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Bee pollination biology: buzzing, behavior, and biomechanics

A dissertation presented

by

Callin Switzer

to

The Department Organismic and Evolutionary Biology

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Biology

Harvard University

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Bee pollination biology:
buzzing, behavior, and biomechanics

Abstract

For millions of years, flowering plants have relied on animals to transfer pollen among flowers; this leads to fertilization, seed set, and ultimately the passing of genetic information to the next generation.

The relationships among pollinators and plants are complex. Pollinators generally visit flowers to collect food rewards. Plants have evolved a variety of mechanisms that manipulate pollinator behavior and increase the probability of successful fertilization. Plants can influence the evolution of pollinators, and pollinators can influence the evolution of plants. In this work, I characterize some of the relationships between plants and pollinators.

First, I focus on buzz pollination (or floral sonication). When a bee performs this behavior, it usually grasps the anthers of the flower and, using its flight muscles, vibrates. When the vibration is transferred to the flower, pollen is released. This behavior is particularly useful when bees collect pollen from plants that have poricidal anthers that release pollen only from small pores. When bees vibrate these anthers, pollen is released, like salt coming out of a saltshaker. Buzz pollination is important for a variety of human foods (e.g. tomatoes). Honeybees, notably, cannot perform buzz pollination, and thus other pollinators, like bumblebees, are more effective at pollinating some plant species.

I answer the following questions about buzz pollination: How do bumblebees change their buzz pollination behavior in different environmental conditions and on different species of plants? How do different species of bees perform buzz pollination differently?

How does marking bees with bee tags affect their ability to perform buzz pollination? How does consuming a pesticide affect bees' ability and likelihood to perform buzz pollination?

Last, I investigate pollination from the plant's perspective. I characterize the explosive pollination mechanism of the mountain laurel. I describe how the catapult mechanism of the mountain laurel may act as a pollen dispensing system – similar to poricidal anthers, the catapult may dispense pollen only to the subset of floral visitors that are likely to successfully transfer the pollen.

Contents

Abstract iii
Acknowledgments vi

Introduction **1**

1 Shakers and head bangers: Differences in sonication behavior between Australian *Amegilla murrayensis* (blue banded bees) and North American *Bombus impatiens* (bumblebees) **5**

2 *Bombus impatiens* (Hymenoptera: Apidae) display reduced pollen foraging behavior when marked with bee tags vs. paint **21**

3 The neonicotinoid pesticide, imidacloprid, affects *Bombus impatiens* (bumblebee) sonication behavior when consumed at doses below the LD_{50} **37**

4 Bumblebee sonication behavior changes with plant species and environmental conditions **56**

5 Dispensing pollen via catapult: explosive pollen release of the mountain laurel (*Kalmia latifolia*) **74**

References **92**

Appendix A Supplemental Material for Chapter 1 **106**

Appendix B Supplemental Material for Chapter 2 **109**

Appendix C Supplemental Material for Chapter 4 **113**

Appendix D Supplemental Material for Chapter 5 **130**

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To my family...past, present, and future

Introduction

Pollination – the transfer of pollen from the anther (male) to the stigma (female) of a flower– is generally used for sexual reproduction in angiosperms. Over millions of years, angiosperms and pollinators, the organisms that carry pollen among flowers, have evolved wide-ranging and intricate relationships. When pollinators visit flowers, they're often seeking food rewards. Bees, for example, collect nectar as a source of food for energy and pollen as a source of protein. As the pollinators move among different flowers, they transfer pollen.

In some cases, pollinators have evolved special morphology that allows them to collect rewards from unique flowers. These specialized relationships have fascinated scientists for hundreds of years. Charles Darwin (1862b) wrote about the extreme morphologies of orchids:

> *In several flowers sent me by Mr. Bateman I found the nectaries eleven and a half inches long, with only the lower inch and a half filled with very sweet nectar... We shall, I think, see that their fertilsation of the plant depends on this length and on nectar being contained only within the lower attenuated extremity... in Madagascar, there must be moths with probosces capable of extension to a length of between ten and eleven inches!*

Over 100 years later, researchers first observed a giant hawkmoth (*Xanthopan morganii praedicta*) feeding on the orchid (*Angraecum sesquipedalia*) and transferring pollen (Wasserthal 1993). In other cases, plants and pollinator morphologies are not nearly so specialized. For instance, bumblebees are known to visit hundreds of different species of flowers, and some flowers are visited by a wide variety of pollinators.

This dissertation investigates the "in-between" relationships among plants and pollinators – I investigate systems that are not extremely specialized, but also not completely general.

For plants and pollinators in this category, a wide variety adaptations exist. For instance, some flowers have evolved special shaped anthers that release pollen only through a small pore (poricidal anthers). This morphology is present in about 8% of angiosperms, and is commonly found in the Solanaceae (for instance, in tomatoes, Figure 1.1). These plants release pollen only to some pollinators that perform certain behaviors. To collect pollen from poricidal anthers, pollinators use buzz pollination, or sonication. During sonication, bees vibrate the flowers, using their flight muscles, to shake out pollen. Insects that visit this flower but don't use sonication usually do not get the floral rewards. Often, the bee contacts the stigma of the flower during sonication behavior, which allows for fertilization.

Another example of an "in-between" relationship is the pollination system of the mountain laurel (Figure 5.1). This plant uses explosive pollination – it catapults pollen onto pollinators that visit it and "trip" the catapult mechanism.

Study Systems

In this work, I primarily explore relationships between bees and flowers. The main bee species of interest is the Common Eastern Bumblebee, *Bombus impatiens*. The Eastern Bumblebee is a eusocial bee that forms colonies of hundreds of individuals (Williams et al. 2014). This species typically nests underground, and worker bees are 9-14 mm in length. They are native to the eastern United States, and often collect pollen using buzz pollination. It has both ecological and economic importance because it uses buzz pollination, whereas the most common pollinator in the United States, the European Honeybee (*Apis mellifera*), does not use buzz pollination. Buzz pollination by bumblebees benefits a variety of native and crop plants in the United States.

I also describe the behavior a charismatic bee that occupies a similar niche to the bumblebee. The blue-banded bee (*Amegilla murrayensis*) is a native species in Australia that is known to collect pollen using buzz pollination (Figures 1.1, 1.4). They have blue bands across the metasoma. They are solitary bees that nest in clay washouts and mud bricks. blue-banded bees are smaller than bumblebees, about 11 mm in length (Leijs et al. 2017)

(see Figure 1.1 for photographs of both of these bees)

I investigated bee behavior on a variety of flowers. I conducted observational studies on accessioned plants in the Arnold Arboretum (See Figure C.1). The main experimental flowers were mountain laurels (*Kalmia latifolia*, See Figure 5.1), and three species in the Solanaceae (*Solanum dulcamara*, *Solanum carolinense*, and *Solanum lycopersicum*). These plants are described in depth in Appendix C, particularly Figure C.3).

Dissertation Outline

Chapter 1 investigates how different species of bees behave differently, when pollinating the same species of flower (tomatoes). In this chapter I conducted a biomechanical analysis; I recorded audio and high-speed videos of bumblebees and blue-banded bees. I found that bumblebees generally sonicated at lower frequencies than blue-banded bees. In addition, high-speed videos showed that bumblebees typically grasped the anthers with their mandibles while vibrating, but blue-banded bees often tapped (or head-banged) the flowers.

Chapter 2 investigates how different methods of marking bees affects their pollination behavior. Because knowing the identity of individual bees in experiments is necessary, scientists often need to attach unique markers to each individual. Numbered, plastic bee tags and paint are two common methods. I found that using numbered bee tags affected bumblebee behavior more than paint – bees marked with bee tags were less likely than painted bees to resume their normal behavior of collecting pollen after they were marked.

Chapter 3 investigates how the common neonicotinoids pesticide, imidacloprid, affects bumblebee pollination behavior. I conducted an experiment to test how the consumption of imidacloprid caused changes in buzz-pollination behavior. I found that bees that consumed modest-to-high doses of imidacloprid were significantly less likely to perform sonication behavior than untreated bees.

Chapter 4 investigates how bumblebees change their sonication behavior on a variety of flower species. I conducted an observational study to record sonication frequency and duration of bumblebees visiting a variety of flowers and I found that different flowers were

associated with bees using different sonication frequencies and lengths. I followed this study with an experiment that allowed individual bees to sonicate on three different species of plants. I found evidence that individual bees are capable of changing their sonication behavior on different species of plants.

Chapter 5 investigates the biomechanical properties of the explosive pollination mechanism used by mountain laurel plants. I used high-speed videos to quantify the maximum speeds and accelerations of the pollen catapult, and I built a heat-map of where pollen is launched. I conducted field observations to record which pollinators and behaviors triggered the catapults, and I conducted a pollination experiment to estimate the amount of pollination limitation in the Arnold Arboretum in Boston MA. I found that the mountain laurel is one of the fastest plants ever recorded and that pollinators are important for pollination (autogamous pollination is low). I also found that large bees are the main pollinators that trigger the mountain laurel catapult. The pollen catapult may have evolved to release pollen only to pollinators that are likely to transfer pollen to another flower.

Chapter 1

Shakers and head bangers: Differences in sonication behavior between Australian *Amegilla murrayensis* (blue banded bees) and North American *Bombus impatiens* (bumblebees)

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Abstract

Many bees collect pollen by grasping the anthers of a flower and vibrating their flight muscles at high frequencies – a behavior termed sonication, or buzz-pollination. Here we compare buzz-pollination on *Solanum lycopersicum* (cherry tomatoes) by two bees that fill similar niches on different continents – in Australia, *Amegilla murrayensis* (blue banded bee), and in North America, *Bombus impatiens* (bumblebee). We collected audio recordings of buzz pollination and quantified the frequency and length of buzzes, as well as the total time spent per flower. We found that *A. murrayensis* buzzes at significantly higher frequencies (∼350 Hz) than *B. impatiens* (∼240 Hz), and flaps its wings at higher frequencies during flight. There was no difference in the length of a single buzz, but *A. murrayensis* spent less time on each flower, as *B. impatiens* buzzed the flower several times before departing, whereas

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A. murrayensis typically buzzed the flower only once. High-speed videos of *A. murrayensis* during buzz pollination revealed that its physical interaction with the flower differs markedly from the mechanism described for *Bombus* and other bees previously examined. Rather than grasping the anther cone with its mandibles and shaking, *A. murrayensis* taps the anther cone with its head at the high buzzing frequencies generated by its flight muscles. This unique behavior, combined with its higher buzzing frequency and reduced flower visit duration, suggests that *A. murrayensis* may be able to extract pollen more quickly than *B. impatiens*, and points to the need for further studies directly comparing the pollination effectiveness of these species.

Keywords

sonication; *Solanum*; vibration; pollination; native bees

Introduction

Over 200,000 plant species depend on insects for pollination (Buchmann 1983). Pollinating insects often consume both nectar and pollen, and they transfer pollen grains among plants as they travel from flower to flower, an essential step in the reproduction of many plants. Understanding the physical interactions between plants and insect pollinators (primarily bees) can provide insight into the requirements and evolution of these critical plant-pollinator relationships.

Although considered a mutualistic relationship, the interaction between plants and pollinating insects is not entirely without conflict. The conflict arises because the ideal behavior of the pollinator is different from the pollinator's perspective vs. the plant's perspective (Gegear and Laverty 2001). Bees attempt to expend the least possible energy for the greatest reward; bumblebees forage for pollen in a manner that increases their probability of maximizing their net energy intake (Zimmerman 1982). Plants, on the other hand, would benefit most if pollinators moved sequentially among flower of the same

species – a strategy that an optimally foraging pollinator would rarely use (Gegear and Laverty 2001). One evolutionary "strategy" for increasing a plant's reproductive success is to dispense only a little pollen at a time, ensuring that its flowers are visited multiple times and that pollinators must visit multiple flowers to obtain sufficient pollen (Harder and Thomson 1989). Plants may also benefit from "messy" bees that cannot clean all the pollen off their bodies, since this excess pollen is not consumed by the pollinator, and is more likely to be transferred to different flowers that the bee visits subsequently; outcrossing plants require a pollinator that accumulates pollen on its body where it has a high chance of fertilizing a conspecific plant ovule (Gegear and Laverty 2001).

These evolutionary strategies are particularly evident in the approximately 20,000 insect-pollinated plants ($\sim$ 8% of angiosperms) that have evolved poricidal anthers (Buchmann 1983) - anthers with only small pores through which pollen is released. Poricidal anthers restrict direct access to pollen (De Luca and Vallejo-Marín 2013; Harder and Thomson 1989), helping to limit the amount of pollen that bees can collect during a visit, and depositing pollen in locations on the bees' bodies that are poorly groomed. Throughout areas with temperate climates, *Bombus* spp. (bumblebees) play a vital role in pollinating plants with poricidal anthers, as they are capable of performing sonication, or buzz-pollination, to release pollen that is largely inaccessible to insects that do not perform this behavior (e.g., honeybees) (King and Buchmann 2003). In warmer areas, species belonging to other taxa, e.g. *Xylocopa* (Hogendoorn et al. 2000) and *Amegilla* (Hogendoorn et al. 2006) perform buzz pollination.

Buzz-pollination has been well described in *Bombus* spp.: the bee lands on a flower, curls her abdomen around the anther tips while grasping the anthers with her mandibles, then uses her flight muscles to vibrate her body without flapping the wings (King et al. 1996). These vibrations are transmitted through the head and body to the flower, and pollen is released from the pores onto the bee's body (De Luca and Vallejo-Marín 2013; Harder and Barclay 1994; King and Buchmann 2003). Due to the bee's position on the anther during sonication, pollen is deposited onto her ventral body surface, and although she

collects some of the pollen grains, several locations on the ventral body surface are poorly groomed (Buchmann 1983; Michener et al. 1978), which facilitates the transfer of pollen to other flowers. Both species of bees groom the pollen from their bodies and place it onto specialized carrying structures (Michener et al. 1978). *Bombus* has basket-like corbiculae while *Amegilla* has brushlike scopae for holding pollen on the hind legs (Michener 2000). *Bombus* moistens the pollen with nectar before packing it into the corbicula (Michener 2000; Michener et al. 1978). *Amegilla* packs relatively dry pollen among the hairs of the scopa (Anderson and Symon 1988).

The asynchronous flight muscles that drive the wings form part of a resonant system, whose vibration frequency depends on the mass it is driving (i.e., the mass of the wings) (Josephson et al. 2000). Thus, when the wings are disengaged during sonication, the vibration frequency of the flight muscles is higher than the bees' flapping frequency during flight (King et al. 1996).

Although much of the previous work on buzz-pollination has focused on *Bombus* spp. (Asada and Ono 1996; Buchmann and Hurley 1978; De Luca et al. 2013; Harder 1990; King 1993; King and Buchmann 2003; Morandin et al. 2001), many other bee genera perform buzz-pollination, including *Protandrena*: Andrenidae (Buchmann and Cane 1989), *Megachile*: Megachilidae (Neff and Simpson 1988), *Augochloropsis*: Halictidae (Thorp and Estes 1975), *Xylocopa*: Apidae (Hogendoorn et al. 2000; King and Buchmann 2003), *Nomia*: Apidae (Anderson and Symon 1988), and *Amegilla*: Apidae (Hogendoorn et al. 2006). Information on the mechanics of buzz-pollination in these genera is far more limited, and comparative studies of buzz-pollination mechanisms among different groups of bees are scarce.

Buzz-pollination is known to be critical for many endangered plants, such as *Dianella longifolia* in Australia (listed on the Advisory List of Rare or Threatened Plants in Victoria in 2014 and the Northern Territory Threatened Species list), which can reproduce only through buzz-pollination. In addition, the economic value of buzz-pollination is very high, as it contributes to increased yields in crops ranging from tomatoes (Asada and Ono 1996; Hogendoorn et al. 2006) to blueberries (Javorek et al. 2002) and cranberries (MacKenzie

1994).

In mainland Australia *Bombus* spp. are not present, and multiple native bees perform buzz-pollination. The potential introduction of *Bombus* spp. to the Australian mainland for tomato pollination (Hogendoorn et al. 2006) is being debated intensively, as *Bombus* spp. have been commercialized in other parts of the world, and their effectiveness at pollinating crops in greenhouses is well established (King 1993). Native Australian bees, like *Amegilla* spp. have not been commercialized to the same degree, but research suggests that they also present a viable method of pollinating tomatoes in greenhouses (Bell et al. 2006; Hogendoorn et al. 2006). However, few studies have compared the mechanisms by which native Australian bees and *Bombus* spp. extract pollen via sonication, and buzz-pollination by *Amegilla* spp. has not been quantified.

Here we compare buzz-pollination on *Solanum lycopersicum* (cherry tomatoes) by two bees that fill similar niches on different continents – in Australia, *Amegilla murrayensis* (blue banded bee), and in North America, *Bombus impatiens* (common Eastern bumblebee). To determine whether these species pollinating the same flower perform buzz pollination in the same way, we collected audio recordings of buzz pollination and quantified the frequency and length of individual buzzes, as well as the total time spent on a single flower (which may encompass multiple buzzes). We also recorded bees during flight, to compare sonication frequency to flight frequency. Finally, we filmed *A. murrayensis* during buzz-pollination using high-speed video, to compare its physical interaction with the flower to the well-described sonication behavior of *B. impatiens*.

Materials and methods

Study species and locations

We collected audio recordings of pollination buzzes by *Bombus impatiens* (bumblebees) on *Solanum lycopersicum* 'Sweet 100' (cherry tomatoes), and by *A. murrayensis* (blue banded bees) on *S. lycopersicum* 'Heirloom Roma Cherry' and *S. lycopersicum* 'Tommy Toe' (cherry

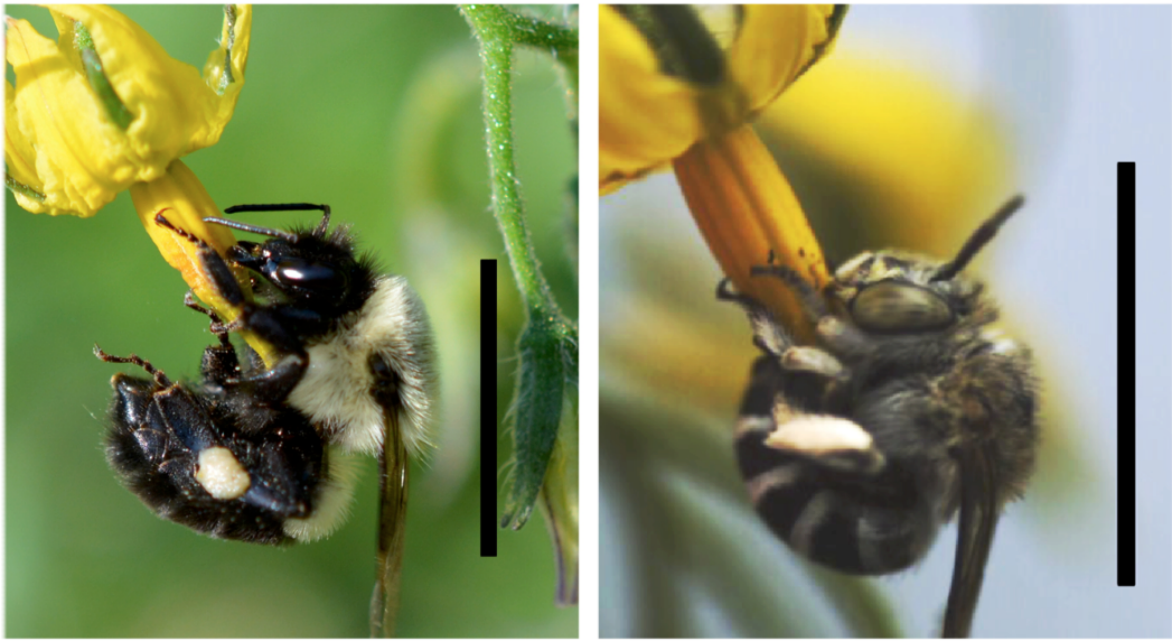


Figure 1.1: Photos of bees used in this study. *Bombus impatiens* workers (left) are typically larger than *Amegilla murrayensis* (right; Table A.1). Both bees are shown on *Solanum lycopersicum* flowers. Black bars indicate approx. 1 cm. Bumblebee Photo credit: Tim Stanley / Native Beeology

tomatoes) (Figure 1.1). Although the varieties of cherry tomatoes (*S. lycopersicum*) used were different, the flowers are very similar in size and morphology, and thus we do not expect that the tomato variety significantly affected buzz-pollination characteristics (Tables A.7 and A.9). Recordings of *B. impatiens* were collected in a community garden in Carlisle, Massachusetts, USA (42°52'N; 71°32'W), and those of *A. murrayensis* at the Adelaide Botanic Garden, Adelaide, Australia (34°92' S; 138°61' E). In Australia, *S. lycopersicum* 'Tommy Toe' plants growing in the garden were supplemented with potted tomato plants (*S. lycopersicum* 'Heirloom Roma Cherry') to provide additional flowers. For the potted plants, we recorded if the flower had been previously visited.

Audio recordings and analysis

We collected audio recordings with a shotgun microphone (SGM-1X, Azden, Tokyo, Japan) attached to a digital recorder (DR-100mkII, Tascam, Montebello, California), held within 3 cm of the bees' bodies. We attempted to position the microphone pointed at each bee's

thorax, approximately orthogonal to the bee's frontal plane. We were not able to maintain that position for all recordings; however, we have no evidence that recording from different angles affects the analysis of sonication frequency or duration. We recorded bees while landing, buzzing flowers, and flying away, to analyze audio characteristics of both flight and buzz-pollination.

Because some bees perform multiple buzzes on a single flower with pauses in-between, we recorded the time of landing and takeoff to calculate the total visit duration. This was considered a suitable estimate of the time spent on a single blossom, since these bees generally did not crawl between tomato flowers. When audio recordings did not span the entire length of a flower visit, we excluded them from the analysis of visit duration.

After collecting audio recordings of landing, buzz-pollination, and take-off flight, we captured bees with a net and noted the time, temperature, and relative humidity. To ensure independent samples, we either marked bees after the first capture (and excluded recaptured bees from the analysis), or collected the bees and pinned them as specimens. We measured intertegular (IT) span with digital calipers on bees that were released and with ImageJ (`http://imagej.nih.gov/ij/`) on photographs of pinned specimens to obtain the average size of each species (Table A.1). When bees performed multiple buzzes while visiting a single flower, the frequency and duration of these buzzes were averaged for statistical analysis.

