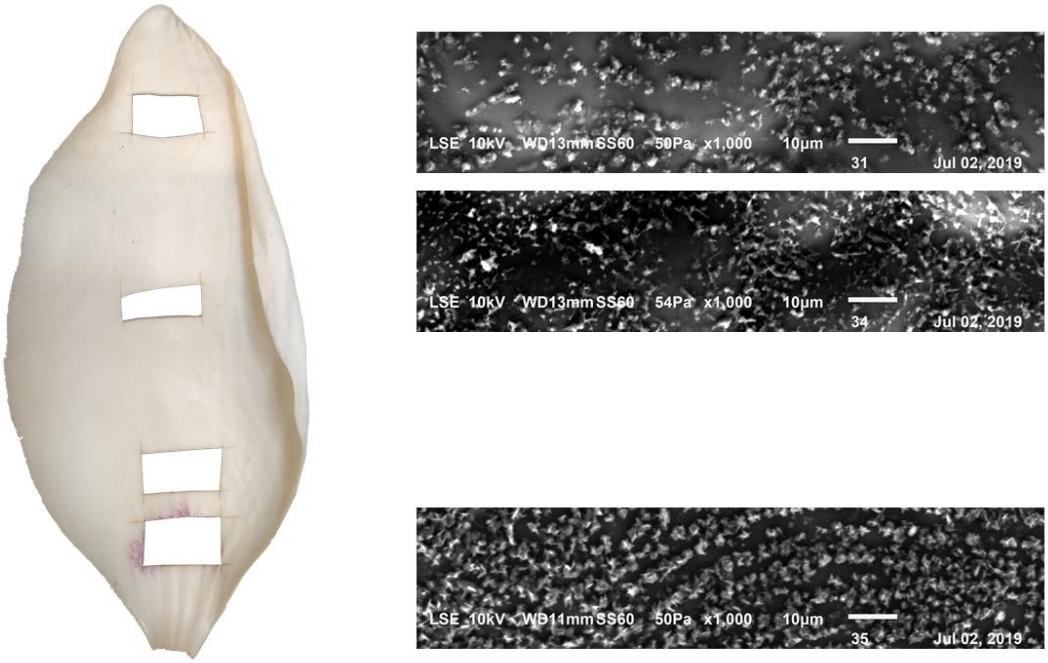

Figure 8. Shown left is a *M. macrophylla* tepal with sections removed corresponding to images on the right. Shown right are cryo-SEM images of axial surface tepal sections from a female *M. macrophylla* ssp. *ashei* flower. The second section up from the bottom of the tepal does not have a corresponding electron micrograph image. All images were captured at 1,000x magnification.

Pollen Surface Imaging

M. macrophylla pollen grains appeared oval-shaped with a pointed end and had an average length of 32 µM and an average width of 16 µM (Figure 9).

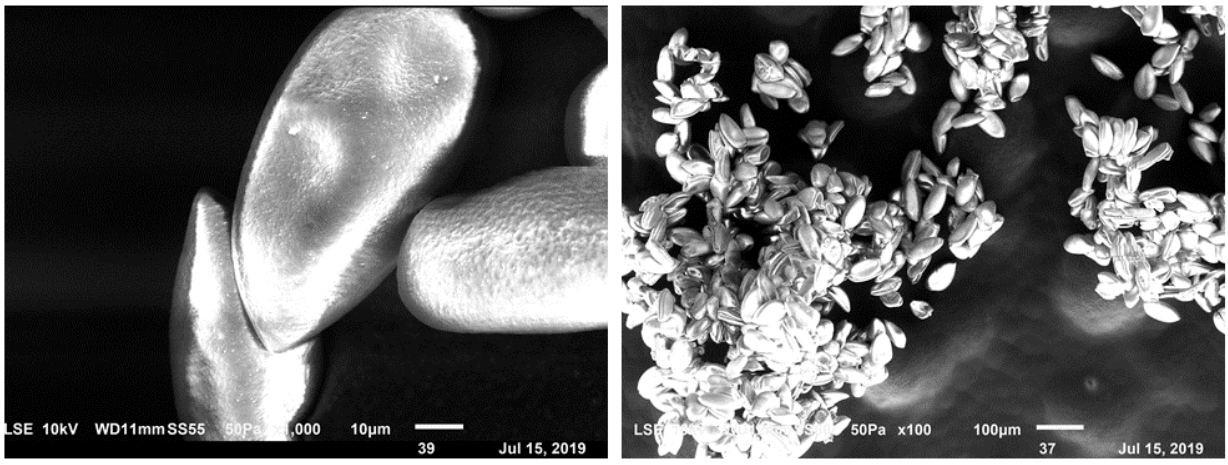

Figure 9. Cryo-SEM images of *M. macrophylla* pollen grains. The left image shows pollen grains at 1,000x magnification. The right image shows pollen grains at 100x magnification.

Liquid Chromatography-Mass Spectrometry Analysis

At writing, liquid chromatography-mass spectrometry analysis performed by Dr. Phil Stevenson's laboratory has not yet determined the compounds present in *M. macrophylla's* stigmatic secretion.

Stigmatic Secretion Assay Scoring Analysis

Application of 20 µL of stigmatic secretion did not affect any of the tested behaviors ($P > 0.05$ for all) except abdomen grooming ($P = 0.05$), where stigmatic secretion caused increased abdominal grooming in treatment bees (Figure 14). There was no statistical difference between control and treatment bees for the total immobile, active duration ($P = 0.439$; Figure 10) or the longest immobile, active period ($P = 0.212$; Figure 11) indicating the application of 20 µL of stigmatic secretion did not affect *B. impatiens* mobility. Additionally, no differences were observed in antennae grooming ($P = 0.08$) or leg flails ($P = 0.49$) indicating the application of 20 µL of stigmatic secretion did not result in *B. impatiens* leg (Figure 12) or antennal agitation (Figure 13).

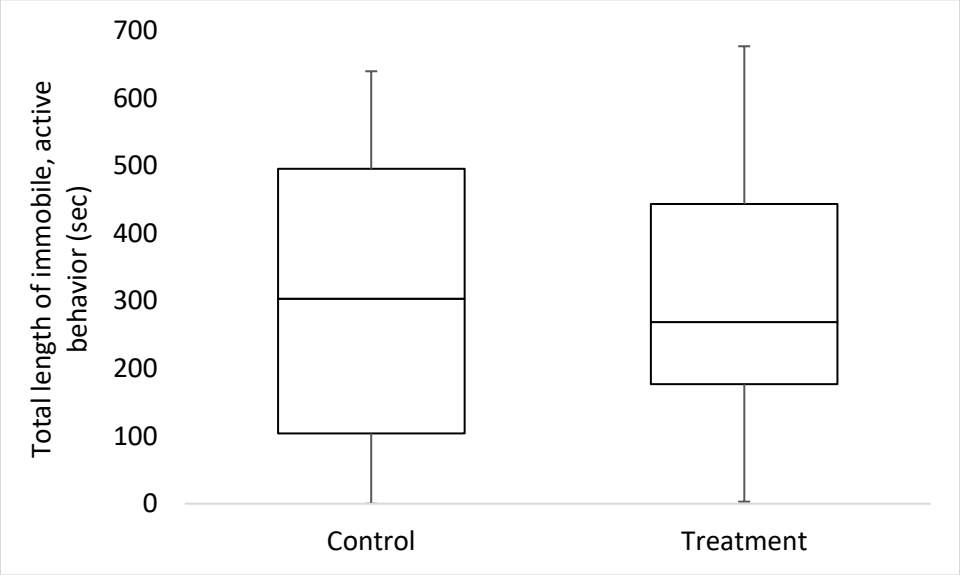

Figure 10. The total length of immobile, active behavior in seconds for control and treatment *B. impatiens* from stigmatic secretion laboratory assays. The upper error bar represents the maximum for the data set, the lower error bar represents the minimum, and the middle line represents the median.