We played recordings in Audacity (`http://audacity.sourceforge.net/`) and identified the start and end of each buzz aurally and visually to determine the buzz length. We defined buzzes that had breaks of less than about 0.1 seconds to be single buzzes. Figure 1.2 a shows an oscillogram from a series of buzzes by *B. impatiens*, with a single buzz expanded in Figure 1.2b. We calculated buzz frequencies in R (R Core Team 2016), using the "seewave" (Sueur et al. 2008) and "signal" (Signal Developers 2013) packages. We first filtered recordings to remove low-frequency noise and then calculated fundamental frequencies within sliding windows of 2048 points, with 80% overlap. Recordings typically contained a small number points that were clearly outliers (Figure 1.2c) – single data points

at frequencies more than one standard deviation beyond the median fundamental frequency. These outliers were most likely artifacts caused by using a relatively small sliding window and/or collecting recordings in noisy, outdoor environments. We removed outliers and then calculated the median of the trimmed distribution to determine the frequency of each buzz (Figure 1.2c).

We calculated wing beat (flight) frequency using the same method as for buzz frequency – identifying flights aurally and visually in Audacity, and then using R to calculate fundamental frequency (Table A.2). For one recording of a pollination buzz by *B. impatiens* and four recordings of flight by *A. murrayensis*, we were unable to obtain an accurate frequency using seewave, so we analyzed these recordings manually, by performing a Fast Fourier Transform (FFT) with the spectrum function in Audacity, using a Hanning window of 2048 points. We then listened to the recording and matched the sound with one of the peaks from the FFT spectrum.

Video Recordings

We collected videos of *A. murrayensis* performing buzz-pollination on *S. lycopersicum* (cherry tomato) flowers in the Adelaide Botanic Garden, using a high-speed camera (TS3, Fastec Imaging, San Diego, California) recording at 2000 fps. We recorded a total of nine videos, four of which are known to be of unique individuals, because we were able to capture these bees after filming.

Statistical tests

All statistical tests were performed in R (R Core Team 2016). We used multiple linear regression to compare flight frequency, average buzz-pollination frequency, average buzz length, and visit duration between *A. murrayensis* and *B. impatiens*; this method allowed us to compare the two species of bees while accounting for environmental variables: temperature, time of day, and relative humidity. To fit the assumptions of linear regression, we squared buzz-pollination frequency, square-root transformed flight frequency, and log-transformed

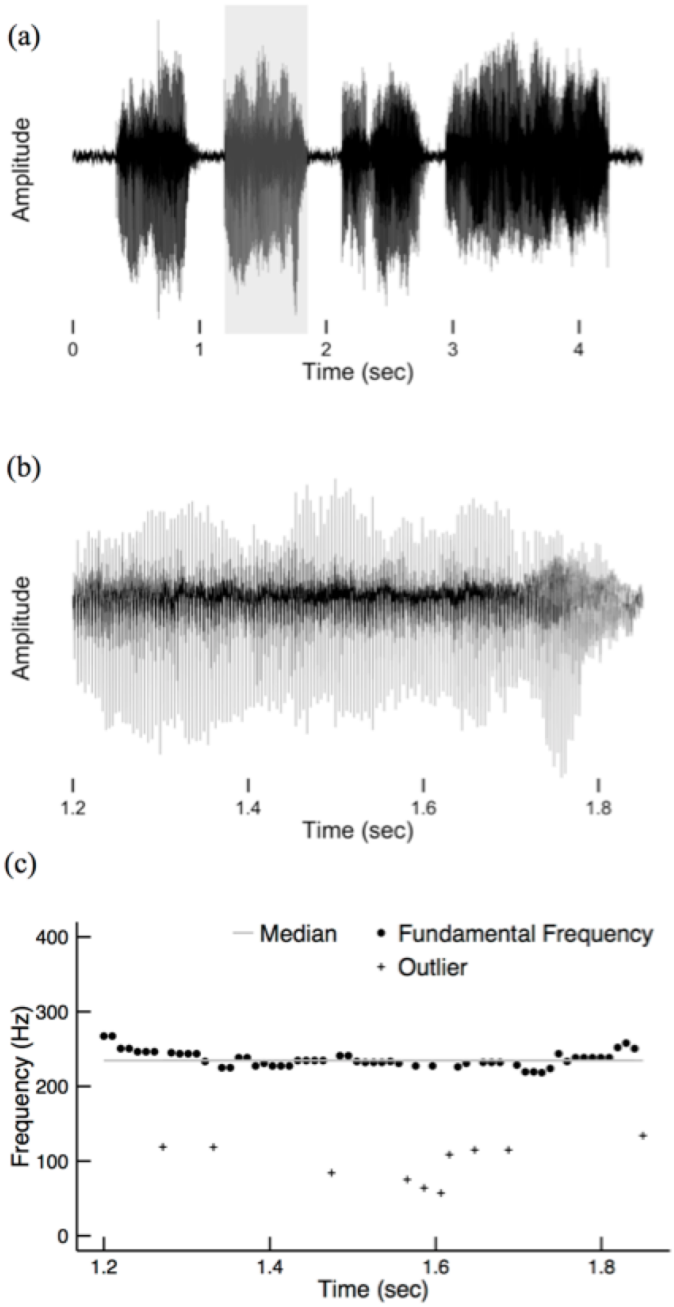


Figure 1.2: Audio recordings of buzz-pollination. **a** Oscillogram showing four pollination buzzes by *Bombus impatiens* (bumblebee) on a flower of *Solanum lycopersicum* "Sweet 100" (cherry tomato). Shaded region indicates a single buzz. **b** Expanded oscillogram of the single buzz shaded in a. **c** Fundamental frequency calculated over the course of the buzz shown in b. Dots represents the fundamental frequencies calculated from overlapping windows of 2048 data points. Frequency measurements that were identified as outliers and removed are indicated by a plus symbol

buzz length and visit duration. We also used paired t-tests to compare flight vs. pollination buzz frequency for individuals within each species.

We used multiple linear regression to compare buzz pollination characteristics for *A. murrayensis* on different tomato varieties, and on virgin vs. nonvirgin flowers (Tables A.7-A.9).

We adjusted significance level using Bonferroni correction, to account for performing multiple comparisons with the same individuals. Because we performed four multiple regressions and one t-test, we adjusted our significance level to 0.05 divided by 5, or 0.01. We did not adjust the significance level to include the four covariates in each of the multiple regressions, because the only variable of interest was the bee species. This correction is overly conservative, but using a less-conservative adjustment would not have changed our conclusions. Graphs were made with ggplot2 (Wickham 2016).

Results

None of the buzz-pollination or flight characteristics quantified were associated with environmental variables (temperature, relative humidity, time of day). We found that buzz frequency was significantly higher for *Amegilla* than for *Bombus* ($t(70) = 8.452$, p-value « 0.001; Figure 1.3a; Table A.3), and wing beat frequency was also higher for *Amegilla* than for *Bombus* ($t(71) = 13.372$, p-value « 0.001; Figure 1.3a; Table A.4). Within each bee species, the wing beat frequency during flight was significantly lower than the buzz-pollination frequency (Amegilla $t(21) = 24.67$, p-value « 0.001; *Bombus* $t(52) = 16.59$, p-value « 0.001).

There was no significant difference between the two bee species in the length of an individual pollination buzz (Figure 1.2b; $t(70) = 1.124$, p-value > 0.2; Table A.5), but *B. impatiens* spent more time on a single flower than *A. murrayensis* (Figure 1.3c; $t(53) = 3.974$, p-value < 0.005; Table A.6). We found no significant differences in buzz characteristics of *A. murrayensis* when pollinating the two different varieties of *S. lycopersicum*, or when pollinating unvisited vs. previously visited flowers (Tables A.7-A.9).

The high-speed videos revealed that *A. murrayensis* differs markedly from *B. impatiens*

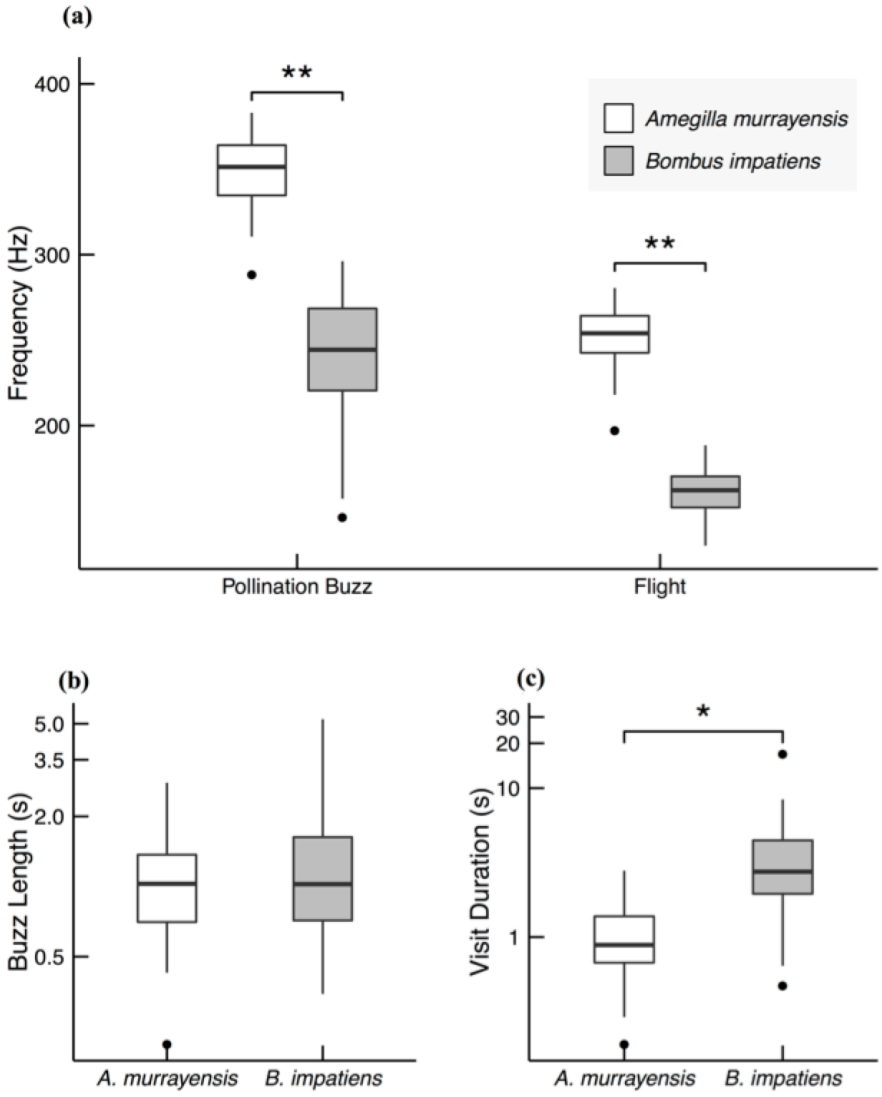


Figure 1.3: Buzz-pollination and flight characteristics of *A. murrayensis* and *B. impatiens*. (a) Pollination buzz frequency of *A. murrayensis* (white, n = 22) vs. *B. impatiens* (gray, n = 53), and flight (wing beat) frequency of *A. murrayensis* (n = 23) vs. *B. impatiens* (n = 53). (b) Average buzz length of *A. murrayensis* (n = 22) vs. *B. impatiens* (n = 53). (c) Flower visit duration of *A. murrayensis* (n = 22) vs. *B. impatiens* (n = 36). Double asterisks and single asterisks indicate a significant difference at $p < 0.0001$ and $p < 0.0005$, respectively.

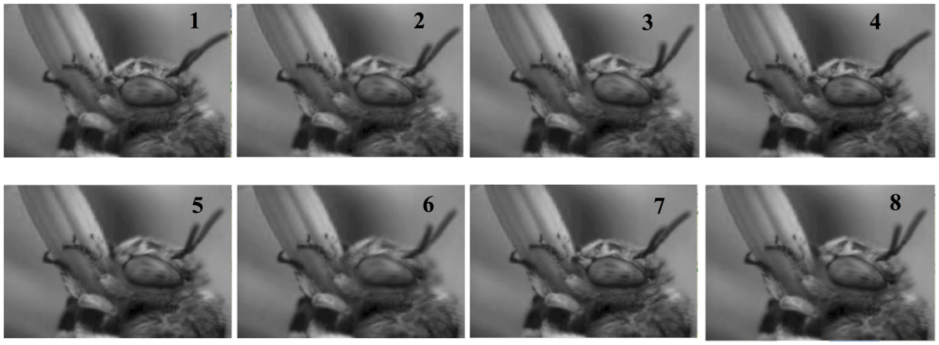


Figure 1.4: Image sequence of *Amegilla murrayensis* during buzz pollination. Rather than grasping the anther firmly with its mandibles like other buzz-pollinating bees, *A. murrayensis* taps its head against the anther of a *Solanum lycopersicum* (cherry tomato) flower at the high buzzing frequency generated by its flight muscles (approximately 350 Hz; Table A.2). The interval between images is 1/1000 of a second. The dark marks on the anthers were made with ink, to help visualize the movement of the anther.

(and many other buzz-pollinating bees described thus far) in how it physically interacts with the flower during buzz pollination. While *B. impatiens* and other bees grasp the flower's anthers with their mandibles as well as their legs, *A. murrayensis* does not. All videos we collected showed *A. murrayensis* grabbing the anther with only its legs, and repeatedly tapping the anther with its head at the high buzzing frequency that is likely generated by its flight muscles (Figure 1.4). Research indicates that the flight muscles are used during sonication in *Bombus occidentalis* (King et al. 1996), and our high speed videos show that the mesosoma of *A. murrayensis* is deforming with each tap of the head – similar to the way that bumblebees' mesosomas deform while they buzz. In particular, the videos for both bumblebees and blue-banded bees show the first segment of the mesosoma, called the pronotum, moving at the same frequency as the head. The mesopleuron can also be seen oscillating during buzz pollination. In two recordings, we saw a bee briefly grasp the anther with its mandibles, but it quickly switched to the head-tapping behavior.

We noticed that *A. murrayensis* left brown marks on the anther cone – these "bee kisses" are interpreted by commercial tomato growers as a sign that bees have visited the flowers (Buchmann and Nabhan 1996). *A. murrayensis* may be damaging the anthers with impact forces, but the resulting "bee kisses" are similar to those left by *B. impatiens*.

Discussion

We found that *Amegilla murrayensis* (blue banded bees) buzz cherry tomato flowers at significantly higher frequencies (∼350 Hz) than *B. impatiens* (∼240 Hz; Table A.2), while accounting for environmental variables. The flight (wing beat) frequencies of both species are lower than their buzz pollination frequencies. This is likely due to the properties of asynchronous muscles, which are part of a resonant system (Josephson et al. 2000). When the mass of the wings is reduced in this system, the wingbeat frequency increases (Roberts and Cartar 2015). Likewise, when the mass on the wings is increased or simply moved further from axis of rotation, the frequency of the resonant system should decrease. The lower frequency during flapping flight is likely because the wings are extended during flapping, but held close to the body during buzz pollination. *A. murrayensis*' flight frequency is significantly higher than that of *B. impatiens*, which is not surprising due to its smaller body size (Burkart et al. 2011). Previous studies have suggested that the amount of pollen released from poricidal anthers increases with buzz frequency (Harder and Barclay 1994), or with buzz frequency and displacement (De Luca et al. 2013; King and Buchmann 1996); thus, the higher buzzing frequency of *A. murrayensis* may be more effective at releasing pollen from the anthers.

Despite the large difference in buzzing frequency between species, the average length of a single buzz was the same in *A. murrayensis* and *B. impatiens*, with both bees buzzing in bouts lasting approximately 1 second – similar to the duration required to eject most of the pollen from *Solanum laciniatum* flowers (King and Buchmann 1996). However, *B. impatiens* spent significantly more time on a single flower (approximately 3.7 seconds), as compared to *A. murrayensis*, which departed after ∼1 second. This difference is due to the fact that *B. impatiens* typically buzzed an individual flower several times (often gathering and cleaning pollen from its body in-between buzzes) before departing, whereas *A. murrayensis* typically buzzed a flower once and then flew away to clean pollen from its body. Occasionally, *A. murrayensis* returned to the same flower after cleaning, but most often it moved onto a new flower.

The fact that *A. murrayensis* spent significantly less time on each flower, typically buzzing the flower only once, combined with its higher (and possibly more effective) buzzing frequency, suggests that *A. murrayensis* may be able to extract pollen from flowers more quickly than *B. impatiens*. An alternative explanation for the difference between bee species in the amount of time spent on each flower is the possibility that tomatoes in Australia could provide different amounts of pollen during buzzing than tomatoes in the United States (due to potential differences in tomato varieties, local environment, visitation rates by local bees, etc.). However, because we recorded *A. murrayensis* performing only a single buzz on both virgin and previously-buzzed flowers – which are known to release less pollen (King and Buchmann 1996) – we do not believe that *A. murrayensis* is adjusting the number of buzzes it performs based on the pollen reward. Whether or not *A. murrayensis* is in fact obtaining more pollen than *B. impatiens* with a single buzz, the behavior of often moving onto the next flower after performing only one buzz appears to be typical for this species, at least when foraging from cherry tomato plants.

We also found that *A. murrayensis* interacts with the flower in a unique way during buzz pollination. Rather than grasping the anthers firmly with its mandibles and shaking (as described for *Bombus* and many other bee genera previously studied; ((Buchmann 1983); (Buchmann and Hurley 1978); Crobet and Huang 2014; Jesson and Barrett 2005; (King 1993)), *A. murrayensis* taps the anthers with its head repeatedly, at the high frequencies most likely generated by its flight muscles. This "head-banging" behavior may be intentional, or it may be a side-effect of the bees being unable to grasp the anthers firmly enough with their mandibles while sonicating, possibly due to their small size or insufficient grip strength - although other, smaller bee species, such as *Lipotriches* (Halictidae) have been recorded grasping anthers firmly with their mandibles during buzz pollination, and we observed *A. murrayensis* grasping onto leaves with its mandibles while grooming its body.

The mechanical features of a buzz that have been proposed to determine how much pollen is dislodged include the length of a buzz and the maximum acceleration (often called amplitude) (De Luca et al. 2013) or velocity of the buzz (Corbet and Huang 2014).

Acceleration and velocity both increase with buzz frequency and with displacement (Corbet and Huang 2014; De Luca et al. 2013). Impact forces, which occur when two objects collide, cause sharp changes in velocity, which in turn produce large spikes in acceleration. Thus, the head tapping observed in *A. murrayensis*, in which the head collides and then disengages with the anther repeatedly at high frequencies, may produce higher accelerations than grasping the anthers firmly and shaking. High accelerations produced by collisions with the anther could lead to higher pollen release rates, or could help break up clumps of wet pollen. These hypotheses could be tested in future studies by manipulating flowers with mechanical shakers that either grasp firmly or collide repeatedly with the anthers, and quantifying the amount of pollen released.

If *A. murrayensis* is capable of removing more pollen with a single buzz (due to its higher buzzing frequency and/or head-tapping behavior), this could be detrimental from the plant's perspective, since a single forager is removing a large portion of its pollen. On the other hand, some researchers have suggested that high pollen removal from poricidal anthers is associated with higher pollen deposition onto stigmas (Harder 1990; Harder and Thomson 1989), although this is not always the case for plants with non-poricidal anthers (Wilson and Thomson 1991). In addition, if *A. murrayensis* routinely spends less time on a single flower, then it may move onto another flower more rapidly than *B. impatiens*, which could lead to higher pollination rates.

The relative effectiveness of *A. murrayensis* and *B. impatiens* as pollinators may also be affected by other aspects of their behavior – for instance, how well each species grooms the pollen from its body (decreasing the probability of transferring pollen between flowers), and whether the behavior of one species brings its body closer to the stigma (increasing the chances of depositing pollen). A definitive answer to the question of pollination effectiveness will ultimately require controlled experiments comparing the yield of tomato plants buzz-pollinated by *A. murrayensis* vs. *B. impatiens*.

Although further work is required to make any claims about the pollination effectiveness of these different bees, our work shows that at least one native Australian bee – *Amegilla*

murrayensis – differs significantly from *Bombus* spp. in several aspects of buzz pollination, including its buzzing frequency and the amount of time spent per flower. Furthermore, our observation that *A. murrayensis* interacts with flowers in a unique way during buzz pollination – by "head-butting" rather than "shaking" the anthers – leads to further questions about the behavioral, mechanistic, and evolutionary roots of this method of buzz pollination.

Acknowledgments

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Chapter 2

Bombus impatiens (Hymenoptera: Apidae) display reduced pollen foraging behavior when marked with bee tags vs. paint

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(For published version, see Switzer and Combes 2016b, *Journal of Melittology*, No.62, pp. 1-13)

Abstract

Numbered bee tags, developed for marking honeybees (*Apis mellifera* Linnaeus), are glued to the mesosoma of many bees to uniquely identify them. We recorded whether or not bees sonicated to collect pollen after being marked, and we compared the sonication frequency, sonication length, and wing beat frequency of *Bombus impatiens* Cresson that were tagged with bee tags vs. marked with paint. We found that bees with tags glued to their mesosoma had no significant change in wing beat frequency, sonication frequency, or sonication length, relative to bees that were marked with paint; however, we found that the probability of collecting pollen via sonication after being marked was much lower for bees marked with bee tags vs. paint.

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Introduction

Behavioral experiments on bees often require that researchers mark individuals so that they can be uniquely identified. If researchers do not know the identities of individual bees, then they risk pseudoreplication, in which they treat each observation as a unique individual when they may actually be collecting repeated measurements on the same individuals. Pseudoreplication can cause incorrect estimates of errors and lead to invalid statistical results (Hurlbert 1984). If researchers treat many observations as independent individuals but have only several individuals, the standard errors associated with coefficients in a multiple regression may be erroneously low, leading to p-values that are also erroneously low.

To remedy this problem, individual bees can be removed from their colonies and placed into experimental cages (Poissonnier et al. 2015). An alternative method of avoiding pseudoreplication while allowing bees to continue to interact with all of their nest-mates is to mark individuals, and then collect only one observation per bee, or account for repeated measurements on the same individual (Milinski 1997). Few studies have investigated how marking insects affects behavior (De Souza et al. 2012; Packer 2005).

Common methods for marking individual bees include marking with dots of paint or attaching uniquely-numbered tags. When marking bees, numbered tags are often used (e.g. Osborne et al. 1999; Osborne and Williams 2001) because paint comes in limited colors, and combinations of paint can quickly become complex and difficult to decipher if researchers wish to mark tens or hundreds of bees. Numbered bee tags, glued to the mesosoma, have regularly been used by beekeepers to identify honeybee queens, and are often used to mark other types of bees.