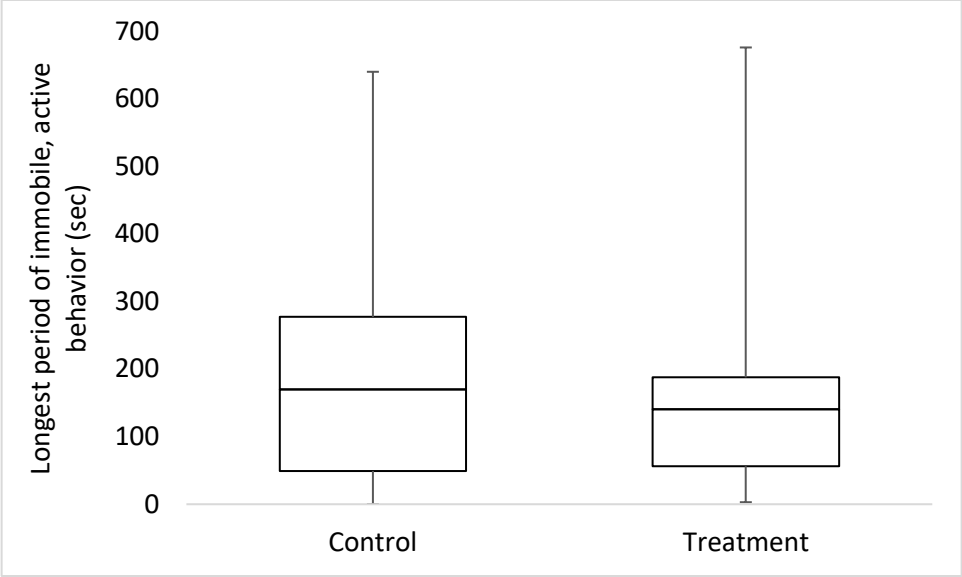

Figure 11. The longest single period of immobile, active behavior in seconds for control and treatment *B. impatiens* from stigmatic secretion laboratory assays. The upper error bar represents the maximum for the data set, the lower error bar represents the minimum, and the middle line represents the median.

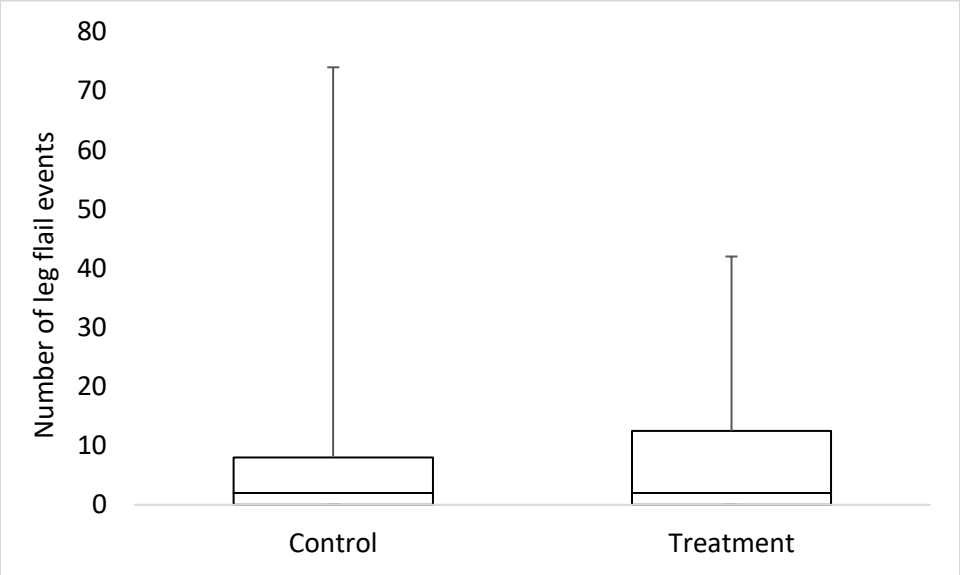

Figure 12. The number of leg flail events for control and treatment *B. impatiens* from stigmatic secretion laboratory assays. The upper error bar represents the maximum for the data set, the lower error bar represents the minimum, and the middle line represents the median.

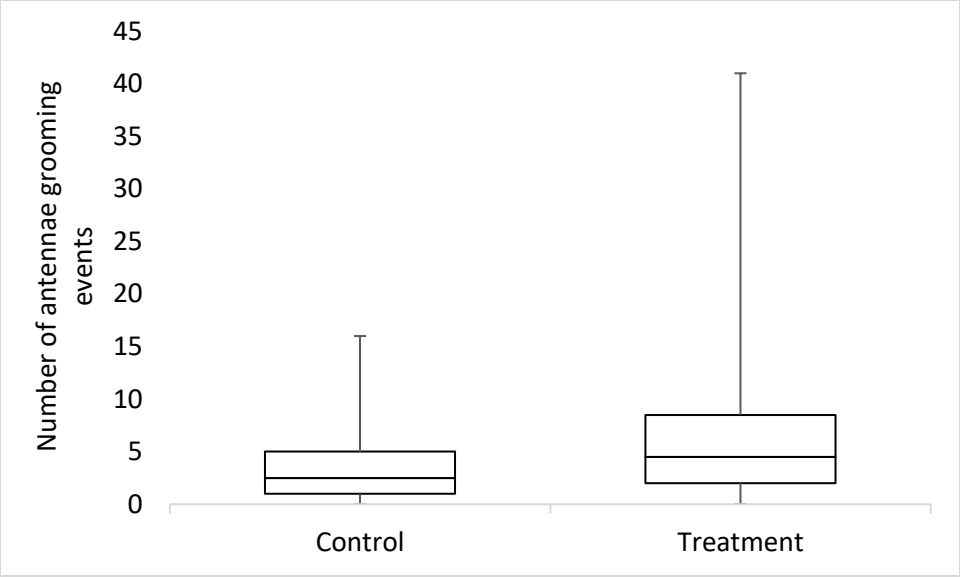

Figure 13. The number of antennae grooming events for control and treatment *B. impatiens* from stigmatic secretion laboratory assays. The upper error bar represents the maximum for the data set, the lower error bar represents the minimum, and the middle line represents the median.

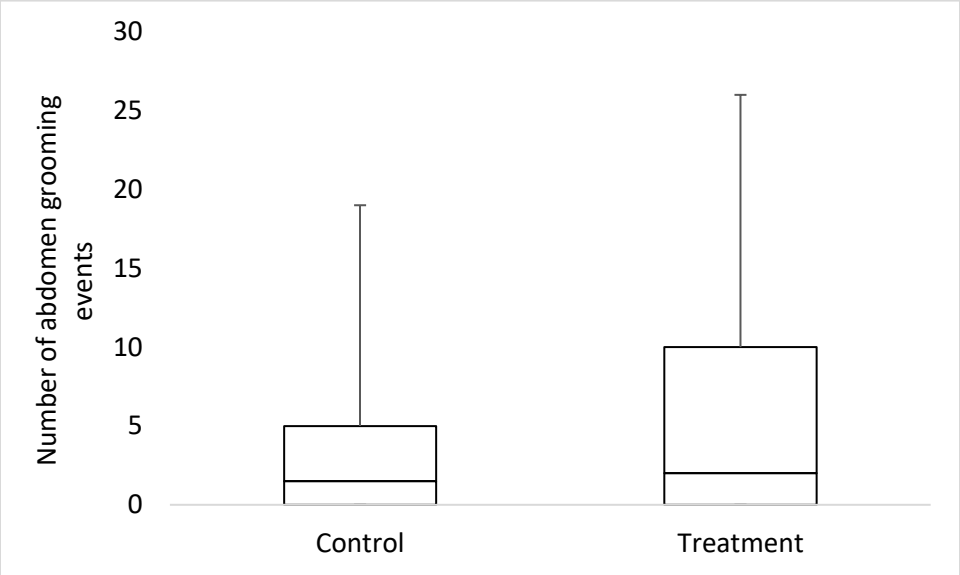

Figure 14. The number of abdomen grooming events for control and treatment *B. impatiens* from stigmatic secretion laboratory assays. The upper error bar represents the maximum for the data set, the lower error bar represents the minimum, and the middle line represents the median.

Discussion

While this study did not confirm stigmatic secretion as the cause of bee lethargy within *Magnolia macrophylla* flowers, field observations and laboratory assays yielded insightful data that provide a basis for future work. During field observations, I most frequently observed lethargic *A. mellifera* and *X. virginica* within female *M. macrophylla* ssp. *macrophylla* and ssp*. ashei* flowers.

Additionally, I observed X. *virginica* chewing the reproductive organs of male flowers which later had lethargic *X. virginica* within them, indicating a potential link between bee lethargy and herbivory of floral reproductive tissue. Using Cryo-SEM I observed a tepal wax layer gradient which I hypothesize may play an adaptive role in preventing bee escape from female flowers. Finally, exposure to stigmatic secretion in laboratory assays increased abdomen grooming in *B. impatiens* but did not increase immobile or lethargic behavior. Here I elaborate on these findings, place them in the context of previous literature, and conclude with a discussion of future research directions.