To attach the tags or apply dots of paint, some researchers narcotize bees with cold or CO2 to keep them from moving while being marked, but Poissonnier et al. (2015) found that these methods affect bee behaviors – activity, brood care, foraging, aggression, and egg production – for up to four days after treatment. In addition, Wilson et al. (2006) found that cold narcosis affects bumblebee foraging recruitment. Because of these potential

confounding factors, alternative methods may be necessary to study bee behavior.

Another method of immobilizing bees is to use a honeybee queen-marking cage (Capaldi et al. 2000; Reynolds et al. 2009), in which a bee is pressed against a mesh grid with a piece of foam. A paint dot or marker can then be placed on the bee, typically on the mesoscutum, by reaching through the grid to access the bee's body. A researcher using a queen-marking cage does not need cold or CO2 narcosis, and thus queen-marking cages are more convenient for field-based experiments.

A variety of glues have been used to affix tags to bees. In general, scientists marking individual insects need an adhesive that is durable, non-toxic in the amount applied, easy to apply, lightweight, and quick drying (Walker and Wineriter 1981). Many bee-tagging kits include lacquer for attachment, but tags attached with lacquer sometimes fall off after a period of time. Superglue (cyanoacrylate) meets many of the qualifications of an effective tag adhesive, and it has been used to attach tags to bees and wasps on many occasions (e.g Crall et al. 2015; Hagbery and Nieh 2012; Medeiros and Araújo 2014; Tenczar et al. 2014; Wilson et al. 2006).

Though commonly used, cyanoacrylate has been reported to affect some aspects of insect behavior. One study documented a high level of mortality when cyanoacrylate was used on the cuticle of corn rootworms, *Diabrotica Chevrolat* (Coleoptera: Chrysomelidae) – the authors suggested the softer cuticle, relative to other unaffected species, as a cause (Boiteau et al. 2009). Other authors describe preparation of honeybees for a flight mill, and recommend not using superglue, because "bees will quickly die" (Scheiner et al. 2013). However, evidence that superglue increases mortality when used on bees is scarce.

Here, we measure the effects of tagging vs. painting bees on their behavior and performance when collecting pollen from plants in large, outdoor enclosures. We measured differences in pollination behavior on tomato (*Solanum lycopersicum*) plants, which release pollen through small pores at the tips of the anthers. Bumblebees collect pollen from poricidal anthers using a behavior termed sonication, or buzz pollination (Buchmann 1983). During sonication, bumblebees grasp the anthers of the flower and vibrate their flight

muscles, without flapping the wings (King et al. 1996). This vibration is transferred to the anthers, and pollen is shaken out of the pores onto the bee's body (King 1993). Because tomato flowers produce no nectar, bees visiting these flowers could collect only pollen.

We measured the sonication frequency and sonication length of unmarked bumblebees during buzz pollination, as well as their wing beat frequency during flight, and then marked bees with either paint or bee tags. Then, we recorded whether these bees sonicated again and recorded the same sonication and flight parameters from marked bees that did resume pollination behavior. We chose to use superglue gel (cyanoacrylate) because it has been used on bees in the past (Tenczar et al. 2014), and because the gel formulation is less likely than liquid superglue to drip into the tegula and interfere with the wings.

Materials and Methods

Study organisms and foraging space

We purchased four, class-A, colonies of *Bombus impatiens* from Biobest (`http://www.biobestgroup.com`). Two colonies arrived on 10 Sept 2015, and two colonies arrived on 22 Sept 2015. Upon receiving the colonies, we verified that queens were present and removed any males. Each colony was placed in a mesh cage that was 1.8 m long by 1.8 m tall by 0.6 m wide. These cages were placed in a pollinator-excluding greenhouse. The greenhouse had mesh walls and a plastic roof – thus the conditions inside the greenhouse were similar to the outdoor conditions. We allowed bees to acclimate to the cages for at least two days prior to starting experiments.

The colonies were insulated by placing them in styrofoam coolers with small holes cut for entry and exit. Each cage contained a nectar feeder (1.0 M sucrose) and pollen feeder to provide nectar and pollen ad libitum. Pollen was purchased from Koppert Biological Systems (`http://www.koppert.com`), ground with a mortar and pestle and placed ($\sim$2 g) in a small, plastic dish. Pollen was replaced approximately every three days.

In addition to the artificial feeders, each cage contained a potted tomato (*Solanum*

lycopersicum). We used two varieties of cherry tomatoes, "Cherry Roma" and "Sweet 100 Hybrid". Each day that we observed the bees, we replaced the plant inside the bees' cage with a different plant that had been kept in a greenhouse that excluded pollinators – thus, we were able to constantly provide freshly-opened flowers for foraging. We observed all four of the colonies until 16 Oct 2015.

We also recorded local weather data – barometric pressure, temperature, relative humidity, and light intensity – at the time of every observation, using a weather station inside the greenhouse.

Marking foragers and collecting audio recordings

During each observation day, we placed a plant with freshly-opened flowers inside a cage, and waited outside the cage, observing bees foraging on the *S. lycopersicum* flowers. When a forager landed on a flower, we reached into the cage with a shotgun microphone (SGM-1X, Azden, Tokyo, Japan) and collected an audio recording that included both sonication and flight behavior (after the bee took off) with a digital recorder (DR-100mkII, Tascam, Montebello, California). After recording an individual bee, we captured it with an insect vacuum (2820GA, Bioquip, Rancho Dominguez, California) and transferred the bee from the aspirator tube into a queen-marking cage with a plunger (The Bee Works, Oro-Medonte, Ontario, Canada). We gently pressed the bee against the mesh at the top of the tube to immobilize her while we marked her mesosoma.

We alternated between marking captured bees with paint or bee tags (Figure 2.1), to randomize the age distribution among bees with each type of mark. In total, we marked 100 bees with paint and 112 bees with tags. We excluded one individual marked with a bee tag from statistical analyses because we later determined that it was a newly emerged queen. We did not use all of the marked individuals for all analyses because we were not able to obtain all type of data for all individuals. We used Sharpie oil-based paint pens (Sharpie, Oak Brook, Illinois), after finding that water-based paints wore off too quickly in preliminary experiments. We used unique colors or combinations of two colors on each

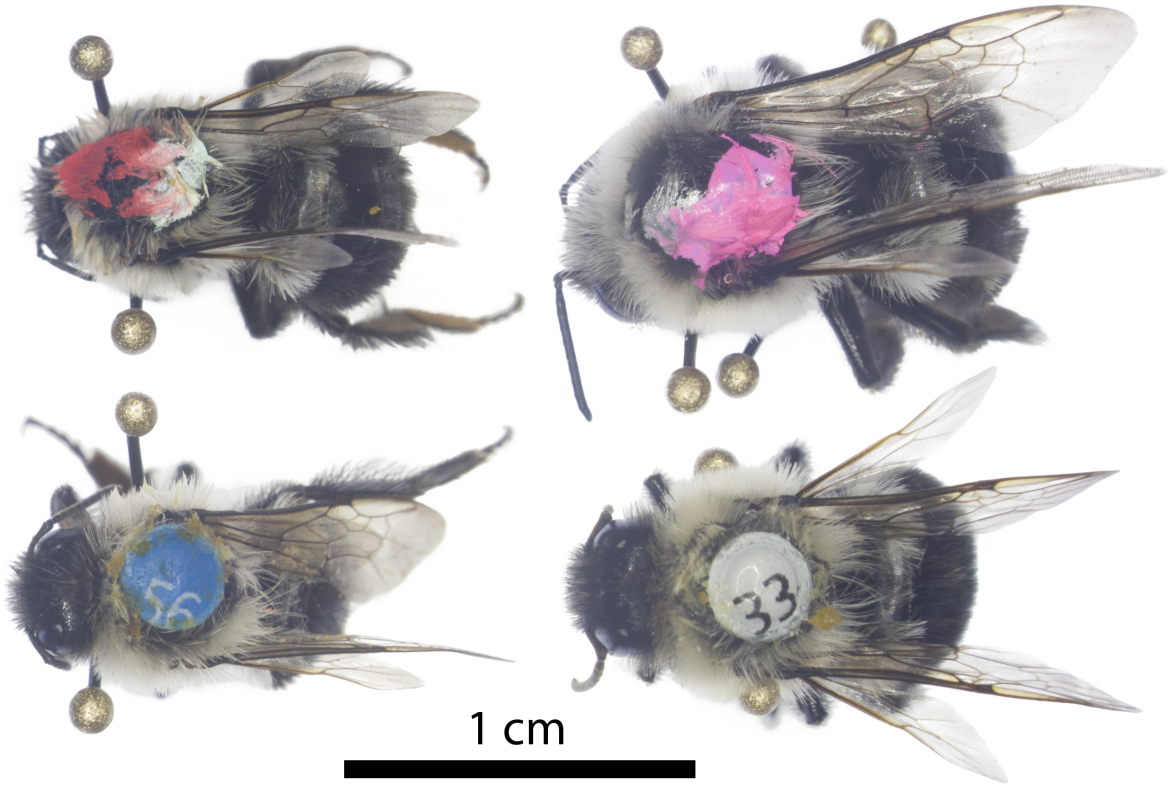


Figure 2.1: *Bombus impatiens* individuals marked with bee tags (bottom) and paint (top). Black bar is 1 cm.

individual. After placing small dots of paint on the dorsal part of the bee's mesosoma, we used the output vent from the insect vacuum to blow air onto the paint for 30 s to dry, before releasing it back into the cage.

For marking bees with tags, we used queen-marking tags, which are small, colored plastic discs (∼3 mm diameter, ∼1.5 mg) that are numbered 1-99 with a variety of background colors (queen marking kit, Abelo, Full Sutton, York, United Kingdom). To apply a tag, we pressed the bee gently into the mesh at the top of the queen-marking cage and applied a small dot of superglue gel (cyanoacrylate, Gel Control, Locktite, Henkel Corporation, Westlake, Ohio). We attempted to apply glue only to the mesoscutum but sometimes covered other areas, especially if the bee was very small. We then pressed the bee tag onto the glue and used the output vent from the insect vacuum as indicated above. We released bees back

into the cage by letting them fly out of the queen marking cage, and thus confirmed that at the time of release they were able to flap all of their wings.

Whenever we observed previously-marked individuals foraging for pollen on *S. lycopersicum* plants, we again collected audio recordings of their sonication and flight behavior, for comparison with the recordings we made before marking. We observed 118/212 bees engaging in sonication behavior after being marked. Of these, 40 were marked with bee tags and 78 were marked with paint. We did not observe each cage every day due to poor weather conditions on some days. Rain hitting the top of the greenhouse or heavy wind shaking the greenhouse interfered with audio recordings by increasing background noise.

At the end of the experiment (16 Oct 2015), we collected all of the bees from the colonies, recorded whether or not they were alive, and used digital calipers to measure their intertegular (IT) span, the minimum distance between the inner margins of the tegula (wing bases). We were unable to collect IT span measurements for all marked bees, as the marks sometimes wore off of the bees before the end of the experiment. We excluded these individuals from our analysis, because we have no evidence that either paint or bee tags were more likely to wear off (paint = 17/100 bees missing at the end of the experiment; bee tag = 17/112 bees missing).

Extracting data from audio recordings

We used R (R Core Team 2016), with the packages, seewave (Sueur et al. 2008) and tuneR, (Ligges 2013) to extract sonication and wing beat frequencies from the audio recordings. We first listened to the recordings to identify the loudest, longest sonication sound. We analyzed only the loudest, longest sonication because during observations we noticed that bees often performed shorter, higher-frequency buzzes on the petals of the flowers. In an effort to compare the same type of sonication (i.e., pollen-collecting buzzes) among all bees, we excluded these short "petal buzzes" from analysis. We classified a bout of buzzing as a single sonication if there were no audible breaks of $\sim$0.2 s or more in the buzzing. After selecting the loudest, longest sonication, we determined the length of the sonication buzz,

and used the "spec" function from the seewave package to calculate the power spectral density, using a hanning window of 2048 points (Sueur et al. 2008). To identify the sonication frequency (the dominant frequency at which the bee was vibrating), we selected the highest peak on the spectrum between 195 Hz and 400 Hz. We chose this range based on results from De Luca and Vallejo-Marín (2013), Switzer et al. (2016), and preliminary observations on commercial colonies of *B. impatiens*, all of which suggest that sonication buzzes of *B. impatiens* fall within this range of frequencies.

To check the accuracy of the frequency identified as the highest peak in the spectrum, we generated a sine wave at this frequency, and C. Switzer aurally compared the sound of the sine wave to the audio recording of sonication by listening to the two sounds, played in close succession. Sometimes the frequency identified as the highest peak in the spectrum sounded very different in pitch from the raw audio recording; this often occurred when the recording had a great deal of background noise. In these cases, we used Audacity (Audacity Development Team 2015) to identify the sonication frequency. Within Audacity, we selected the sonication portion of the audio recording and plotted the spectrum (hanning window, 2048 points). We then generated sine waves at each of the frequencies corresponding to the peaks in the spectrum. C. Switzer compared each of these sine waves to the recording, aurally, and chose the peak that corresponded most closely in frequency to the audio recording of the sonication.

We used the same process to quantify wing beat frequency during flight – selecting a portion of the recording that contained the bee flying, plotting a spectrum, and selecting the highest peak. We changed the range to 120 Hz to 220 Hz for selecting the peak – based on Switzer et al. (2016) and preliminary data collected from similar commercial colonies – and checked all wing beat frequencies aurally in the same way as for sonication frequency.

Statistical Analysis

To determine whether the two marking methods affected sonication frequency, sonication length, or wing beat frequency, we subtracted the value of each variable recorded after

marking bees from the value recorded before marking. Thus, if bees had the same value for these variables before and after marking, the change in behavior would be zero. If a bee had a lower value after marking, then the difference in behavior would be negative.

We performed multivariate multiple regression to determine if there were significant changes to the bees' behaviors – wing beat frequency, sonication frequency, and sonication length. We were able to make comparisons only on bees that performed sonication behavior again after being marked (num. tag= 30, num. paint=62). Since we suspected that environmental variables such as temperature might affect some of these behaviors (Unwin and Corbet 1984), we initially included the following weather covariates in our models: temperature, pressure, light intensity, and relative humidity. We also included the following variables: mark type, IT span, tomato variety, number of days between initial recording and post-mark recording, and colony number (since we used four colonies). We used the "vif" (Variance Inflation Factor) function from the car package to check for multicollinearity, and found no problems with our data (Fox and Weisberg 2010). We used the "mStep" function from the qtlmt package in R to drop terms from the model sequentially, using Akaike Information Criterion (AIC) (Cheng 2013). We conducted stepwise procedures for backward stepwise regression, starting with all of the covariates listed above and the interaction of mark type * intertegular span. We included this interaction because, prior to collecting data, we suspected that bee tags might affect smaller bees more severely. We had no prior reasons to include any other interactions. We forced all of the models to contain mark type as a covariate. We report the model with the lowest AIC from the stepwise procedure.

To determine if the marking method affected whether or not bees continued foraging for pollen from tomato plants after being marked, we used survival analysis techniques from the R package, survival (Therneau and Grambsch 2013). This type of analysis is often used in clinical studies that are right-censored. The data recorded includes the amount of time since diagnosis, and whether or not an event (often death) occurs. The data are right-censored because the event does not occur for all participants in the study. Survival analysis can be performed with many events. For instance, it has been used to model

the amount of time until seeds germinate (Manso et al. 2013). Seed germination time is right-censored because some of the seeds may die, whereas others are not dead, but do not germinate by the end of the study. Here we use "collecting pollen from *S. lycopersicum* after being marked" as our event. Our data are right-censored because some of the marked bees died, whereas others stayed alive, but were never observed sonicating on *S. lycopersicum* after being marked, within the time limits of the study.

We used Cox proportional hazards regression to determine if there was a significant difference between the two mark types in the probability of bees sonicating after being marked. We used Cox regression so we could include IT span and colony number as covariates. We centered the IT span variable before modeling to make interpretation easier. We also suspected an interaction between mark type and IT span, so we included an interaction: IT span * mark type. We used a likelihood ratio test to determine whether including colony number in the model made it significantly better.

We report no p-value corrections to account for multiple comparisons because available correction methods would not change our results (i.e. the significant results we report regarding the effects of mark type on behavior are all with p-value « 0.05). We used the R packages, ggplot2 (Wickham 2016) and ggfortify (Horikoshi and Tang 2015) to make Figure 2.2.

Results

Does marking affect flight or sonication mechanics?

The final model for sonication frequency, sonication length, and wing beat frequency included only mark type and the number of days between observations as explanatory variables – i.e., none of the other covariates significantly improved the model (i.e. no other covariates reduced AIC). Our overall model showed no significant differences in wing beat frequency, sonication frequency, or sonication length depending on the mark type (MANOVA; Pillai test stat = 0.133; approx. $F(6,91) = 2.09$, p-value = 0.056). This model

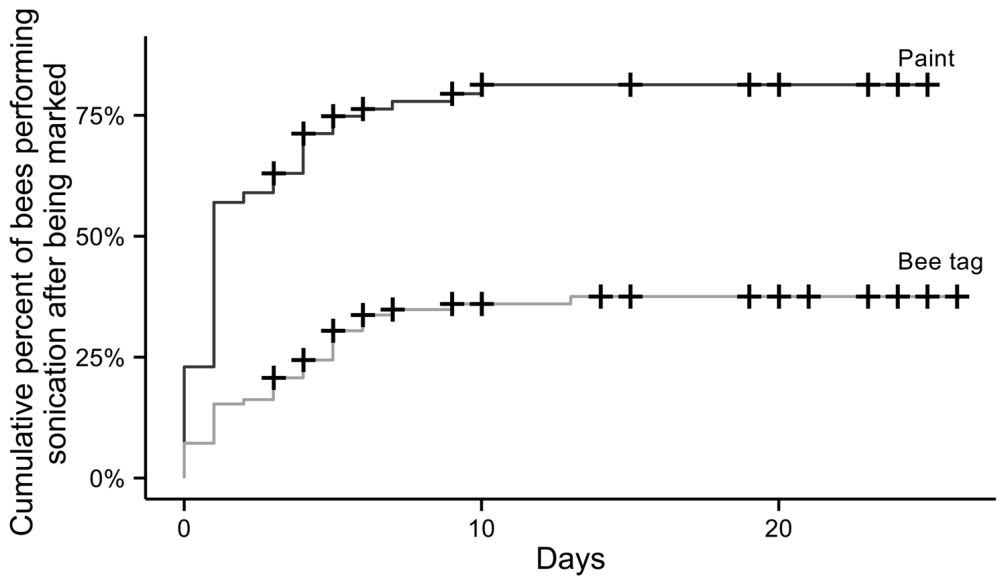


Figure 2.2: Curves showing the cumulative percentage of bees that used sonication on *Solanum lycopersicum* after being marked with paint vs. bee tags, out of the total number of marked bees recovered by the end of the experiment (npaint = 83; ntag = 94; nmissing = 34). The "+" symbols indicate censored data – bees that never were observed collecting pollen after being marked, within the time constraints of the experiment.

included only bees that sonicated after being marked (ntag= 30, npaint=62). Generally, when the overall model is not significant, researchers do not investigate further comparisons (Hsu 1996). However, because of the nearly significant p-value (0.056) for the overall model, we chose to skeptically investigate the separate multiple regressions. When we investigated the coefficients of separate multiple regressions for wing beat frequency, sonication frequency, and sonication length with mark type and days between observation as independent variables, we found no significant effects of bee tags vs. paint marks on any of the variables. However, the regression for wing beat frequency may warrant further investigation (β(mark = tag) = -3.2; t(89) = 1.76; p-value = 0.081), with tagged bees displaying a slightly lower ($\sim$ 5 Hz) wing beat frequency than painted bees. See Table B.1 for mean and standard deviations for each of these behaviors.

Does marking affect the likelihood of engaging in further sonication?

Figure 2.2 shows the cumulative percentage of bees that were observed sonicating on *S. lycopersicum* after being marked with paint or a bee tag, out of the total number of marked bees that were recovered by the end of the study (ntag = 94; npaint = 83; nmissing= 34). We started with a model that included colony number as a covariate, but removed this based on the results of a likelihood ratio test. Our final model is based on the following significant covariates: mark type, IT span, and the interaction between these two variables (Likelihood Ratio Test: $\chi^2(3) = 59.05$, p-value < 0.001). The hazard ratio for being marked with a bee tag is 0.25, which means that for a fixed point in time, individuals of average IT span that were marked with bee tags were about one fourth as likely to engage in further sonication behavior, as compared to bees that were marked with paint (β(Mark: Bee tag) = -1.37; z = 5.97; p-value < 0.001).

Though IT span was not a significant predictor of whether bees would engage in further sonication for bees marked with paint (β(IT Span) = 1.73; z = 1.83, p-value = 0.067), we included it in the model because there was a significant interaction of IT span * mark type. The interaction term suggests that the effects of different types of marks vary depending on bee size. In particular, for bees marked with bee tags, a larger IT span has a larger an effect on the probability of bees engaging in further sonication behavior than it does for bees marked with paint; for bees marked with tags, a one-mm increase in IT span corresponds to being 2.77 times more likely to engage in further sonication behavior (β(Mark: Bee tag * IT Span) = 1.02; Hazard ratio = 2.77; z = 2.12; p-value = 0.034). We discuss possible explanations for the interaction below.

Bee mortality

After collecting all bees on 16 Oct 2015, we classified them as either dead or alive. Out of all the bees we marked, about half (118/212) were observed engaging in further sonication behavior. These were used in another study and thus excluded from the mortality results. Of the bees that did not sonicate after marking (94/212), 47 were alive, 31 were dead, and

16 no longer had a mark. Of the painted bees that did not engage in further sonication behavior after being marked (22/94), we found 5 dead and 14 alive, and we were unable to recover 3 (23% dead, 64% alive, 13% N/A). Of the bees marked with bee tags that did not engage in further sonication behavior, (72/94), we found 26 dead and 33 alive, and we were unable to recover 13 (36% dead, 46% alive, 18% N/A). However, because bees that engaged in sonication behavior again after being marked were excluded from the mortality analysis, a formal test for differential mortality is inappropriate in this case.

Discussion

We investigated how two common methods of marking individual bees – paint and bee tags – affected the sonication behavior of bumblebees. For bees that engaged in further sonication behavior after being marked we found that tagging resulted in no significant differences in wing beat frequency, sonication frequency, or sonication length, relative to paint markings. We found a trend, though not a statistically significant one, suggesting that bee tags may result in a decreased wing beat frequency, relative to marking with paint. Thus, future experiments aimed at testing the effects of mark type on wing beat frequency are warranted; these tests would likely require a larger sample size of wing beat frequencies collected before and after marking than we were able to collect in the current study (ntag= 30, npaint=62), to determine whether results are significant.

Though we found no significant differences in the mechanical behavior of bees that engaged in further sonication after being marked, we did find that the probability of observing bees engaging in further sonication behavior on *S. lycopersicum* after being marked was greatly affected by the mark type (Figure 2.2). Bees with bee tags were much less likely to sonicate on *S. lycopersicum* again after being marked.

We did not have enough evidence to determine whether bees marked with bee tags are more likely to die than those marked with paint, and we acknowledge that our sample may be biased, because we were only able to quantify mortality in bees that were not observed sonicating again after being marked. Our sample sizes for recording mortality differed

greatly between mark types, with 26 dead of 72 individuals marked with bee tags and 5 dead of 22 individuals marked with paint. Our results do not provide evidence to link mortality with the mark type. We also did not compare our marked bees to bees that are completely unmarked, because we needed to mark bees in some way to be able to identify them throughout the experiment. We report our mortality results, however, because they may suggest follow-up studies to determine whether different marking methods affect mortality.

Many studies have glued markers and other devices to bees, but the effects of these manipulations have rarely been examined. Hagen et al. (2011) glued radio transmitters (200 mg) to *Bombus* spp. to track their foraging behavior, and they reported a significant behavioral change due to the transmitter. They suggested that the large mass (about 100 times more than a bee tag) may be the cause of the behavioral change, but they did not directly test this hypothesis.

Bee tags have also frequently been used in mark-recapture studies (e.g. Eltz et al. 1999). In our study, we would have overestimated the population size if we had been using bee tags as markers, because far fewer of the bees marked with tags were "recaptured" (i.e., observed engaging in further sonication behavior), relative to painted bees.

We do not know why applying bee tags had such a dramatic effect on the likelihood of bees engaging in further sonication, but we can speculate about several potential explanations. First, due to the difficulty of immobilizing a bee in a queen-marking cage, errors in marking are common – smeared glue or paint, off-center tag placement, etc. These errors are likely to be more problematic with glue than with paint, as glue transferred to other parts of the body may have more negative effects. Sometimes the bee can reach the tag with her legs while in the queen cage, before the glue is dry. Glue smeared accidentally onto other body parts could be a cause of some bees changing their behavior after being tagged. Future studies could test for the effect of superglue alone by marking bees with superglue (perhaps colored) without a bee tag to determine if the glue or the tag is more problematic.

A second potential source of the behavioral differences in our experiments is the length

of time we allowed for the glue to dry before releasing bees from the queen cage. We chose to dry the glue for 30 s before releasing the bee because we were trying to minimize handling time – but drying the glue for a longer period of time may have helped prevent any potential glue smearing. Third, the size and solid shape of the bee tags themselves, relative to the paint markings, could have contributed to the behavioral change. Though we did not quantify the amount of time bees spent trying to clean their dorsal mesosoma after being marked, we noticed that bees that had been marked with bee tags tended to spend a lot of time using their middle legs to try to remove the tag. This agrees with past research. De Souza et al. (2012) found that marking social wasps with water-based ink caused an increase in short-term grooming behavior. Finally, the difference in whether bees engaged in further foraging/sonication behavior could potentially be related to colony dynamics. Are the bees inside the colony excluding or acting aggressively toward bees that have bee tags, as compared to those with paint markings? Packer (2005) found that marking solitary and semisocial halictid bees on the top of the head with paint affects their interactions with conspecifics, in terms of aggression and cooperation. Future, in-colony observations may provide an answer to this question.

We can also propose a few potential explanations for the significant interaction term between mark type and IT span in the Cox regression. One is that the bee tag is proportionally larger for smaller bees – in the smallest individuals, sometimes the bee tag is wider than the intertegular span – and this relatively larger tag may lead to greater behavioral changes in smaller bees. Second, smaller bees may be more likely to get glue smeared onto their wings. This could happen because of operator error: tagging small bees is more difficult than tagging large bees, because they are more difficult to hold in the queen-marking cage. Since the wing bases are closer together, the researcher may be more likely to place glue onto them than with a large bee. The same type of operator error may have happened with paint, but the consequences for the bee may be less severe when the bee is marked with paint, rather than glue.

This work has several implications for future experiments that involve marking bees.

First, since we found no significant mechanical differences (sonication frequency, sonication length, or wing beat frequency) in bees that engaged in further sonication behavior after being marked, we can justify using tags or paint to mark bees for experiments aimed at measuring these variables. Furthermore, although CO2 and cold narcosis have been shown to cause behavioral changes in activity level, brood care, foraging, aggression, and egg production (Poissonnier et al. 2015), these methods may still be preferable to immobilizing bees with queen cages when applying bee tags. If bees are narcotized with cold or CO2, then tag position is more precise and the glue has longer to dry, so the risk of bees smearing glue into the tegula should be decreased. However, based on our current results showing that bee tags decrease the probability that a bee will engage in further sonication, researchers studying this behavior may get better returns if they mark bees with paint, rather than bee tags. Most importantly, we suggest that experiments be carried out one or two days after tagging bees, so that researchers perform experimental treatments only on marked bees that have resumed normal behavior following the marking treatment.

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Chapter 3

The neonicotinoid pesticide, imidacloprid, affects *Bombus impatiens* (bumblebee) sonication behavior when consumed at doses below the LD_{50}

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Abstract

We investigated changes in sonication (or buzz-pollination) behavior of *Bombus impatiens* bumblebees, after consumption of the neonicotinoid pesticide, imidacloprid. We measured sonication frequency, sonication length, and flight (wing beat) frequency of marked bees collecting pollen from *Solanum lycopersicum* (tomato), and then randomly assigned bees to consume 0, 0.0515, 0.515, or 5.15 ng of imidacloprid. We recorded the number of bees in each treatment group that resumed sonication behavior after consuming imidacloprid, and re-measured sonication and flight behavior for these bees. We did not find evidence that consuming 0.0515 ng imidacloprid affected the sonication length, sonication frequency, or flight frequency for bees that sonicated after consuming imidacloprid; we were unable to test changes in these variables for bees that consumed 0.515 or 5.15 ng because we did not observe enough of these bees sonicating after treatment. We performed Cox proportional hazard regression to determine whether consuming imidacloprid affected the probability

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of engaging in further sonication behavior on *S. lycopersicum* and found that bumblebees who consumed 0.515 or 5.15 ng of imidacloprid were significantly less likely to sonicate after treatment than bees who consumed no imidacloprid. At the end of the experiment, we classified bees as dead or alive; our data suggest a trend of increasing mortality with higher doses of imidacloprid. Our results show that even modest doses of imidacloprid can significantly affect the likelihood of bumblebees engaging in sonication, a behavior critical for the pollination of a variety of crops and other plants.

Keywords

buzz pollination; native bees; pollination; *Solanum*; tomato; vibration

Introduction

Crops and wild plants depend on insects for pollination (Klein et al. 2007; McGregor 1976). Honeybees pollinate many crops, but wild bees are valuable pollinators for a significant number of crops globally (Klein et al. 2007). Because much of the human food supply depends on bees, concern has been expressed about recent population declines in honeybees (*Apis mellifera*) (Allen-Wardell et al. 1998; vanEngelsdorp et al. 2009) and wild bees (Gallai et al. 2009; Goulson et al. 2015). Pesticides may be one factor contributing to the wide-scale decline of pollinators (Potts et al. 2010), and many studies have investigated the effects of sub-lethal and chronic exposure on bees (Desneux et al. 2007; Gill and Raine 2014; Gill et al. 2012; Henry et al. 2012; Whitehorn et al. 2012).

One common pesticide used in the United States and much of the world is imidacloprid (Pollak and Vouillamoz 2011). This is a systemic and contact insecticide in the class of neonicotinoid (mimicking nicotine) pesticides (Gervais et al. 2010; Mullins 1993; Tomizawa and Casida 2005). It acts on acetylcholine receptors in the nervous system (Nagata et al. 1997) and has low mammalian toxicity (Mullins 1993), but causes paralysis and eventual death in insects (Kagabu et al. 2004). Imidacloprid kills freshwater arthropods at concentrations of

between several μg L^{-1} to several thousand μg L^{-1} (Beketov and Liess 2008). Neonicotinoid pesticides have delayed effects that lead to death, but not within the time span of typical LD50 (lethal oral dose) measurements of 24 or 48 hours (Beketov and Liess 2008; Rondeau et al. 2014). Concerns about the effects of neonicotinoids on pollinators led to an EU-wide ban on three neonicotinoid pesticides in 2013, including imidacloprid (Decourtye et al. 2013; European Commission 2013).

Neonicotinoids and their metabolites (also toxic to insects) have been reported in all tissues of plants, including pollen and nectar (Pohorecka et al. 2012). When applied to the soil, imidacloprid can be incorporated into nectar for up to $\sim$230 days after application (Byrne et al. 2014). When imidacloprid is applied to seed coatings, the majority of the pesticide (up to 90%) remains in the soil (Goulson 2013). The amount of pesticide found in pollen and nectar varies. The following values have been reported for imidacloprid residues: 10 ppb nectar and 14 ppb in pollen from squash (Stoner and Eitzer 2012), 16 ppb in buckwheat nectar (Krischik et al. 2007), and 12.8 ng mL^{-1} in nectar of citrus trees (Byrne et al. 2014). In the citrus experiment, Byrne et al. (2014) found that when imidacloprid was applied at the full label rate (1.02 L ha^{-1}), the highest reported value in nectar was 21.9 ng mL^{-1}; however, taking into account the total residues of imidacloprid and its toxic metabolites, the highest amount reported was 37.1 ng mL^{-1}. Furthermore, the highest amount of total residues in uncapped nectar from the hive comb of nearby honeybees was reported as 95.2 ng mL^{-1} (Byrne et al. 2014). Other researchers have estimated that honeybees foraging for nectar could consume between 1.1 and 4.3 ng of imidacloprid over their lives (Rortais et al. 2005).

The lethal oral dose (LD50) of imidacloprid for bees has been measured in several studies, with significant variation. The LD50 for bumblebees has been reported at 40 ng per bee (24-hour) and 20 ng per bee (72 hour) (Marletto et al. 2003). For honeybees (*A. mellifera*), a more recent review highlights several studies that report the 48-hour LD50 for honeybees to be in the range of about 4 to 104 ng per bee (Blacquiere et al. 2012).

Some studies report that imidacloprid irreversibly blocks nicotinic acetylcholine receptors

in insects (Rondeau et al. 2014; Tennekes and Sanchez-Bayo 2011). Scarce data exists about whether a single, concentrated dose or a chronic, low dose of imidacloprid is more harmful to insects (Van den Brink et al. 2016). Different arthropods show different sensitivities to acute vs. chronic doses (Roessink et al. 2013), and pesticides affect arthropods differently in summer vs. winter (Van den Brink et al. 2016). Suchail et al. (2001) report that chronic exposure to imidacloprid was toxic to honeybees at a 60-6000 times lower dose than those required to produce the same acute effect.

Neonicotinoid pesticides are also known to have sub-lethal effects on bees. Learning ability in bumblebees was affected by even a small dose of imidacloprid (10 μl of 10 ppm imidacloprid in sugar solution) (Stanley, Smith and Raine 2015), and imidacloprid-treated pollen has been shown to reduce visual learning capacity in honeybees (Han et al. 2010). Asian honeybees (*Apis cerana*) showed reduced olfactory learning after consuming as little as 0.1 ng of imidacloprid (Tan et al. 2015). Furthermore, neonicotinoid pesticides have sub-lethal effects on bumblebees at the colony level – colonies consuming pollen and sugar water containing 6 mg kg^{-1} and 0.7 mg kg^{-1} imidacloprid, respectively, had an 85% reduction in production of new queens (Whitehorn et al. 2012).

Chronic, sub-lethal doses of neonicotinoids have also been shown to alter some aspects of pollination behavior in bees. Bumblebees experiencing chronic exposure to neonicotinoids change their pollination behavior by returning with less pollen (Feltham et al. 2014). Imidacloprid ingestion by honeybees has been shown to reduce the number of returning foragers and the number of foraging bouts per bee (Karahan et al. 2015). Other research suggests that pesticides impair the pollination services provided by bumblebees – bumblebee colonies that were exposed to field-realistic doses of pesticides for 13 days showed lower visitation rates to apple blossoms, which resulted in these apple trees producing fewer seeds than trees that were pollinated by untreated bees (Stanley, Garratt, Wickens, Wickens, Potts and Raine 2015).

Experiments investigating the effects of chronic pesticide exposure are valuable in that they may mimic field-realistic experiences, but these types of experiments are typically

unable to measure the amount of pesticide actually ingested by bees, because measuring the amount of contaminated nectar that a bee consumes in the field is difficult. The estimates for the amount of nectar a foraging bee consumes in a day vary widely. The consumption of sugar depends on the bee's energetic needs – Beutler and Loman (1951) suggests that honeybees consume 11 mg sugar per hour of flight, and Rortais et al. (2005) estimated that a honeybee foraging for nectar during the summer would consume 32–128.4 mg of sugar per day.

Bumblebee workers consume more nectar per day than honeybees. Furthermore, foragers consume sugar at a higher rate than nest workers (Rortais et al. 2005), likely due to the energetic demands of flying and collecting resources. Incubating *Bombus terrestris* queens metabolize approximately 600 mg of sugar per day, according to respiration rate data (Silvola 1984), and male bumblebees (*Bombus lucorum*) foraging for 4 hours per day consume an average of 180 μl of 50% sugar solution in 24 hours – about 90 mg of sugar per day (Bertsch 1984). Laycock et al. (2012) reported individual bumblebees (*Bombus terrestris*) consuming 400 mg of sugar syrup (1.27 kg L^{-1} fructose/glucose/saccharose) in a day.

All of these reports suggest that a B. impatiens worker, foraging for a full day, could reasonably consume 150 - 300 μl of nectar (50% w/w sugar) per day. If that nectar had a field-realistic concentration of imidacloprid (10 μg L^{-1}) (Stoner and Eitzer 2012), then a forager could ingest 1.5 to 3 ng of imidacloprid during a day of foraging. This estimate is similar to Laycock et al. (2012), which reported *B. terrestris* individuals consuming 3 ng of imidacloprid in a day when they fed on sugar syrup with imidacloprid (10 μg L^{-1}).

Here, we investigate the effects of imidacloprid on an important aspect of bumblebee pollination behavior, upon which many crops and other plants depend – sonication, or buzz pollination. We examined bumblebees (*Bombus impatiens*) sonicating on the blossoms of tomato plants (*Solanum lycopersicum*); these flowers produce only pollen (no nectar), enclosed within tube-like anthers with small pores that release pollen when sonicated, or vibrated, by bees (Buchmann 1983; Thorp 2000). Bumblebees and other wild bees (but not honeybees) can produce these vibrations by contracting their flight muscles at high frequencies without

flapping their wings.

We provided bees with doses of imidacloprid that were well below reported LD50 values in a single, concentrated treatment (Marletto et al. (2003) reported a 48-hour LD50 of 20 ng per individual *B. terrestris*). Two of our treatment groups represent amounts of imidacloprid that a bee could reasonably ingest in a single day (0.0515 ng and 0.515 ng), and the highest treatment, represents an amount that a bee may consume over several days (5.15 ng). We quantified several aspects of sonication and flight behavior, including sonication frequency, sonication length, and flight frequency, before treatment. After treatment, we observed bees for up to several weeks, recording which bees resumed sonication behavior, and re-measuring sonication and flight variables in bees that did perform buzz pollination. Unlike past studies, which treated whole hives of bumblebees with chronic doses of imidacloprid (e.g. Gill and Raine 2014; Stanley, Garratt, Wickens, Wickens, Potts and Raine 2015), we controlled the amount of imidacloprid ingested by individual bees by providing a single, measured dose in sucrose solution to each bee.

Material and Methods

Study organisms and foraging space

We purchased four class-A hives of *Bombus impatiens* from Biobest (`http://www.biobestgroup.com`). Two hives arrived on 10 Sept 2015, and another two hives arrived on 22 Sept 2015. Upon receiving the hives, we verified that queens were present in each, and we removed any males. Each hive was placed in a mesh cage that was 1.8 m long by 1.8 m tall by 0.6 m wide. These cages were placed in a pollinator-excluding greenhouse. The greenhouse had mesh walls and a plastic roof – thus the conditions inside the greenhouse were similar to outdoor conditions. We allowed bees to acclimate to the cages for at least two days prior to starting experiments.

The hives were enclosed in foam coolers for insulation, with small holes cut for entry and exit. Each cage contained a pollen feeder and a nectar feeder, providing pollen and nectar ad

libitum. Nectar consisted of 342 g organic cane sugar per liter of water, or $\sim$ 1 M sugar water. Pollen was purchased from Koppert Biological Systems (`http://www.koppert.com`) and ground with a mortar and pestle before placing $\sim$2 g in a small, plastic dish. Pollen was replaced approximately every 3 days.

In addition to the artificial feeders, each cage contained a potted tomato plant (*Solanum lycopersicum*). We allowed bees to visit two varieties of cherry tomatoes with similar floral morphology, "Cherry Roma" and "Sweet 100 Hybrid". We used "Sweet 100 Hybrid" only on days when we did not have enough fresh "Cherry Roma" flowers. Each day that we observed the bees, we replaced the plant inside the bees' cage with a different plant that had been kept in a greenhouse that excluded pollinators. Thus, we were able to rotate tomato plants into the bee cages, constantly providing freshly-opened flowers for pollen foraging each day. We observed all four of the hives, typically between 10am and 4pm, until 16 Oct 2015. Because new individuals were treated daily, different numbers of individuals were observed for a range of dates.

We also recorded local weather data – pressure, temperature, relative humidity, and light intensity – at the time of each observation, using a weather station inside the greenhouse.

Marking individual foragers

We observed hives on each day with good weather (sunny and relatively still) until 16 Oct. When a bee was first observed foraging on *S. lycopersicum*, we captured her with an insect vacuum (2820GA, Bioquip, Rancho Dominguez, California) and transferred her from the aspirator tube into a queen-marking cage with plunger (The Bee Works, Oro-Medonte, Ontario, Canada). We gently pressed the bee against the mesh at the top of the tube to immobilize her while we marked her mesosoma.

We alternated marking the bees with paint or bee tags, to evaluate whether the marking method affects foraging behavior (Switzer and Combes, in review, Journal of Melittology). We marked about half of the bees with paint, using oil-based paint pens (Sharpie, Oak Brook, Illinois), and the other half with bee tags - small, numbered plastic discs ($\sim$3 mm

diameter; Queen marking kit, Abelo, Full Sutton, York, United Kingdom) attached to the mesosoma with superglue (Gel Control, Locktite, Henkel Corporation, Westlake, Ohio). After applying paint or affixing the tag with superglue, we used the outward vent from the insect vacuum to blow air onto the paint/glue for 30 seconds to dry it before releasing the bee back into the cage.

When we observed previously-marked individuals foraging for pollen on the *S. lycopersicum* plants, we reached into the cage with a shotgun microphone (SGM-1X, Azden, Tokyo, Japan) to collect an audio recording that included sonication and post-sonication flight sounds with a digital recorder (DR-100mkII, Tascam, Montebello, California). We then recaptured the bee for treatment with imidacloprid. We treated new bees with imidacloprid almost every day of the experiment and therefore did not have the same number of post-treatment observation days for each bee. This is accounted for by using Cox regression (below).

Imidacloprid treatments

After recapture, each marked bee was transferred to a clear, one-liter, plastic container with a vented lid, and held indoors without access to nectar or pollen for an average of 140 minutes. After being deprived of food, bees were randomly assigned to one of four treatment groups, and fed 10 μL of sugar water mixed with different amounts of imidacloprid (Pestanal, Sigma-Aldrich, St. Louis, Missouri), using a micropipette.

To prepare solutions for the treatment groups, we dissolved imidacloprid in deionized water and performed a series of dilutions to obtain the correct doses. Solutions were mixed and stored out of UV light, since imidacloprid breaks down quickly in water that is exposed to light at wavelengths between 200-300 nm (Zheng and jin Lee 2004). We fed 10 μL of sugar solution mixed with imidacloprid to bees, resulting in the bees consuming 0.0515, 0.515, or 5.15 ng of imidacloprid. After feeding bees with 10 μL of imidacloprid solution, we used a clean micropipette to feed additional, untreated 1 M sugar water to the bees until they stopped drinking (stopped extending their proboscis). We deprived treated bees of food for

at least one hour after consuming the imidacloprid solution and additional nectar, and then released them back into the foraging cages.

The first time we observed a bee foraging on *S. lycopersicum* after treatment, we again recorded sonication and flight sounds with a microphone, then collected the bee and removed it from the experiment. The amount of time that elapsed between pre and post-treatment recordings was different for each bee – we account for the differences when we analyze the sounds, and we analyze the differences in time directly, using Cox regression (below).

At the end of the experiment (16 Oct 2015), we collected all of the remaining bees from the hives, recorded whether they were alive or dead, and used digital calipers to measure their intertegular (IT) span (the distance between the wing bases). We were unable to collect IT span measurements for all bees, as a small proportion of the paint marks or tags wore off during the course of the experiment – we marked a total of 212 bees during the experiment, and were unable to identify 17 of 100 marked with paint and 17 of 112 marked with tags. These individuals were dropped from our analysis.

Extracting data from audio

We used R (R Core Team 2016), with the packages seewave (Sueur et al. 2008) and tuneR (Ligges 2013), to process the audio recordings and quantify sonication and flight sounds. We first listened to the recordings to identify the loudest, longest sonication sound, and recorded its length. We focused on the loudest, longest sonication, because we observed that bees often performed shorter, higher-frequency sonications on the petals of the flowers, and we wanted to exclude these from analysis. We classified sounds as a single sonication if there was no audible break for > 0.2 seconds. After selecting the sequence for analysis, we used the "spec" function from the seewave package to calculate power spectral density, using a hanning window of 2048 points (Sueur et al. 2008). To determine the sonication frequency (the frequency at which the bee was vibrating), we selected the highest peak on the spectrum between 195 Hz and 400 Hz (a reasonable range for sonication frequency).