Field observations yielded ample data for *A. mellifera* and *X. virginica*; infrequent observations of the remaining four taxa may have been caused by lack of lethargy and escape from female flowers. Observations of lethargic *A. mellifera* within female flowers are consistent with previous literature (Thien 1974). Lethargic *A. mellifera* displayed several behaviors not seen in observations of other taxa including rapid leg flailing. I speculate that this may be caused by the lethargic compound acting as a neurotoxin, although further evidence of the compound's identity is needed to conclusively state this. Additional behaviors included frequent abdomen and antennae grooming which seemed to be caused by agitation from stigmatic secretion contact. Observations of non-lethargic *A. mellifera* within male flowers may provide some support to the hypothesis that stigmatic secretion causes lethargic behavior, since stigmatic secretion is never found on the stigmas of male flowers. However, observations of lethargic *X. virginica* in male flowers are inconsistent with this hypothesis. While stigmatic secretion may not be the cause of lethargy, I speculate that when stigmatic secretion is reabsorbed at the end of the female floral phase the potential compound responsible for lethargy remains in floral tissue. Therefore, *X. virginica* chewing floral reproductive organs may ingest the lethargy-causing compound. Further observations that lethargy follows reproductive organ herbivory and that the lethargy-causing compound remains in floral reproductive tissue are needed to confirm this speculation. The remaining four taxa, *B. impatiens*, Formicidae, Halictidae, and Coleoptera, were infrequently observed ($n < 4$ for each). I speculate that observations were low because these visitors did not become trapped in female *M. macrophylla* flowers like *A. mellifera* and *X. virginica* did. Therefore, they may have entered and left flowers before I could observe them.

Adaptive and non-adaptive hypotheses exist for the presence of a wax layer on the axial surface of *M. macrophylla* inner whorl tepals. One adaptive possibility is that the wax layer could trap pollinators between floral reproductive phases. *M. macrophylla* flowers do not seal closed due to their large size, and therefore, the wax layer may serve the same function as closing petals or tepals in trapping pollinators between floral phases. The tepal wax layer may also be physiologically adaptive and function to prevent water loss from tepals much like the cuticle on a leaf. However, further evidence on tepal water transpiration rates would be needed to support this speculation. If non-adaptive, the tepal wax layer may have once played a role in floral functioning and no longer does or may be a trait carried down from an ancestral species.

Laboratory assays indicated that stigmatic secretion application increased abdomen grooming (Figure 14) but not the other four scored behaviors (Figures 10 – 13). I propose two main hypotheses as to why tests failed to reject the null hypothesis for these behaviors, each concerning the role of stigmatic secretion as the cause of lethargy.

First, stigmatic secretion may be the cause of bee lethargy, but assays failed to detect this. If true, I speculate that several sources of experimental error may account for this outcome. Most importantly, the volume of stigmatic secretion applied to bees may have been too small to cause lethargy. During field collection of stigmatic secretion an average flower contained 100 µL of stigmatic secretion, a five-fold increase from the 20 µL volume applied during laboratory assays. Furthermore, the small application volume only allowed stigmatic secretion to be targeted to a bee's underside, whereas in a flower the bee's entire body may have contacted stigmatic secretion. In future studies, I would apply the average volume of stigmatic secretion collected from a flower to a single bee and target application to the bee's entire body to better match field conditions. The second source of error may be the rapid degradation of the compound responsible for lethargy after collection and before storage in a -80 ℃ freezer. During field collection stigmatic secretion was stored in microcentrifuge tubes in an icebox, however, during this period compounds such as proteins may have degraded. Therefore, if the compound responsible for lethargy was a protein, the stigmatic secretion would no longer cause behavioral changes during laboratory assays. Future studies collecting stigmatic secretion should avoid this possibility by storing collected stigmatic secretion immediately in liquid nitrogen before freezing at -80 ℃.

A second possibility is that stigmatic secretion is not the cause of lethargy. If true, I speculate that two main hypotheses may explain this. First, a volatile produced during flowering may result in lethargic behavior. Due to the cup shape formed by the female flower, volatiles may collect in the cup and once a bee enters it would breathe in the volatiles and become lethargic. However, during male flowering, air movement across the open flower may remove volatiles and therefore bees visiting during male flowering would not become lethargic. However, this hypothesis fails to explain instances of lethargic *X. virginica* in male flowers. A second hypothesis is that a compound emitted from tepals during female flowering but not male flowering causes lethargy. Again, this hypothesis fails to explain observations of lethargic *X. virginica* in male flowers and currently no studies have found evidence for *M. macrophylla* floral compound production and excretion.

With increased funding and time, I would continue this research in two main directions. First, I would conduct field observations in the native ranges of both *M. macrophylla* ssp. *macrophylla* and ssp. *ashei*. Although the eight mature *M. macrophylla* trees at the Arnold Arboretum were useful for field observation of floral visitors, observing visitors in *M. macrophylla's* native ecosystems would shed light on naturally abundant visitor taxa. Additionally, *M. macrophylla* trees in their native range flower from April through June (Thien 1974) and may attract visitor communities that begin foraging earlier than those found at the Arnold Arboretum. I would also aim to better match stigmatic secretion application during laboratory assays to field conditions such as discussed above. By better replicating field conditions, lab assays may be able to detect behavioral changes not previously observed.

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