To check the accuracy of the frequency obtained by this method, we generated a sine wave at the frequency identified as the highest peak, and compared it aurally to the audio recording of sonication. If the frequency returned from the spectrum was noticeably different from the audio recording (which can occur due to background noise), we used Audacity (Audacity Development Team 2015) to obtain the correct sonication frequency. Within Audacity, we selected the sonication portion of the audio recording, and plotted the spectrum (hanning window, 2048 points). We then generated sine waves at each of the frequencies corresponding to the peaks in the spectrum. We compared each of these sine waves to the recording, aurally, and chose the peak that corresponded most closely to the audio recording of the sonication.

We used the same process to quantify wingbeat frequency – selecting a portion of the recording that contained the bee flying, plotting a spectrum, and selecting the highest peak. We used a range to 120 to 220 Hz for selecting the peak of wingbeat frequency, as wingbeat frequencies are typically lower than sonication frequencies (Switzer et al. 2016). We checked all wingbeat frequencies aurally in the same way as for sonication frequency.

Statistical analysis

To determine whether imidacloprid affected wingbeat frequency, sonication frequency, or length of sonication, we calculated the change in each behavior by subtracting post-treatment values from pre-treatment values. We observed a few bees sonicating on "Sweet 100 Hybrid" flowers (when "Cherry Roma" flowers were not available), and we initially excluded these individuals from our analysis of sonication and flight sounds, because we have previously found that bumblebees change sonication frequency and/or length on different species of plants (Switzer and Combes 2017). We reran the analysis, including the few bees that sonicated on *S. lycopersicum* "Sweet 100 Hybrid", and we found no differences in significance of coefficients associated with imidacloprid treatment; thus, these bees were included in the final analysis.

We performed multivariate multiple regression to determine if there were significant

changes in the bees' behaviors – wingbeat frequency, sonication frequency, and sonication length. We were able to make comparisons only on bees that resumed sonication behavior after being treated with imidacloprid. Because very few bees in the 0.515 and 5.15 ng imidacloprid treatment groups were observed foraging for pollen on *S. lycopersicum* flowers after treatment, we dropped those two treatments from our analysis. Our initial model included imidacloprid treatment as the only dependent variable, and we used a series of likelihood ratio tests to determine if adding other covariates made the model significantly better. We chose this forward stepwise procedure – starting with a small model, and adding covariates – because we started with a small dataset and wanted to find variables that influenced the response variables while excluding variables that made small contributions (Armstrong and Hilton 2010). We suspected that environmental variables such as temperature might affect behaviors (Unwin and Corbet 1984), so we investigated the following weather covariates: temperature, atmospheric pressure, light intensity, and relative humidity. We also investigated mark type (paint or bee tag), intertegular span, the number of days between pre-treatment recording and post-treatment recording, and the hive number.

To evaluate whether imidacloprid treatment affects the probability that bees would resume sonication behavior, we used survival analysis techniques from the R package, survival (Therneau and Grambsch 2013). The data recorded includes the amount of time since diagnosis/treatment and whether or not an event occurs. The data are right-censored. For example, survival analysis has been used to examine the amount of time until seeds germinate (Manso et al. 2013). Here we used "collecting pollen from *S. lycopersicum* after being treated with imidacloprid" as our event. Our data are right-censored because some of the bees died, and others stayed alive but were never observed on *S. lycopersicum* after treatment, within the time limits of the study.

We used Cox proportional hazards regression to determine if there was a significant difference in the probability of bees resuming sonication behavior among the treatment groups. The coefficients from the Cox model can be used to estimate hazard ratios – the chance of the event occurring in the treatment group, relative to the control group. For

example, in the highest treatment group, the coefficient is -2.73; exp(-2.73) = 0.065, meaning that for a fixed point in time, individuals in the highest treatment group are about 0.065 times as likely to sonicate as bees in the control group.

We used Cox regression so we could include intertegular span and hive number as covariates. We centered the intertegular span variable before modeling to make interpretation easier. We also suspected an interaction between mark type and intertegular span, and between treatment and intertegular span, so we included interaction terms: intertegular span * mark type + intertegular span * treatment. We started with a full model (all covariates) and stepped backward, using likelihood ratio tests to determine if each covariate improved the model. We chose a backward stepwise procedure – starting with a large model and removing terms – because we had a priori reasons to include the interaction terms, and a forward procedure would not investigate interactions when the main effects are not significant. We used the R packages, ggplot2 (Wickham 2016) and ggfortify (Horikoshi and Tang 2015), to make figures for survival curves.

Though the experiment was not designed to quantify mortality rates among the treated bees, we report the numbers of treated individuals that were observed resuming sonication behavior (and then removed from the experiment), that were alive at the end of the experiment but not observed sonicating after treatment, and that were dead, in each of the treatment groups. We did not use statistical tests to assess differences among groups, because the experiment was not designed to test mortality, and we were unable to identify all of the bees at the end of the experiment.

We report un-adjusted p-values, with no corrections to account for multiple comparisons. However, we discuss the possible tests that may be interpreted skeptically, due to relatively high p-values.

Results

Sample sizes

After removing newly emerged queens and bees that were incorrectly marked or treated, we report 199 bees from four hives that were marked in our study – 106 with bee tags and 93 with paint. We observed 105 of the marked bees resuming sonication behavior after being marked – 34 with bee tags and 71 with paint. Of the 105 bees that received one of the imidacloprid or control treatments, 45 were observed resuming sonication behavior after being treated. The remaining 60 bees remained in the cages, but were not observed preforming sonication after being treated.

Wingbeat frequency, sonication frequency, and sonication length

We included only bees in the control and smallest dosage group (0 and 0.0515 ng imidacloprid, respectively) in this analysis, since we did not have large enough samples of the other two groups for analysis. Beyond the imidacloprid dose, no additional covariates improved the model, so we report wingbeat frequency, sonication frequency, and sonication length as dependent variables, with imidacloprid dose as the only independent variable. We found no evidence that wingbeat frequency, sonication frequency, or sonication length were affected by consuming 0.0515 ng of imidacloprid (MANOVA; Pillai test stat = 0.08; approx. F(3,31) = 0.9; p-value = 0.45).

Probability of resuming sonication

Figure 3.1 shows the cumulative percentage (inverse Kaplan-Meier curves) of bees that were observed sonicating on *S. lycopersicum* after consuming different doses of imidacloprid. For this analysis, we used bees that sonicated on both varieties of *S. lycopersicum*. When we reran this analysis, either including the tomato variety as a covariate or excluding bees that sonicated on *S. lycopersicum* "Sweet 100 Hybrid", we found no difference in the significance of coefficients associated with imidacloprid treatment. Our final Cox proportional hazard

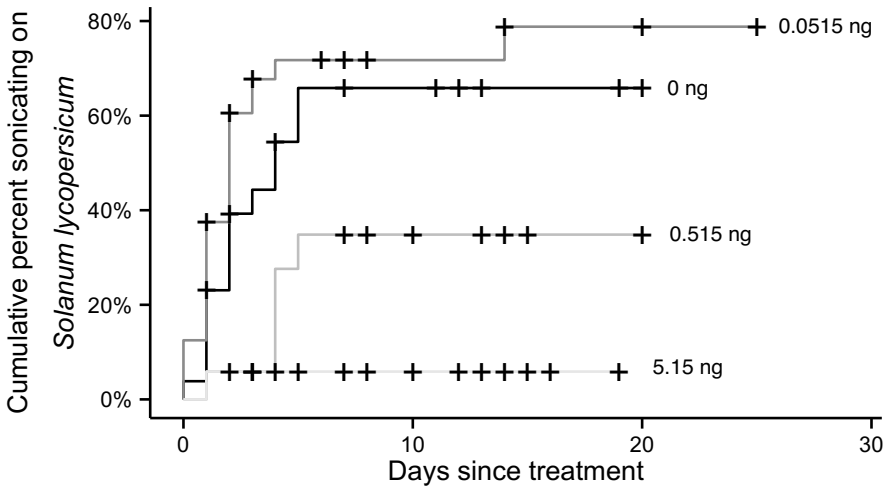


Figure 3.1: Curves showing the cumulative percentage of bees that performed sonication on *Solanum lycopersicum* after ingesting different amounts of imidacloprid (n(0 ng) = 26, n(0.0515 ng) = 32, n(0.515 ng) = 17, n(5.15 ng) = 17). The "+" symbols indicate censored data – bees that were never observed collecting pollen after treatment.

regression model included only imidacloprid treatment as a predictor variable ($\chi^2(3) = 23.58$; p-value < 0.00001). No other covariates made the model better. This model did not include individuals for which we could not record intertegular span, because we needed to have the same dataset in all models for likelihood ratio tests to be valid. However, we reran the final model with all individuals (including those without IT span measurements), and found no change in significant or non-significant coefficients.

Though the bees that ingested 0.0515 ng of imidacloprid did not show a marked difference in their probability of resuming sonication behavior (β(0.0515 ng) = 0.44; z = 1.326; p-value = 0.18), the bees that ingested 0.515 ng of imidacloprid were marginally different than the control group (β(0.515 ng) = -1.02; z = 1.98; p-value = 0.048) – this borderline p-value would become non-significant if we adjusted for multiple comparisons, using the Bonferroni method. The bees that ingested 5.15 ng of imidacloprid showed markedly different behavior (β(5.15 ng) = -2.73; z = 2.64; p-value = 0.0084).

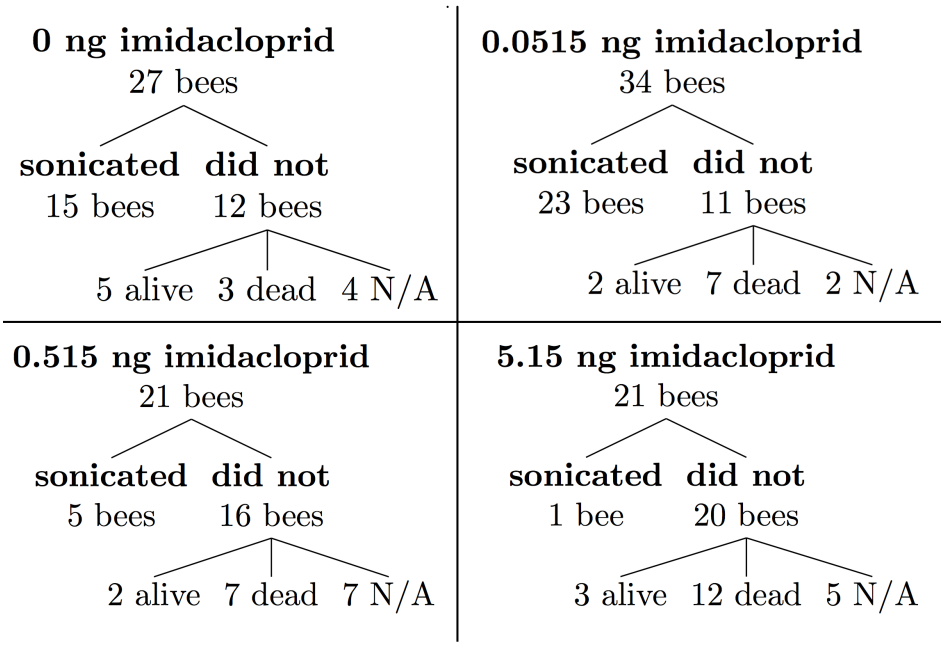


Figure 3.2: The number of bees in each treatment group, and their status at the end of the experiment.

Bee mortality

Figure 3.2 shows the number of bees that received each treatment, and their status at the end of the experiment. Bees that were observed sonicating after treatment were removed after observation, so they could not be classified as dead or alive at the end of the experiment. The sample sizes in Figure 3.2 are different from the sample sizes used in the Cox regression, because we were unable to find some individuals at the end of the experiment, so we dropped them from the Cox regression.

Discussion

Based on previous research about the effects of pesticides on other aspects of pollen foraging (Feltham et al. 2014)), we expected to see a significant difference in the buzz-pollination behavior of bumblebees that ingested imidacloprid. We did not find evidence that any of the mechanical aspects of behaviors we investigated – sonication frequency, sonication length, or wingbeat frequency – were different for bees that ingested 0.0515 ng vs. 0 ng

of imidacloprid. We were unable to analyze data for bees in groups that received higher doses of imidacloprid, because we rarely observed them performing buzz pollination after ingesting imidacloprid – this reflects that imidacloprid has sub-lethal or delayed lethal effects when consumed at doses above 0.5 ng per bee. However, in a separate study, we found that wingbeat frequency was not affected by doses of imidacloprid up to 1 ng per bee (unpublished data).

The probability of observing bees sonicating on *S. lycopersicum* after treatment was significantly lower for bees that consumed 0.515 ng or 5.15 ng of imidacloprid, relative to bees that consumed no imidacloprid (Figure 3.1). We acknowledge that the p-value for the 0.515 ng group was relatively high (p-value = 0.048) – had we adjusted the p-value to account for multiple comparisons, using the conservative Bonferroni method, this group would have a p-value above the α=0.05 level. We also acknowledge that our data were likely not completely independent for each individual – treating individuals with imidacloprid and removing foragers from the experiment (after they were observed sonicating again) likely influenced other bees within the hives, making them more likely to forage. Overall, though, it is clear that imidacloprid does affect the likelihood of bees continuing to engage in buzz pollination when ingested doses are above 0.5 ng bee^{-1}.

Though we were unable to analyze mortality in each of the treatment groups statistically, our data suggest a trend in mortality with increasing treatment doses (Figure 3.2). We estimated mortality rates as follows: 11% (3/27) for control, 21% (7/34) for 0.0515 ng, 33% (7/21) for 0.515 ng and 57% (12/21) for 5.15 ng doses. The amounts of imidacloprid fed to bumblebees in this study were well below the LD50 values previously reported for bumblebees, which range from 20-40 ng per bee for 24 to 48 hours after consumption (Blacquiere et al. 2012; Decourtye et al. 2003; Marletto et al. 2003).

The probable cause of death in our study was acute or delayed lethal effects of the pesticide, though other possible explanations exit. One hypothesis is that treated bees may not have been able to find the hive after leaving the nest to forage; studies on honeybees suggest that imidacloprid reduces navigation abilities (Bortolotti et al. 2003; Fischer et al.

2014), but a study on bumblebees suggests that navigation may not be the cause of impaired foraging (Feltham et al. 2014).

We saw no evidence that bees were able to clear imidacloprid from their systems after a period of time. If bumblebees in our study had cleared the imidacloprid from their bodies, we would have expected to see no difference in the pollination behavior of treated bees vs. untreated bees, as post-treatment observations of pollination behavior did not begin until at least 24 hours after treatment, and continued for up to several weeks. Alternatively, bees in our study may have in fact cleared imidacloprid from their bodies, but the effects of the treatment lasted well beyond the point when they had cleared the pesticide.

Our study had several limitations. First, treating bees with imidacloprid in a single dose does not represent the manner in which bees would be exposed in natural environments, but this method had the benefit of allowing us to precisely control the amount of pesticide ingested by bees. Second, though our study investigated only the effects of imidacloprid in nectar, pollen also can contain imidacloprid and its residues (Rogers and Kemp 2003; Schmuck et al. 2001; Stoner and Eitzer 2012). The *Solanum lycopersicum* plants in our study were never treated with neonicotinoid pesticides. However, the supplemental pollen feeder was filled with pollen collected from honeybees (`http://www.koppert.com`), and we cannot be sure that this pollen was free of neonicotinoid pesticides. However, we do not expect the pollen to significantly affect forager behavior for several reasons. First, foragers do not consume large amounts of pollen, relative to nectar intake, as pollen is primarily collected for the larvae. In honeybees, foragers eat only small amounts of pollen (Crailsheim et al. 1992). In bumblebees (*Bombus terrestris*), Malone et al. (2000) recorded workers eating 3.3 to 35.3 mg of pollen per day – a small amount, relative to the reported 125 to 215 μL of sugar syrup (50% w/v) consumed per day. The concentration of neonicotinoid pesticides in pollen is often reported to be 8-20 ppb (Bonmatin et al. 2003; Rogers and Kemp 2003; Schmuck et al. 2001), though Mullin et al. (2010) found 206 ppb imidacloprid in honeybee pollen.

An interesting follow-up experiment would be to test bumblebees' ability to learn how

to perform buzz pollination after consuming imidacloprid. Compared to nectar foraging, pollen collection is a more challenging behavior that has been shown to require a substantial time to learn (Raine and Chittka 2007), and imidacloprid is known to hinder learning in bees (Han et al. 2010; Stanley, Smith and Raine 2015; Tan et al. 2015). In our experiment, bees were observed collecting pollen from *S. lycopersicum* at least two times before consuming imidacloprid (once prior to marking and again prior to imidacloprid treatment). Thus, bees had already learned how to process *S. lycopersicum* flowers before consuming imidacloprid. However, we might predict that bees who consume imidacloprid before performing buzz pollination would have a more difficult time learning to collect pollen by this method, or would have trouble collecting pollen as effectively as non-treated bees.

Conclusions

To sum up, our results show that imidacloprid in small doses can affect the buzz-pollination behavior of bumblebees. We did not find evidence that consuming 0.0515 ng imidacloprid affected bumblebees' wingbeat frequency, sonication frequency, or sonication length, and we were unable to test these variables in bees that consumed higher amounts of imidacloprid. However, we found that bumblebees who consumed 0.515 or 5.15 ng of imidacloprid were significantly less likely to resume sonication behavior after treatment, compared to bees subjected to a control (0 ng) treatment. In addition, we noted that many (but not all) of the bees that had consumed 5.15 ng of imidacloprid were dead at the end of our experiment, suggesting that future experiments are needed to test whether 5.15 ng imidacloprid - which is significantly below reported LD50 values for bumblebees - increases the mortality of foragers within a nest through either acute or delayed lethal effects.

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Chapter 4

Bumblebee sonication behavior changes with plant species and environmental conditions

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Abstract

Bumblebees collect pollen from some plants by grasping flowers and vibrating their flight muscles – a behavior termed buzz pollination, or sonication. The extent to which bees can and do alter their sonication has been scarcely documented. We show that bumblebees are capable of changing their vibration frequency. In two field studies we found that relative humidity, date, and bee size are associated with sonication frequency and/or duration. We found that the frequency and duration of sonications differed on different plants. In a greenhouse study, we found that individual bumblebees change their sonication frequency and duration when collecting pollen from flowers of three different Solanum plants. This suggests that bees may change their sonication behavior to optimize pollen release from different types of flowers.

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Keywords

buzz pollination, Solanum, vibration, audio, *Bombus impatiens*

Introduction

Approximately 8% of flowering plants (∼20,000 species) have poricidal anthers, which release pollen only through small pores (Buchmann 1983). These anthers release pollen when they are vibrated by bees performing buzz pollination, or sonication (Buchmann 1983; King and Buchmann 2003) - a behavior in which bees grasp the anthers with their mandibles (usually) and vibrate their bodies by activating their flight muscles while "decoupling" the wings (King et al. 1996). This transfers the vibration to the flowers, and pollen pours out of the anthers onto the bees' bodies, where it can be groomed and brought to the nest to provision the larvae (Buchmann and Cane 1989).

Not all bees perform buzz pollination. Notably, honeybees (*Apis mellifera* L.) do not sonicate to collect pollen (King and Buchmann 2003), which makes them relatively poor pollinators of plants like tomatoes (Greenleaf and Kremen 2006), peppers (Kwon and Saeed 2003), blueberries (Javorek et al. 2002), and cranberries (Ortwine-Boes and Silbernagel 2003). Bumblebees, however, perform sonication on many different flowers, and are important commercial pollinators of tomatoes grown in greenhouses (Greenleaf and Kremen 2006).

Some physical characteristics of sonication, including the vibration frequency, amplitude, and duration, affect the rate of pollen release from plants (De Luca et al. 2013; De Luca and Vallejo-Marín 2013). There is evidence that bees can change their sonication behavior, possibly to optimize foraging efficiency. Previous work has shown that two types of buzzes (defensive vs. pollination) are different in terms of frequency, amplitude, and duration in several bumblebee species (De Luca et al. 2014). In addition, Morgan et al. (2016) found that *Bombus terrestris* fine-tunes its floral sonication behavior – both frequency and amplitude – as it gains experience with handling flowers.

However, because the flight muscles of bees are asynchronous (i.e., a single neural

impulse triggers several contractions through stretch activation; Josephson et al. 2000), the extent to which bees are capable of changing their contraction frequency is understudied, though frequency is thought to be limited by physical and physiological properties, rather than being under behavioral control (De Luca and Vallejo-Marín 2013). If a bee's mesosoma operates as a resonant system during buzzing (when the wings do not flap) as well as during flight, several of their sonication characteristics may be correlated – for instance, an increase in buzzing frequency may be associated with a decrease in amplitude. Finally, the duration of sonication may be energetically or thermally constrained (Kammer and Heinrich 1974).

Sonication behavior in bumblebees has been documented multiple times on different types of flowers (De Luca et al. 2013; King and Buchmann 2003; Knudsen and Olesen 1993; Switzer et al. 2016), but the question of whether individual bees alter their sonication behavior on different plants remains unresolved. One study suggested that bees change the length of their sonication bouts in response to pollen output (Kawai and Kudo 2009). Another study demonstrated that two wild bumblebee species (*Bombus friseanus* Skorikov and *Bombus festivus* Smith) sonicate at different frequencies on different plants (Corbet and Huang 2014). However, environmental factors and potential differences in body size were not randomized or experimentally manipulated by Corbet and Huang (2014). Furthermore individual bees were not identified. Thus, the possibility exists that different populations of these wild bees (with different sonication behaviors) were visiting the different types of plants.

We conducted two large field studies (involving > 400 wild bumblebees, *Bombus impatiens* Cresson), as well as a greenhouse experiment that allowed for repeated observations on known individuals, to investigate whether bumblebees can and do alter their sonication behavior on different types of plants. We documented environmental conditions (temperature and relative humidity) and body size during field studies, allowing us to account for these additional factors and determine whether they may affect sonication. This variety of approaches allowed us to determine (1) whether bees are capable of changing their

sonication frequency (comparing individual bees' irritation buzzes vs. sonication buzzes), (2) whether environmental conditions and body size are associated with how bees sonicate, and (3) whether bees vary their sonication frequency and/or duration when sonicating on different species of plants.

Materials and Methods

Field studies on wild Bombus impatiens

Field investigations were conducted in an urban environment (Arnold Arboretum, Boston MA, 42.29988, -71.12344) and a relatively rural environment (Concord Field Station, Bedford MA, 42.50779, -71.29311). Approximately 40 days of observations were conducted on wild *Bombus impatiens* between 14 June 2013 and 17 September 2013, typically between the hours of 08:00 and 13:00. We obtained weather information from a weather station at the Arnold Arboretum and from a weather station at Hanscom Air Force Base, near the Concord Field Station.

During each day of observation, we recorded audio clips of bees' sonication buzzes on one or two different species of flower (see Appendix C for flower descriptions and Figure C.1-C.2 for photos). We analyzed sonication data collected only on plant species for which we recorded at least 20 individuals of *B. impatiens*.

After recording an individual bee sonicating for pollen collection, we captured the bee with a net. We placed the net on the ground and while the bee was still in the net, we gently pressed against the bee with the foam tip of the microphone to elicit and record irritation (also called "defense" or "alarm") buzzing – another behavior in which the bee makes an audible buzzing sound without flapping its wings. We excluded irritation buzzes that occurred while the foam on the microphone was touching the bee, because pressure on the bee may cause a change in irritation buzz frequency. After recording irritation buzzes, we transferred the bee to a plastic container in a cooler, to narcotize the bee for later measurement and identification. We also recorded wingbeat frequency of flying bumblebees

prior to capture, as they approached or flew between flowers; however, we do not report differences between wingbeat frequency and sonication frequency here, because research has already established that sonication frequency is significantly higher than wingbeat frequency in wild *B. impatiens* (Switzer et al. 2016).

After returning to the lab, we measured each bee's intertegular (IT) span (distance between wing bases) and mass, and we marked the bee to avoid performing repeated observations on the same individuals in the field; thus, sonication data was not collected from bees that had previously been captured.

In total, we collected data from 356 wild *B. impatiens* workers sonicating on five different plants (*Callicarpa cathayana* Chang, *Callicarpa japonica* Thunb., *Hypericum* 'Hidcote', *Rosa* 'Bucbi', and *Rubus odoratus* L.) at the Arnold Arboretum, and from 90 workers sonicating on three different plants (*Rosa multiflora* Thunb., *Coronilla varia* L., and *Physalis philadelphica* Lamarck) at the Concord Field Station (see Appendix C for further descriptions of the plants).

Greenhouse study on commercial B. impatiens

We further investigated sonication behavior on different species of plants by performing an experiment with two class-A colonies of *B. impatiens* that were purchased from Biobest (`http://www.biobestgroup.com`). Both experimental hives arrived on 22 July 2015. Upon receiving the hives, we verified that queens were present in each, and we removed any males. Each hive was placed in a mesh cage (1.8 x 1.8 x 0.6 m wide) within an outdoor, pollinator-excluding greenhouse with natural airflow (i.e., exposed to outdoor temperature and humidity).

We allowed bees to acclimate to the cages for at least two days prior to marking foragers, and we conducted the experiment over six days, from 13-20 Aug 2015.

The hives were enclosed in foam coolers for insulation, with holes for entry and exit. Each cage contained feeders that provided pollen and nectar ad libitum. Nectar consisted of 1.0 M sugar water (organic cane sugar). Pollen was purchased from Koppert Biological

Systems (http://www.koppert.com), and ∼2 g of pollen ground with a mortar and pestle was placed in the feeder approximately every 3 days. In addition to the artificial feeders, each cage contained a potted tomato plant (*Solanum lycopersicum* "Cherry Roma"). This plant was replaced each day with a different plant that had been kept in a pollinator-excluding greenhouse, to constantly provide freshly-opened flowers for pollen foraging (See Appendix C and Figure C.3 for descriptions and photos of plants).

Before collecting experimental data, we marked bees that were observed sonicating on *S. lycopersicum* by applying numbered bee tags (queen marking kit, Abelo, Full Sutton, York, United Kingdom) to their mesosoma with superglue. The first time a bee was observed foraging on *S. lycopersicum*, we captured her with an insect vacuum (2820GA, Bioquip, Rancho Dominguez, California) and transferred her from the aspirator tube into a queen-marking cage with plunger (The Bee Works, Oro-Medonte, Ontario, Canada). We gently pressed the bee against the mesh at the top of the tube to immobilize her while we glued a bee tag to her mesosoma. We used the outward vent from the insect vacuum to blow air onto the glue for 30 seconds to dry it before releasing the bee back into the cage. We collected experimental data only on bees that had been observed sonicating, were tagged, and then subsequently resumed sonication behavior; this prevented the tagging process from confounding our results (unpubl. data).

After the majority of sonicating bees had been marked with bee tags, we began collecting audio data from marked bees sonicating on different plants. During each audio recording session, we rotated pots of three different Solanum species (Solanaceae) into the bee cages – *Solanum lycopersicum* L., *Solanum dulcamara* L., and *Solanum carolinense* L. (see Appendix C for a description of all plants). The *S. lycopersicum* plants were kept in cages overnight, but we rotated fresh *S. lycopersicum* plants into the cages before collecting data. We estimated that the *S. carolinense* plants had fewer flowers than the other two species, but in general the plants had less than 10 open flowers. We rarely recorded bees sonicating on virgin flowers, since all flowers would usually be visited within a few minutes of placing the plant into the cage. We used at least three individual plants of *S. carolinense* and *S. duclamara*, and we used

∼15 individual plants of *S. lycopersicum*.

We started each session of data collection by placing a potted *S. lycopersicum* plant that had been outside the cage overnight into the cage. After we noticed a decrease in foraging activity on the plant in the cage (median time in cage: ∼20 min), we removed the *S. lycopersicum* plant and replaced it with either *S. dulcamara* or *S. carolinense*, not always in the same order. We again collected recordings until we noticed a decrease in activity (∼20 min for all plants), and then we replaced the plant in the cage with the species that had not yet been used.

Collecting and analyzing audio recordings

In both field and greenhouse studies, we used a shotgun microphone (SGM-1X, Azden, Tokyo, Japan) to collect audio recordings of sonication with a digital recorder (DR-100mkII, Tascam, Montebello, California). We held the microphone as close to the bee as possible, without touching the bee, attempting to hold the microphone orthogonal to the bees' frontal plane. We were not able to maintain this precise position for all recordings, but we have no evidence that recording from different angles affects the analysis of sonication frequency or duration. When more than one bee was on a flower, we gently brushed one bee off with the foam tip of the microphone, ensuring that each recording contained only one sonicating individual.

We used R (R Core Team 2016), with the packages seewave (Sueur et al. 2008) and tuneR (Ligges 2013), to process the audio recordings and quantify sonication frequency and duration. See Appendix C for further descriptions of processing audio recordings.

Statistical analyses

Field studies

We compared irritation buzzes with pollination buzzes by pooling data from the Arnold Arboretum and the Concord Field Station, excluding individuals for which we were unable to process irritation buzz frequency. To evaluate how much individual bees could vary their

buzz frequency between pollen-collection and irritation buzzing, we used a paired t-test to compare sonication frequency and irritation buzz frequency.

To evaluate whether sonication behavior differed on different types of plants, we analyzed field data collected at the Arnold Arboretum and the Concord Field Station separately, because we had no plants that were observed in both locations.

We conducted two stepwise regression procedures for each location – one regression for sonication length and one for sonication frequency. We log-transformed (base e) sonication length to help it fit the assumptions of linear regression. See Appendix C for model selection, evaluation of model assumptions, and multiple comparison adjustments.

Greenhouse study

We used a cross-over design for the experiment with colonies of bumblebees foraging in cages within the outdoor greenhouse. In this experiment, bees were allowed to forage on multiple plants, but not every bee foraged on all three of the plant species (*S. lycopersicum*, *S. dulcamara*, and *S. carolinense*).

We analyzed bees' sonication frequency and sonication length using two separate multilevel models, with the R package, lme4 (Bates et al. 2015). We log-transformed (base e) sonication length to help it fit the assumptions of linear regression. After finding significant models, we conducted three post-hoc tests to compare all pairwise combinations of plants for differences in sonication frequency and sonication length. See Appendix C for model selection, evaluation of assumptions, and multiple comparison adjustments.

Results

Field studies on wild B. impatiens

We first investigated the question of whether bees can change their sonication frequency by comparing the irritation buzzes of individual bees (n = 399) with the pollen-collecting buzzes they produced when foraging. Bees recorded at the field sites were capable of

producing irritation buzzes that were an average of 90 Hz higher in frequency than those produced during pollen collection (t(398) = 44.47; two-sided p-value < 2.2 x 10^{-16}; n = 399; Table C.1).

At the Concord Field Station, we recorded buzz pollination by 90 wild *B. impatiens* workers on three species of plants (all different from the plants in the Arnold Arboretum), over 13 days of recording. We report models with lowest BIC (Bayesian Information Criterion). We found that sonication frequency was significantly associated with plant species, while accounting for differences in bees' masses and IT spans (F(4, 85) = 4.022; p-value = 0.0049; See Table C.2 for coefficients and sample sizes). Sonication length was associated with plant species, while accounting for relative humidity (F(3, 86) = 6.367; p-value = 0.0006; Table C.3). Both models had overall p-values well below the adjusted significance level of 0.025. Holding other variables constant, larger masses were associated with higher sonication frequency. Holding other variables constant, larger intertegular (IT) spans were associated with lower sonication frequency at the Concord Field Station (Table C.2). Higher relative humidity was associated with longer sonication bouts at the Concord Field station (Table C.3). Post-hoc tests revealed that R. multiflora was sonicated at a significantly higher frequency than the other plants at the Concord Field Station, after accounting for bee mass and IT span (Figure 4.1a; Tables C.4); however, we found no significant differences in the length of sonication between these plants after accounting for relative humidity (Figure 4.1b, Table C.5).

At the Arnold Arboretum, we recorded buzz pollination by 356 wild *B. impatiens* workers on five species of plants, over 29 days of recording. These recordings revealed that both sonication frequency and sonication length were significantly associated with plant species, while accounting for differences in relative humidity and date (Frequency: F(6, 349) = 10.98; p-value = 3.24 x 10^{-11}. Length: F(7, 348) = 7.321; p-value = 3.34 x 10^{-8}; See Tables C.6-C.7 for regression coefficients and sample sizes). These p-values are well below the adjusted significance level of α = 0.025. Both models at the Arnold Arboretum included relative humidity and date. The model for log (base e) sonication length also included date2.

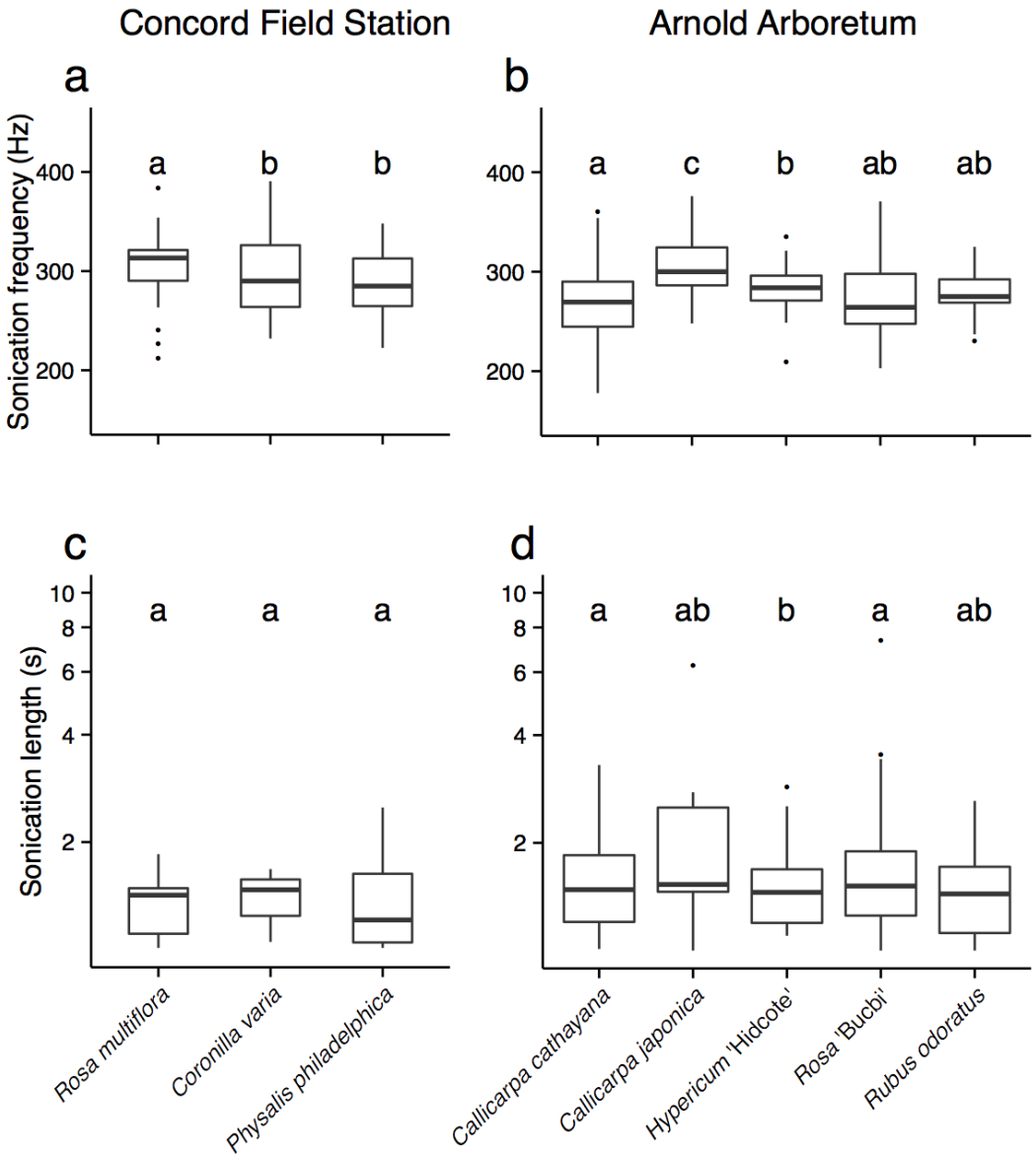


Figure 4.1: Boxplots showing differences in sonication frequency and length at the Concord Field Station (a and c) and at the Arnold Arboretum (b and d). Different letters above the boxes indicate plants that were sonicated at significantly different frequencies or lengths of time by bumblebees ($p < 0.017$ for a and c, $p < 0.005$ for b and d). Note that differences in behaviors were calculated after accounting for associations with other covariates. At the Concord Field Station, differences in frequency (a) account for bee mass and IT span, while differences in length (c) account for relative humidity. At the Arnold Arboretum, differences in frequency (b) and length (d) account for differences in relative humidity and date. Whiskers indicate the highest or lowest values that are within 1.5 * interquartile range (IQR), and dots represent individuals outside 1.5 * IQR.

Higher relative humidity and recordings later in the summer were associated with higher sonication frequencies at the Arnold Arboretum (Table C.6). For sonication length at the Arnold Arboretum, higher relative humidity was associated with longer sonication bouts, and bees tended to perform shorter sonication bouts during the middle of the summer than at the beginning or end (Table C.7). After accounting for these variables, post-hoc tests revealed that bumblebees at the Arnold Arboretum displayed significantly different sonication frequencies and durations on different plants (Figure 4.1b and 4.1d; Tables C.8-C.9).

Greenhouse study on commercial B. impatiens

We recorded 237 bouts of buzz pollination by 64 *B. impatiens* workers on three different species of plants over 6 days of recording in the greenhouse. Of the 64 workers, 18 individuals were recorded while sonicating on all three species of plants. Twenty-three individuals were recorded on two different plant species, and 23 were recorded on only one plant species. This study was designed as a cross-over experiment – ideally every bee in the experiment would have sonicated on all three types of plants, but we were unable to record every bee foraging on each type of plant. However, when we examined only the subset of bees that were recorded on all three plants, we found very similar results as when the entire dataset was used – the regression coefficients were almost identical, but the standard errors were larger for the smaller dataset (Tables C.10, C.11, C.12, C.13); thus, the entire dataset was used for further analysis.

Our greenhouse recordings revealed that plant species significantly affected bees' sonication frequency. Based on a likelihood ratio test to compare two multilevel models – one with plant and one without plant as a covariate – we found that plant was a significant predictor of sonication frequency ($\chi^2(2) = 18.788$; $p = 8.32 \times 10^{-5}$; Tables C.14-C.15). We used the same process for sonication length, and found that plant was also a significant predictor of sonication length, while accounting for the bee colony ($\chi^2(2) = 9.216$; $p = 0.00997$; Tables C.16 - C.17). Bee colony was a significant predictor for sonication length, but did

not make the model for sonication frequency significantly better (according to a likelihood ratio test). Post-hoc tests revealed that *Solanum carolinense* was buzzed at significantly higher frequencies than the other two plants (Figure 4.2a; Table C.18), and that sonication bouts on *Solanum dulcamara* were significantly longer than those on Solanum lycospericum (Figure 4.2b; Table C.19).

Discussion/Conclusion

Our paired comparison of irritation vs. pollen-collecting buzzes in nearly 400 wild *B. impatiens* workers shows definitively that bumblebees are capable of changing their buzzing frequency. These types of buzzes are superficially similar, in that bees buzz by contracting their flight muscles without flapping their wings; however, bees' highest-frequency irritation buzzes were approximately 90 Hz ($\sim$30%) higher than pollen-collecting buzzes, demonstrating that bees can buzz at a wide range of frequencies. Our findings support those of De Luca et al. (2014), who found that defense buzzes and pollination buzzes are different. In general, they found that defense buzzes are typically greater in amplitude (louder) than pollination buzzes. In addition, defense buzzes may not always be higher in frequency than pollination buzzes – different bee species show different relationships between buzz type and buzz frequency (De Luca et al. 2014). Results from De Luca et al. (2014) and the current study suggest that the sonication buzzes used while collecting pollen are substantially different from the irritation (also called "alarm", or "defense") buzzes that bees produce in lab settings (see King et al. 1996) and that irritation buzzes may not be a suitable proxy for sonications used to collect pollen.

Furthermore, our studies of wild *B. impatiens* sonicating on different species of plants at the two sites showed that different buzz characteristics are associated with plant species, while accounting for other covariates (discussed below) (Figure 4.1). While one previous study suggested that sonication characteristics may vary on different plants (Kawai and Kudo 2009), we were able to show this more definitively, by controlling for pseudoreplication of data, accounting for environmental variables and bee size, and collecting data on several

hundred bees. The data from the field studies showed that plant species was associated with different sonication frequencies and lengths (Figure 4.1).

In some cases, differences in flower morphology suggest a potential explanation for the observed differences in buzzing characteristics. For example, flowers of *Coronilla varia* (also called Vetch) are bilaterally symmetric, and we noticed that bees at the Concord Field Station were interacting with this flower differently than with the other flowers. Bees tended to push their heads into the center of *C. varia* flowers while buzzing, suggesting that they may be using sonication to press their proboscis deeper into the flower to reach the nectar. Clearly, further investigation is necessary to understand the mechanics of this interaction.

In other cases, we could not detect any obvious differences in floral morphology to explain buzz pollination behavior. At the Arnold Arboretum, for example, *Callicarpa cathayana* was sonicated at a significantly lower frequency than *Callicarpa japonica*, although these flowers appear superficially similar; however, other aspects of their biology (amount of pollen produced, number of available flowers, etc.) may underlie the observed differences in sonication characteristics.

Our large dataset of sonication by wild bumblebees in two field sites also allowed us to determine which environmental variables or other covariates may help explain how bees interact with flowers. At the Arnold Arboretum, humidity and date were significant covariates for sonication frequency and sonication length (Tables C.6-C.7). At the Concord Field Station, the date variable was confounded with plant, and so date was dropped from the analysis. However, relative humidity was also found to be a significant covariate for sonication length at the Concord Field Station (Table C.3). For sonication frequency measurements at this site, bee mass and IT span were significant covariates (Table C.2).

Although stepwise procedures are known to sometimes select independent variables that may not actually be causing differences in the dependent variables (Burnham and Anderson 2003), our results suggest that relative humidity, bee size, and date may affect sonication characteristics. Our findings on bee size are not surprising, as previous studies have shown that larger bees generally contract their flight muscles at lower frequencies than small bees,

particularly during flight (Burkart et al. 2011). This agrees with the coefficient associated with IT Span (Table C.2). Holding bee mass and flower species constant, an increase in IT span was associated with a decrease in sonication frequency at the Concord Field Station (Table C.2). However, we found the opposite relationship for bee mass – holding IT span and flower species constant, an increase in bee mass was associated with an increase in sonication frequency. This relationship may warrant further investigation in future studies.

Our finding that relative humidity is associated with sonication behavior suggests several interesting hypotheses. Relative humidity may affect sonication behavior through its effects on how much pollen is released – daily cycles of decreasing relative humidity and rising temperature are known to accelerate changes in plant tissue and lead to pollen release (Pacini 2000). In addition, dehydration of tapetal fluid makes pollen easier to remove, and high humidity may affect the rate at which the tapetal fluid evaporates (King and Buchmann 1996; King and Ferguson 1994). It is likely that a bee can sense how much pollen gets caught in its pile – flower handling time and grooming time increase when more pollen is available(Burkart et al. 2014; Cane and Buchmann 1989; Shelly and Villalobos 2000). In addition, the properties of pollen grains may change with relative humidity; for example the volume of pollen grains may increase as relative humidity increases (Gilissen 1977). Future studies investigating the mechanism(s) that allow bees to gauge the amount of pollen landing on their bodies during pollen foraging would be informative. If the weight of the pollen caught in the bee's pile is an indication of the amount of pollen released from the flower, then high humidity may change the bee's behavior because the high-volume pollen grains may be heavier than desiccated pollen grains.

Relative humidity may also affect how well pollen grains attach to bees. Past research has shown that both electrostatic charges and the outer coating on pollen may be important for allowing pollen to attach to pollinators (Buchmann and Hurley 1978; Pacini and Hesse 2005; Vaknin et al. 2000). A change in relative humidity may affect the electrostatic charge on the bee or the pollen grains, and it may also affect the properties of any coating on the pollen grains. These changes could also affect the size of pollen clumps that are released from the

flower. Thus, bees may change their buzz characteristics in response to the amount of pollen sticking to the pile, which may in turn be affected by relative humidity – a hypothesis that warrants future investigation.

Although the results of our field studies clearly showed that different plants were associated with different sonication characteristics, we could not be sure from the field data alone that the plants were actually the cause of the changes in bee behavior. Because we could not observe the same individual on more than one type of plant in the field, the possibility remained that different populations of bees (which sonicated differently) may have been visiting the different plants, rather than individual bees changing their sonication behavior on different plants. To rule this out and to find a causal relationship, we performed the greenhouse study in which known individuals could be observed sonicating on more than one species of plant.

The results of the greenhouse study provided definitive evidence that individual bumblebees alter both their sonication frequency and sonication length on different plants. Bees buzzed at a significantly higher frequency on *S. carolinense* than the other plants, and buzzes lasted significantly longer on *S. dulcamara* than on *S. lycopersicum* (Figure 4.2).

One thing that we cannot determine from this study is whether bees are actively changing their buzzing behavior (e.g., by altering the contraction frequency of their flight muscles) to optimize pollen release, or if something about the mechanics of the flower leads to a passive change in the observed buzzing mechanics. The sonication buzzes that bees produce on a flower may be related to not only the bees' muscle contractions and the mechanical properties of its body, but also to the resonant properties of the entire system – in other words, mechanical properties of the flower's filaments and anthers (mass, stiffness, and damping) could impact the frequency and amplitude of oscillations produced when the bee interacts with it, even if the bee was trying to behave the same way on all of the plants (King and Buchmann 1996). However, passive mechanics would be unlikely to affect how long bees buzz on different types of flowers, which our results show did vary with the type of plant.

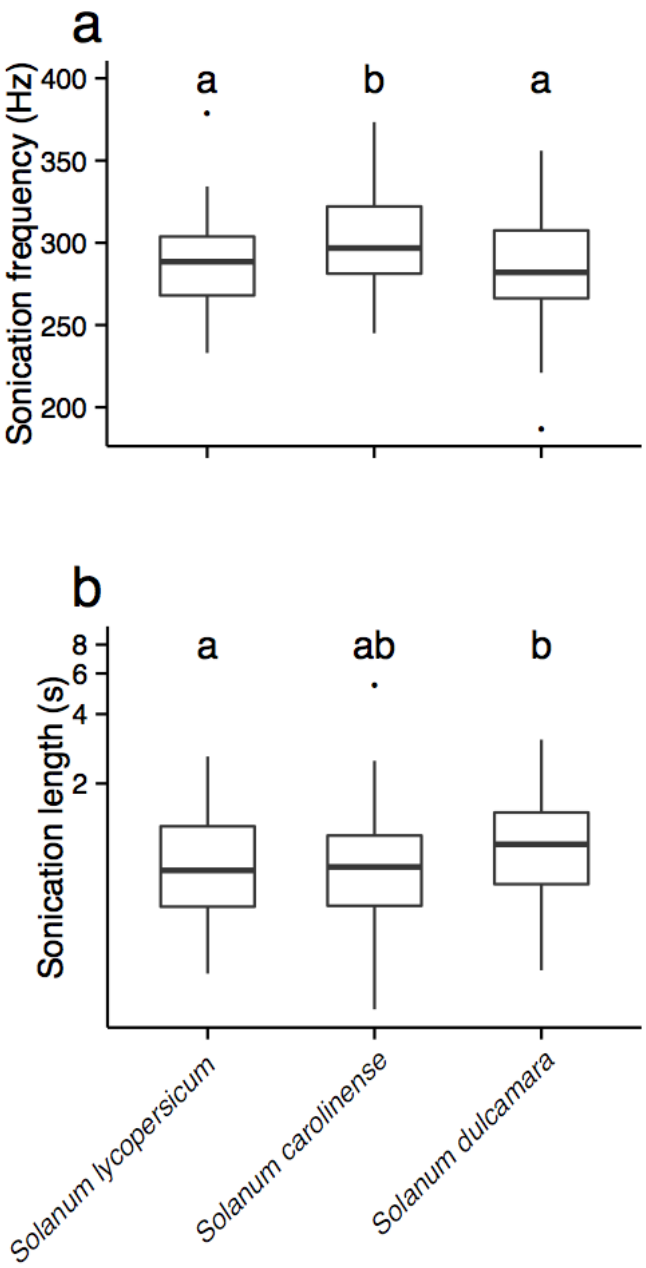


Figure 4.2: Differences in a) sonication frequency and b) sonication length among known *Bombus impatiens* individuals that were observed sonicating on different Solanum plants in the greenhouse study. Different letters above the boxes indicate plants that were sonicated at significantly different frequencies or over different lengths of time ($p < 0.017$, the adjusted significance level to account for multiple comparisons). Differences in b) sonication length account for the different bee colonies. Whiskers indicate the highest or lowest values that are within 1.5 * interquartile range (IQR), and dots represent observations outside 1.5 * IQR.

We hypothesize that bees change their sonication behavior in response to the pollen rewards they receive. Evidence in the past literature to suggests that bees may change their foraging behavior in response to the amount or quality of the reward received from a plant. This has been well studied in nectar foraging. For example, Roubik et al. (1995) suggested that bees forage for optimal nectar concentration, and Manetas and Petropoulou (2000) found that increasing nectar volume causes bees to increase floral visit duration.

Fewer studies have investigated how bees learn and change their foraging behavior when pollen is the reward. Bumblebees have been shown to increase handling time when more pollen is available (Buchmann and Cane 1989), and this could explain why bees sonicated for longer durations on *S. dulcamara*, which may release more pollen than *S. lycopersicum* (unpubl. data). In addition, bumblebees have been shown to 'tune' their vibration frequency and amplitude as they gain experience with flowers (Morgan et al. 2016). The current study extends findings from the previous literature concerning pollen foraging behavior, and suggests that bees may be able to change their vibration characteristics when foraging for pollen via sonication, in order to optimize pollen collection on different plants (i.e. maximize the amount of pollen collected per buzz).

This study brings up several intriguing questions concerning 1) how the different characteristics of sonication buzzes (e.g., frequency, amplitude, duration) are related and whether these characteristics are constrained to particular ranges, 2) how quickly bees can modify their sonication behavior, and 3) how long their memory of optimal buzzing behavior for different plants persists. Future studies may help shed light on these questions.

Acknowledgements

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Chapter 5

Dispensing pollen via catapult: explosive pollen release of the mountain laurel (*Kalmia latifolia*)

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Abstract

The mountain laurel, *Kalmia latifolia*, is a charismatic plant found in the eastern United States that is well known for its explosive pollination mechanism – when a pollinator triggers the mechanism, the flower catapults pollen into the air. Past literature suggests that this pollen catapult acts as a pollen dispensing mechanism, which restricts the amount pollen available to a flower visitor. However, the extent to which the catapult acts as a dispensing mechanism has not been rigorously quantified. In this study, we conducted four investigations to determine how (if at all) the pollen catapult acts as a pollen dispensing mechanism. First, we conducted a kinematic analysis of the catapult mechanism. To our knowledge we are the first to report the high speeds and accelerations attained by this plant (3.5 m s^{-1} and 4100 m s^{-2}, respectively). This is one of the fastest movements measured for a plant organ. Second, we quantitatively described pollen dispersal when the catapult is triggered, by constructing a heat map in three-dimensional space. We found that the flying pollen would most likely contact large bees, but may miss small bees and butterflies. Third, we conducted field observations to record the pollinators and behaviors found on

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the *K. latifolia* plants. We observed that insects visiting these flowers were relatively rare, and that only bees triggered the catapult mechanism. Bumblebees, *Bombus* spp., were the most common visitor. Last, we conducted a pollination experiment to investigate the role pollinators play in fertilization. We found little evidence for autogamous fertilization, but we found evidence that pollinators play a key role in fertilizing these plants. In summary, our results strongly support the claim that the pollen catapult is a pollen dispensing system that releases pollen only to pollinators that are likely to transfer it to the stigma of another flower.

Keywords

explosive pollination, pollination biomechanics, plant-pollinator interactions, bumblebee, *Bombus*, honeybee, carpenter bee, Lepidoptera, Hymenoptera, pollinators, video analysis, automated measurements, automated counting, bootstrap, resampling, Ericaceae, fruit set, pollination limitation, Arnold Arboretum

Introduction

The transfer of pollen is a necessity for most flowering plants. An estimated 85 - 90% of the 352,000 species of flowering plants depend on living organisms, mostly insects, to transfer pollen among flowers (Ollerton et al. 2011). Plants have evolved a variety of mechanisms to manipulate pollinators to transfer pollen successfully among conspecifics, rather than among plants of different species (heterospecifics).

For example, some plants have evolved mechanisms to increase the probability that a pollinator will transfer pollen among conspecifics by requiring pollinators to learn a complex behavior. The energy required to learn complex motor routines is thought to discourage pollinators from visiting a variety of flowers – instead, after pollinators have learned to collect a reward from one or two types of flowers, they continue to visit those types of flowers over and over, even if they bypass other food rewards (Chittka et al. 1999; Heinrich

2004). The repeated visit to a certain type of flower is called constancy.

In addition to constancy, self-incompatible plants are under another evolutionary pressure – to get the pollen from the one individual plant to another. When insects visit flowers, there are three possible fates for the pollen: (1). It can remain in the anthers (removal failure) (2). It can be dislodged, but not carried away (sloppy visitor) (3). It can be removed successfully and carried away; then it has a chance of being involved in fertilization (Hargreaves et al. 2009). Changes in pollen removal, transport, and deposition on stigmas can all improve pollen donation and therefore can be under selection (Harder and Thomson 1989). In many instances, insects fail to remove the pollen, fail to carry the pollen away, or fail to deposit the pollen onto the stigma. Insects may learn to collect rewards from flowers without ever contacting the reproductive organs – in this case, the insect could have very high constancy, but fertilization rates would be low, due to failure to remove pollen. When a flower is visited by an animal for which it is not adapted, this results in a mismatch in morphologies (Inouye 1980). For example, when small bees or butterflies visit a flower that is adapted for bumblebee pollination they may never contact any reproductive parts of the flower.

Pollen theft is when an animal collects pollen from flowers without ever contacting the stigma (Inouye 1980). Honey bees (*Apis* spp.) have been documented pollen thieves, due to their flexibilty to manipulate flowers (Hargreaves et al. 2009)). Pollen thievery, in contrast to nectar thievery, directly affects plant reproduction by reducing fertilization opportunities and/or by causing pollen limitation by depleting the overall pool of male gametes available to fertilize ovules (do Carmo et al. 2004; Hargreaves 2007; Hargreaves et al. 2009).

Plants have evolved several methods to ensure that insects successfully transfer pollen (Whitney and Federle 2013)) – notably pollen packaging and pollen dispensing mechanisms (Harder and Thomson 1989). Pollen packaging is the division of pollen into separate units, which sequentially become available to pollinators, whereas pollen dispensing is the restriction on the amount of available pollen (Harder and Thomson 1989). One example of a dispensing mechanism is poricidal anthers, which limit access to pollen and require

specialized behavior (buzz pollination or floral sonication) to remove pollen (Buchmann 1983; Harder and Barclay 1994; King and Buchmann 1996). Because this is a special behavior that positions the bee so that it catches pollen and transfers that pollen to the stigma of the next flower it visits, this enables that plant to increase the probability that its pollen will fertilize another flower.

Another pollen dispensing mechanism has been described in *Kalmia latifolia*, the mountain laurel (Harder and Thomson 1989). This is one of only a few plants that release pollen via catapult (see other examples in Edwards et al. 2005; Nicholson et al. 2008; Taylor et al. 2006). This explosive mechanism of pollination has interested scientists far back as the mid-1800's (Beal 1867; Rothrock 1867)

The structure of the *K. latifolia* flower is such that the anthers are inserted into pockets in the corolla and are held under tension until something triggers their release (Jaynes 1988; Nimmo et al. 2014; Rathcke and Real 1993). Figures 5.1A and 5.1B show photos of *K. latifolia*. When the filaments are jostled, the anthers are released from the pockets, and they launch pollen into the air. If no pollinator is present, the pollen has the possibility to land on the sticky stigma of the flower or another surrounding flower, though the stigma of the flower is typically off-center, and some populations of *K. latifolia* are largely self-incompatible (Rathcke and Real 1993). Each anther contains two locules that release pollen in the form of tetrads, connected with viscin threads (Sarwar and Takahashi 2012). This means that pollen flies out of the anthers in stringy clumps.

Here we describe the explosive pollination mechanism of *K. latifolia*. We explain the ways that this biomechanical adaptation acts as a pollen dispensing mechanism. Using explosive pollination as a pollen-dispensing mechanism has been suggested in past literature (Edwards et al. 2005; Harder and Thomson 1989; Raju and Reddi 1995; Raju and Rao 2006; Ruan and da Silva 2011; Whitaker et al. 2007), but a quantitative description of the mechanism in *K. latifolia* has received little attention (Jaynes 1988; Nimmo et al. 2014). We detail how this mechanism restricts pollen removal only to pollinators that are likely to transfer that pollen to the stigma of a conspecific flower. We broke our investigation up into four questions:

1. How fast (velocity and acceleration) is the pollen catapult?

2. Where does the pollen catapult launch pollen?

3. What pollinators and behaviors trigger the anthers?

4. What role do pollinators play in fertilization?

The results of these four investigations support our hypothesis and the claims from past literature (Harder and Thomson 1989; Ruan and da Silva 2011) that the catapult mechanism in *K. latifolia* acts as a pollen dispensing system to restrict pollen removal to certain insects (i.e. large bees) that are likely to transfer that pollen to the stigma of another flower.

Methods

Pollen speed and acceleration

We characterized the kinematics of the *K. latifolia* pollen catapult system by manually triggering filaments, with a needle, and we recorded pollen release with high-speed video in the lab. The videos were recorded at 5000 frames s^{-1} with an SA3 camera (Photron, San Diego, California) and a 105mm f/2.8 lens (Nikon, Melville, New York). The camera was positioned orthogonal to the plane of pollen release. It was necessary to remove a section of petals to visualize the pollen. We recorded 32 pollen catapults from 7 flowering individuals collected at Arnold Arboretum (Boston, Massachusetts).

Our goal was to quantify the maximum speed and acceleration of pollen. Each anther released multiple aggregations of pollen, but we focused our analyses on just the fastest pollen clump. We calculated the position of pollen at every time step by digitizing the pollen from each video using a MATLAB-based digitization program, DLTdv5 (Hedrick 2008; The MathWorks Inc. 2014). Although we also digitized the anther tip as it released pollen, the maximum acceleration and velocity did not differ significantly from the pollen, and thus these data are not reported.

To remove digitization errors, we smoothed the digitized files with a smoothing parameter that was selected using 10-fold cross validation and minimized the Euclidian distance between predicted and actual position (See Appendix D). Smoothing lowered our estimates of both accelerations and velocities and thus was a conservative transformation of our data. We then calculated maximum speed and linear acceleration for each trial (description and equations in Appendix D), using R (R Core Team (2016), version 3.3.2). We used the R package lme4 (Bates et al. 2015) to estimate average values for the max speed and linear acceleration. We log-transformed (base e) maximum acceleration for analysis, because it was right-skewed, and we report 95% profile-likelihood confidence intervals.

Calculating pollen trajectories in 3D space

We characterized the trajectory of catapulted pollen using a second set of high-speed videos. Our goal was to determine where the pollen travels when released in order to calculate where a pollinator should be to be hit by pollen. As described above, we triggered pollen release using a needle from 29 flowers removed from 20 individuals from the Arnold Arboretum. For these analyses, we visualized the entire region that pollen traveled within 2-3 cm of the flower throughout the course of each video (Figures 5.1C and 5.1D). We digitized a polygon around this area of released pollen with custom-written code in MATLAB. Figure 5.1D shows an example of the region that pollen occupied during a trial. We scaled and aligned all of the digitized polygons so that the flower was vertical and the distance between the anther pocket and the flower center was 1 unit (see Figure D.4) using custom-written scripts in R and Python (Python Software Foundation, version Python 2.7.13, `http://www.python.org`).

We extruded the digitized pollen polygons to a thickness of 2 voxels, allowing us to visualize them into 3D space (200^3 voxels). We chose this thickness because it is approximately the width of the anther. Finally, we performed a resampling or bootstrap analysis to generate a heat map of pollen location in 3D space. We resampled the 3D extruded polygons (treating each as an independent observation), with replacement, and randomly rotated each one in increments of 1/10 of a full rotation (multiples of 36 degrees). Each

K. latifolia flower has 10 anthers, but we only triggered one per flower. Thus, the random rotation allowed for a 3D representation of pollen trajectory from the entire flower. We conducted this resampling routine 500 times. Each resampled representation was a 200^3 array with voxel values that were between 0 and 29. The value of 0 meant that no pollen was ever seen in that area, and the value of 29 meant that pollen from every flower passed through that voxel. We averaged all of the 3D positions over the resampled arrays, to produce a 3D heat map of pollen trajectory. The heat map and resampling were done with custom-written scripts in Python with the module, Mayavi (Ramachandran and Varoquaux 2011). The custom-written software used in this project is available online at `https://github.com/callinSwitzer/KalmiaCatapult`.

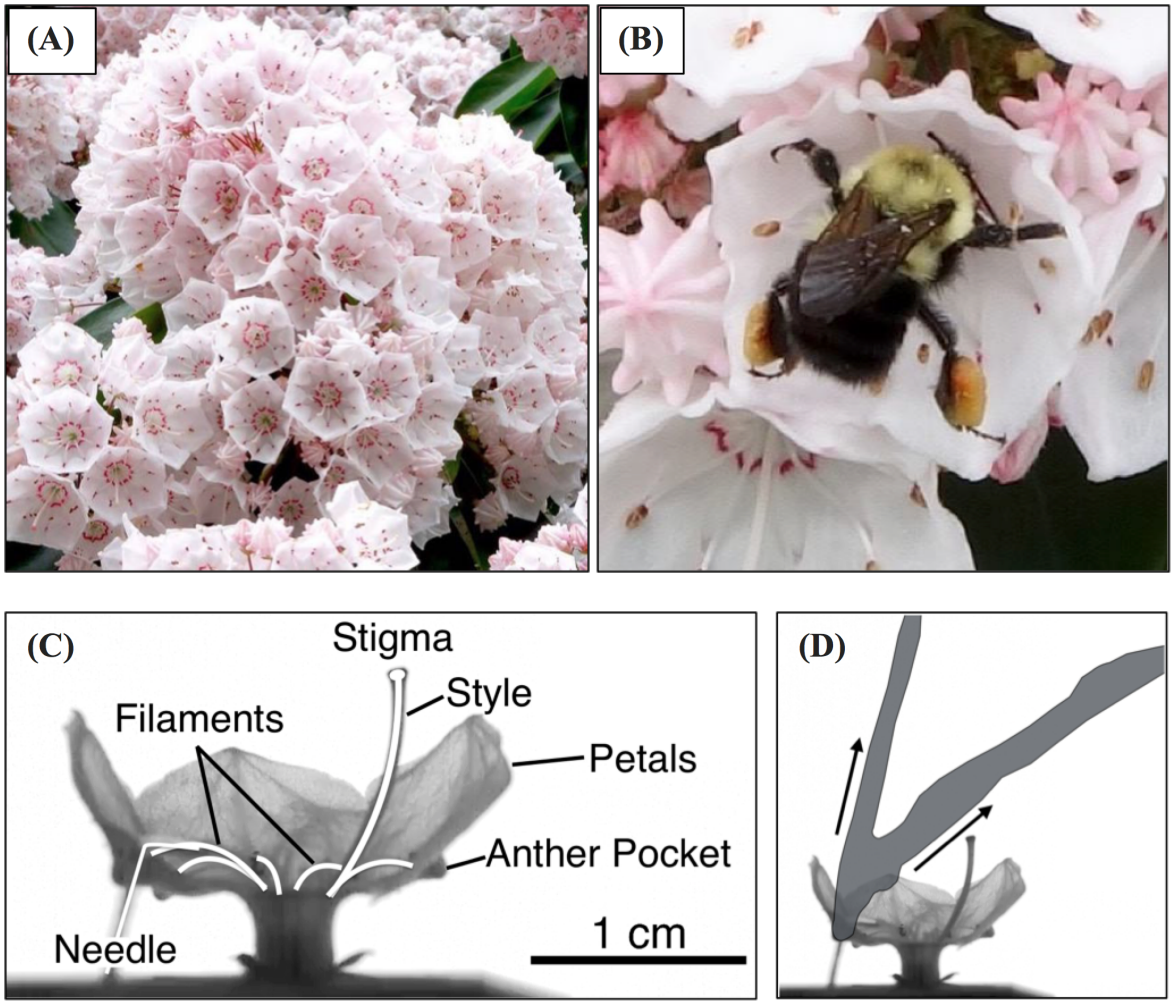


Figure 5.1: Photos of (A) *Kalmia latifolia* inflorescences and (B) a bumblebee visiting a single *K. latifolia* flower. Photos by William (Ned) Friedman. (C) Side view of *K. latifolia* flower before releasing pollen. The stigma, style, filaments, and the needle used for manually triggering the catapult have been traced in white. Note that the stigma is off-center and that half of the petals have been removed to allow for visualization of the pollen release. (D) *K. latifolia* flower with the region that pollen occupied after the catapult was triggered shown as a grey polygon. The arrows indicate the direction that pollen flew from the catapult.

Monitoring insect visitors and causes of catapulting pollen

We sampled insect visitors by collecting videos (2000 frames s^{-1}), using a high-speed camera (TS4, Fastec, San Diego, California) and a 105mm f/2.8 lens (Nikon, Melville, New York). We recorded insects visiting *K. latifolia* plants in the Arnold Arboretum, on five sunny days in June 2016 between 08:00 and 12:00. For each video, we identified the insect visitors and

observed when visitors triggered anthers. We recorded a total of 69 insect visits.

Pollination experiments

To determine if pollinators are necessary for successful pollination of *K. latifolia*, we conducted a pollen manipulation experiments with 4 treatments. On each of 22 plants in the Arnold Arboretum (see Appendix D for accession information) we manipulated four inflorescences (each with multiple flowers) with one of the following randomly assigned treatments: 1.) Control flowers were left open to all pollinators and not manipulated. 2.) Autogomous-selfed flowers were bagged with tulle (fine netting) to exclude all pollinators that were large enough to trigger the catapult mechanism 3.) Manipulatively-selfed flowers were bagged with tulle to prevent pollinators' access and had self pollen applied to receptive stigmas using forceps (forceps were sterilized with isopropyl alcohol between flowers). 4.) Supplemental-outcrossed flowers were left open to all pollinators and had additional, non-self pollen added to receptive stigmas using forceps. Supplemental pollen was collected from 10-20 other individuals, mixed together, and than placed on the stigmas of flowers. We continuously monitored and pollinated the plants (every 3-4 days) as all new flowers opened between June 9, 2016 and July 7, 2016. On October 20, 2016, we collected the fruits from all treatments.

We cleaned and photographed the fruits. We used custom-written software and the module, OpenCV (Bradski 2000) to automatically count fruits from images and measure diameters (See Appendix D for more information). We measured 1305 fruits, in total. We used linear mixed models (LMM's) to compare fruit size and generalized linear mixed models (GLMM's) compare the number of fruits in each of the treatments. For all mixed models, we used the library, lme4 (Bates et al. 2015). We included plant accession number and plant lineage as random effects in these models. We used a negative binomial model to compare the counts of fruits in each treatment, and a Gaussian model to compare the size of fruits. After constructing models, we calculated 95% bootstrap confidence intervals for the means (using 1000 replications). The confidence intervals were based on fixed effects only.

We used the size of fruits as a proxy for the number of seeds produced. We confirmed that larger fruits produce more seeds by counting the number of seeds in one carpel of the five carpels in each of 19 fruits collected from randomly selected individuals (fruits were from flowers that were untreated). We used a Poisson GLMM, with plant lineage as a random effect to confirm that fruit size was a significant predictor of the number of seeds. See Appendix D for more information and code for the statistical models.

Results

Pollen speed and acceleration

Our first goal was to characterize the pollen catapult kinematics by digitizing pollen trajectories. We found that the average max speed for *K. latifolia* pollen was 3.5 m s^{-1}, 95% CI [3.1-4.0] and the average max acceleration of pollen was 4100 m s^{-2}, 95% CI [3300-5300]. Figure 5.2 shows a time-series of position, speed, and acceleration of pollen for a single trial.

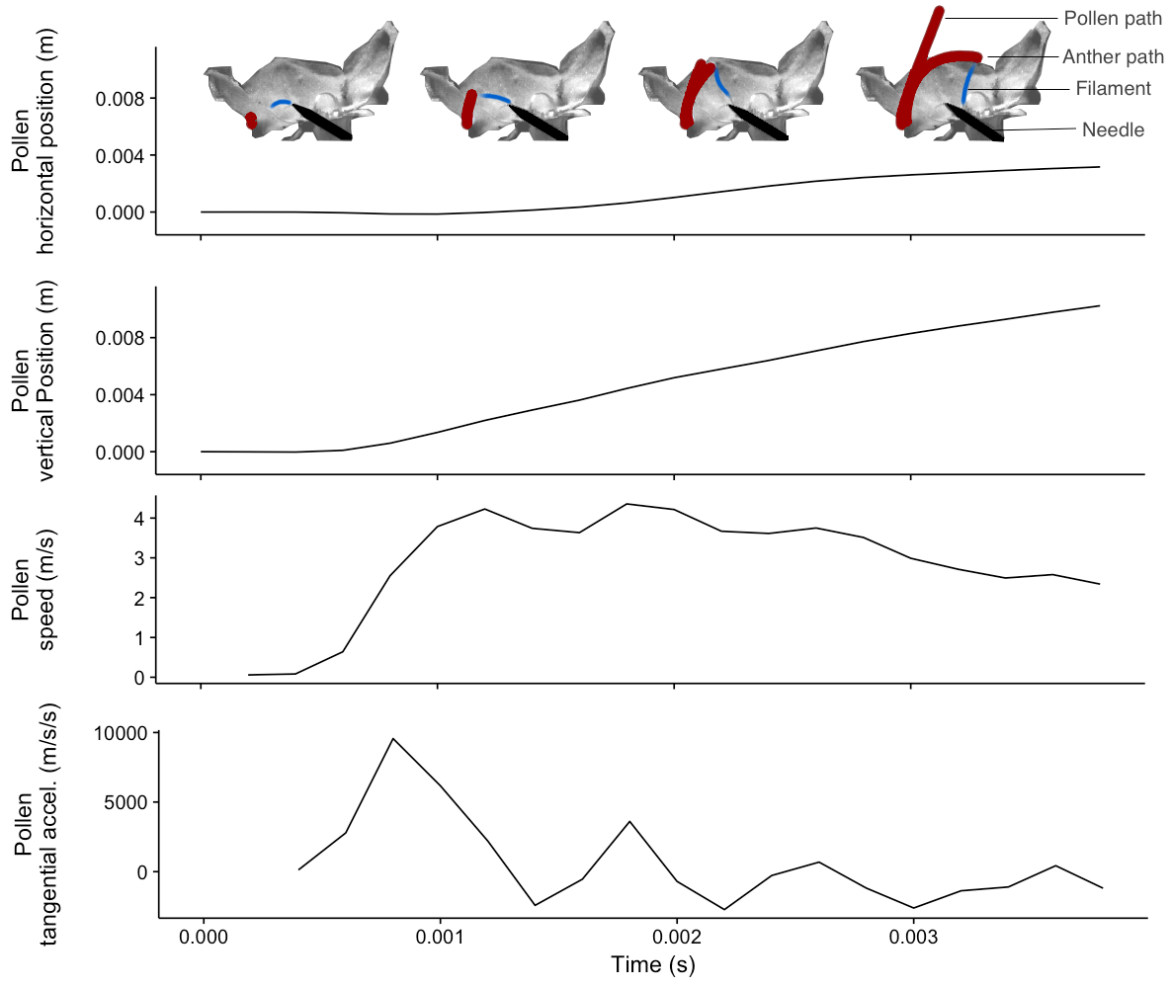


Figure 5.2: Position, speed, and acceleration of *Kalmia latifolia* pollen for a single trial. Top photos show pollen and anther paths, along with the filament and the needle used to trigger the catapult mechanism.

Pollen location in 3D space

Our second goal was to determine the spatial trajectory of released pollen. Figure 5.3 shows a 3D contours and a contour map of a single slice through the 3D heat map. The "choppiness" of the 3D contours in Figure 5.3 is due to the low resolution of the 3D space (200^3 voxels). We found that catapulted pollen was most likely to cross through the center of the flower, at a height a little greater than the length of a stamen.

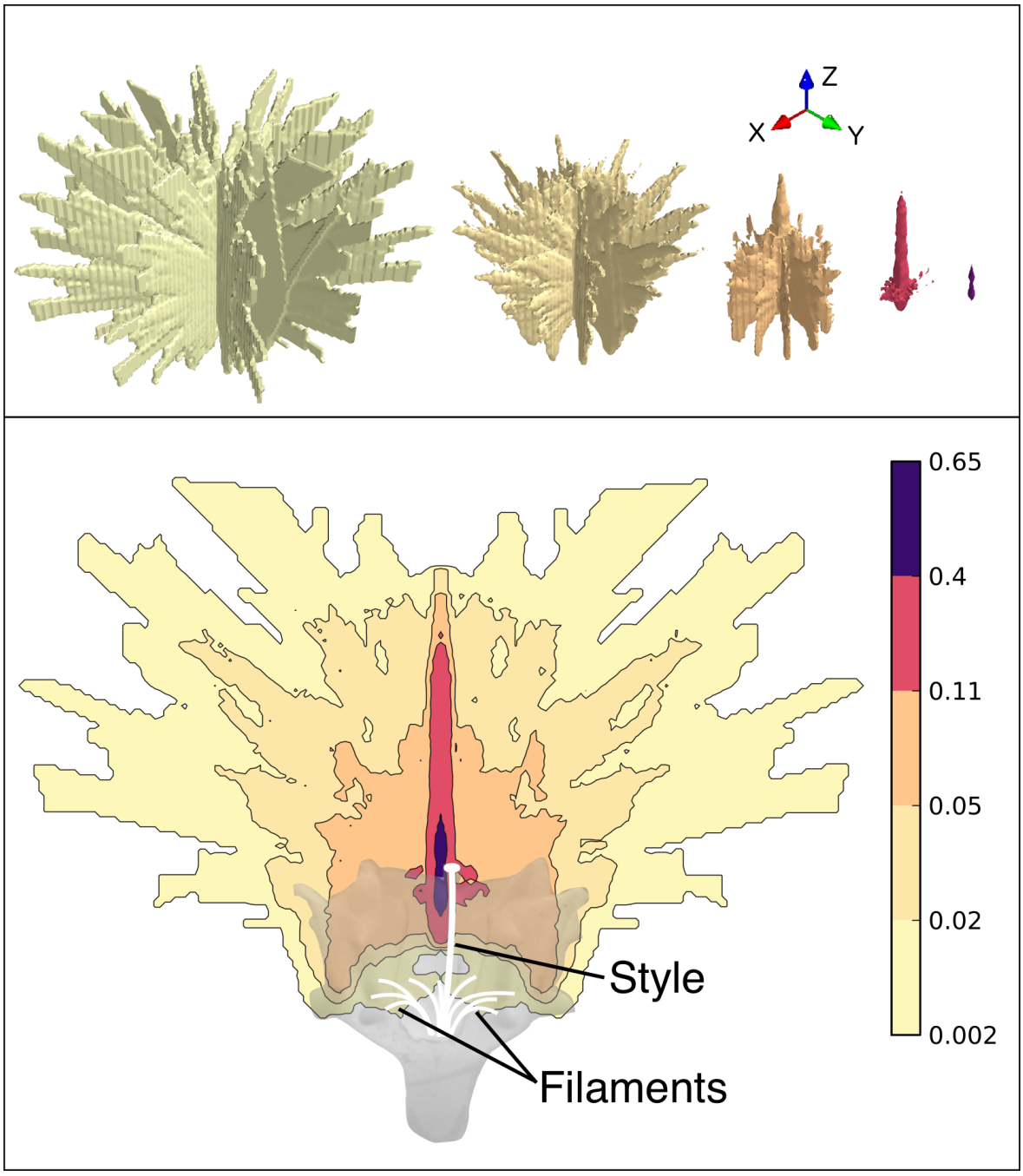


Figure 5.3: Top: 3D visualizations of contours of pollen trajectories of *Kalmia latifolia*. **Bottom:** Contour map of resampled pollen tragectories showing that pollen generally launches toward the center of the flower. The contours represent probability that pollen will fly through that area. For instance, the darkest area in the center indicates that in all of the resampled representations, pollen flew through this area 40-65% of the time. The overlayed flower is a guide to show the approximate position of the flower, and the filaments and style have been roughtly traced with white lines.

Table 5.1: Counts of insect visitors filmed on *Kalmia latifolia*. The majority of visitors were bumblebees, and only the bees were observed triggering the catapult to release pollen.

Insect	Visits	Visits that triggered catapult
Bumblebees (*Bombus* spp.)	54	22
Honeybees (*Apis mellifera*)	7	2
Butterflies and moths (Order: Lepidoptera)	4	0
Potter wasps (Subfamily: Eumeninae)	3	0
Carpenter Bee (*Xylocopa virginica*)	1	1
TOTAL	**69**	**25**

Insect visitors and causes of catapulting pollen

Our third goal was to quantify and characterize the insect visitors to *K. latifolia* and describe the behaviors that triggered the pollen catapults. We reviewed each of the 69 videos and report a brief summary of the pollinators (Table 5.1). We found a variety of insect visitors, but the most common visitors were bumblebees, or *Bombus* spp. (54 of the 69). We observed that only bees (bumblebees, honeybees, and carpenter bees) triggered the anthers to release pollen. Bees generally triggered the anthers with their front or mid legs as they landed on the flower or tried to push their proboscis deeper into the floral nectary. Large bees also typically contacted the stigma of the flower while they were searching for nectar. Figure 5.1B shows a typical visit from a bumblebee, where the stigma is underneath the bee in the photo. Wasps (Potter wasps, Subfamily: Eumeninae) were observed collecting insect larvae from around the *K. latifolia* flowers but not inside. Butterflies did not trigger the anthers, because they could easily insert their long, slender proboscis from a distance without triggering the anthers with their legs.

Pollination experiments

Our fourth goal was to determine if pollinators were necessary for successful pollination of these flowers. We found that the treatment (Control, Autogamous-selfed, Manipulated-selfed, and Supplemental-outcrossed) significantly affected the number of fruits, while accounting for variation that was due to individual plants and plant lineage (Negative

Binomial GLMM, $\chi^2(3) = 52.73$, $p < 0.001$). Figure 5.4A shows the comparisons of the number of fruits that were collected among the different pollination treatments. Treatment also significantly affected the size of fruits that were produced, while accounting for the variation that came from individual plants ($\chi^2(3) = 389.3$, $p < 0.001$) (Figure 5.4B).

Inflorescences that were excluded from pollinators and only allowed to autogomously self (excluding pollinators) had smaller and fewer fruits than any of the other treatments. Adding supplemental outcrossed pollen increased both fruit number and fruit size. Inflorescences that were manually self-pollinated produced fruit numbers and sizes similar to those of the control (un-manipulated, open-pollinated) inflorescences. We found that the size of the fruit (diameter) was a significant predictor of the number of seeds per carpel (Poisson GLMM, $\chi^2(1) = 21.0$, $p < 0.001$). Larger fruits contained a greater number of seeds.

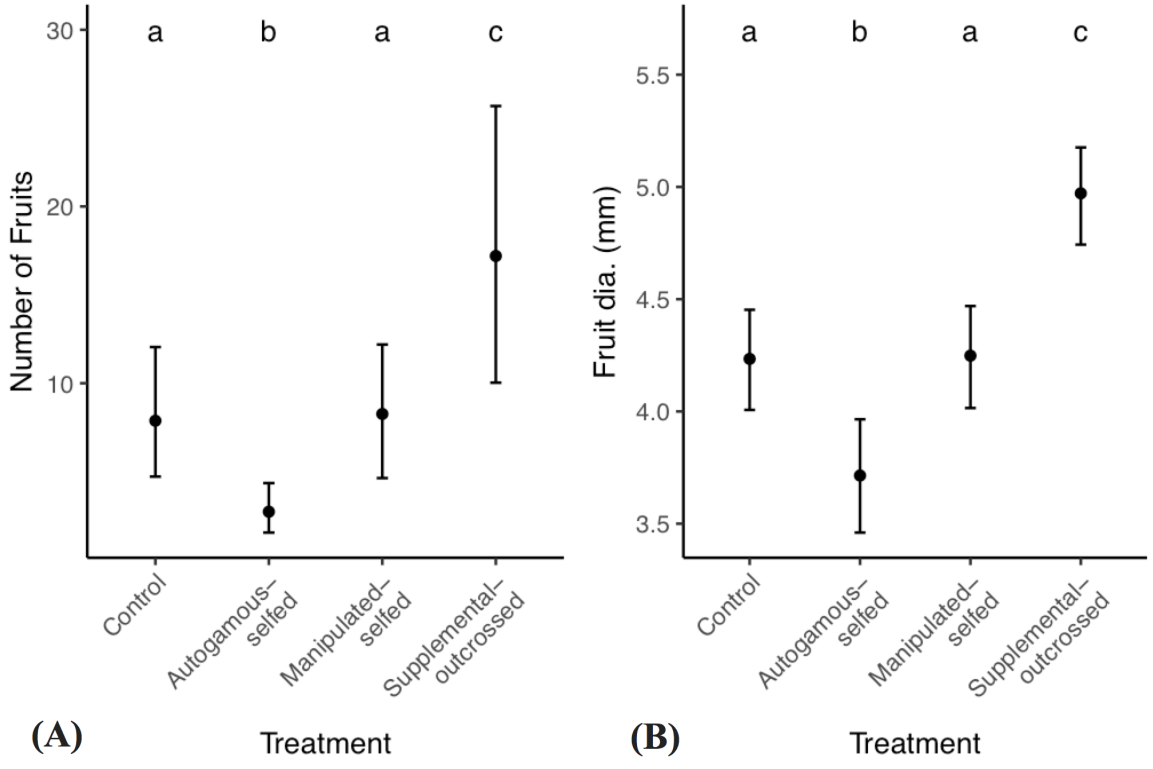


Figure 5.4: (A) Mean and 95% bootstrap confidence interval for the mean number of fruits collected per plant for each treatment. (B) Mean and 95% bootstrap confidence interval for the mean diameter of fruits collected for each treatment. Different letters above the treatments indicate significantly different groups (unadjusted p-values < 0.005).

Discussion

The results from our experiments quantitatively support the claim that *K. latifolia* is adapted to releasing pollen only to the pollinators that routinely touch the stigma of the flower.

In our first experiment, we found that the catapult mechanism launches pollen with high speeds and high accelerations. These quick movements ensure that the pollen launches and lodges in the pollinator's hair (called pile on bumblebees) while the pollinator is still on the flower. The high speed and acceleration of the catapult also ensures that pollen will fly out of the anthers, rather than remaining stuck inside the anther locules.

To put the movements of *K. latifolia* into perspective, we compare them to some other organisms that are known to move fast. To our knowledge, this is the first study reporting the speed or acceleration of catapulted *K. latifolia* pollen. The pollen of *K. latifolia*, is launched differently than some other extreme pollen-catapults – for instance, *Cornus canadensis* has been reported to launch dry grains of pollen toward pollinators with a maximum speed of 3.1 m s^{-1} and accelerate pollen at 24,000 m s^{-2} (Edwards et al. 2005). In comparing *K. latifolia* to *C. canadensis*, we see that *K. latifolia* pollen reaches a comparable speed, but has lower acceleration. Futhermore, *C. canadensis* pollen is grainy, whereas *K. latifolia* pollen is launched in stringy clumps. Another plant that launches pollen very quickly is the white mulberry tree, *Morus alba* (Taylor et al. 2006). This plant is wind-pollinated, so the catapult is not generally triggered by a pollinator (Taylor et al. 2006). The pollen of *K. latifolia* is faster than the reported values for *Catasetum* orchids that fascinated Charles Darwin (1862a). These orchids have been reported to launch pollen sacs onto foraging insects at a speed of 2.65 m s^{-1} with a maximum acceleration 925 m s^{-2} (Nicholson et al. 2008). The mantis shrimp (*Odontodactylus scyllarus*), which is one of the fastest moving animals alive, moves its feeding appendages much faster than *K. latifolia* reaching peak speeds of 14-23 m s^{-1} and has peak accelerations betwee 65-104 km s^{-2} (Patek et al. 2004). However, *K. latifolia* moves faster than several species of snakes (*Pantherophis obsoletus*, a rat-snake, and *Crotalus atrox*, a rattlesnake), that are known to be fast strikers. These snakes produced maximum velocities of around 3 m s^{-1} and accelerations of $\sim$280 m s^{-2} (Penning et al. 2016).

In our second experiment, we found that the pollen tends to fly toward the center of the flower (see Figure 5.3). This was not obvious when we recorded videos of insects visiting *K. latifolia* in the field. Because the pollen is launched in multiple clumps (that cover a wide fan of angles), we often saw pollen fly past the pollinators' bodies in our field observations. Our heat map suggests that pollen may occasionally miss the pollinators, but on average if the pollinator in the center of the flower, it will get hit with pollen.

From the heat map (Figure 5.3), we can make predictions about the size of pollinators that would be the most effective at pollinating the *K. latifolia* flowers. A notable observation from the heat map (Figure 5.3) is that a very small pollinator may not be hit by the pollen. Small honeybees, for instance, can be too close to the floral nectary to get hit with any of the flying pollen; the pollen sometimes launches over the top of their bodies. Alternatively, butterflies with very long proboscises may feed from too far away from the center of the flower to routinely get hit with flying pollen; the proboscis of a butterfly may also be too fragile to trigger the release of pollen. Overall, our heat map (Figure 5.3) is consistent with the hypothesis that a *K. latifolia* is adapted to for pollination by large bees, like bumblebees.

The third part of our investigation (observing pollinators and behaviors in the field) further corroborates our claim that the pollen catapult of *K. latifolia* is an adaptation that releases pollen only to insects that are likely to transfer pollen to the stigma of another flower. In our observations, we recorded that only bees triggered the anthers. The bumblebees were typically positioned with their bodies in the center of the flower, near or touching the stigma, so it is most likely that they routinely got hit by flying pollen.

Our final experiments, which investigated the role of pollinators in determining reproduction suggest that pollinators play a critical role in pollination of *K. latifolia*. We found that fertilization rates were low in the pollinator-excluding (bagged) treatment – both in terms of fruit number and size (Figure 5.4). The open-pollinated treatment and the self-pollinated treatment were very similar in terms of fruit set and fruit size (Figure 5.4). Both treatments were higher than the bagged (pollinator-excluding) treatment. These results indicate that the mechanical release of the anthers by a pollinator is important for fruit set, but that

self-pollination is common in the population of *K. latifolia* in the Arnold Arboretum. The results suggest that pollinators are important for triggering the release of the pollen that self-pollinates some of the flowers. These conclusions agree with some previous research, which found that autogamous self-pollination does not significantly contribute to fruit production (Nagy et al. 1999), though others have reported that a population of *K. latifolia* in Virginia has the ability to self-fertilize without a pollinator (Rathcke and Real 1993). The population of *K. latifolia* in our study is not representative of the geographical location, but rather a variety of accessioned plants from different locations (See Appendix D for accession information). In addition, we found that outcrossed *K. latifolia* had higher fruit set and larger fruits than any of the other treatments (Figure 5.4). Our findings suggest that the *K. latifolia* plants in our study were pollination limited – that is, more pollinators transferring pollen among different plants would result in higher seed set. Past literature also suggests that *K. latifolia* is rarely visited by pollinators (Jaynes 1988; Rathcke and Real 1993; Real and Rathcke 1991). In our study, pollinators were so rare that we collected only 69 videos of insect visitors in $\sim$20 hours of filming, even though individual *K. latifolia* plants can have thousands of flowers open at the same time (Rathcke and Real 1993).

We hypothesize several reasons why the *K. latifolia* in our study were pollination limited. First, the flowers produce very little nectar (Jaynes 1988; Real and Rathcke 1991), and bumblebees may be more attracted to coflowering plants (Rathcke and Real 1993). In the Arnold Arboretum, there are a variety of plants in flower at the same time as *K. latifolia*. Furthermore, literature suggests *K. latifolia* nectar and possibly pollen is toxic to bees (Adler 2000), and especially to honeybees (Eckert 1955; Oertel 1980). Though literature suggests bumblebees cannot detect the ecologically relevant levels of toxins in the nectar in a laboratory setting (Tiedeken et al. 2014), the poisonous nectar may still play a role in this system.

Summary

In summary, our results are support the claim that the pollen catapult of *K. latifolia* is an adaptation that acts as a pollen dispensing system – restricting pollen removal to certain insects (i.e. large bees) that are likely to transfer that pollen to the stigma of another flower. The explosive pollen release launches pollen toward the center of the flower, near the stigma, which is where the bumblebees are when they are probing for nectar. We found that other insects rarely visit *K. latifolia* and trigger the pollen catapults. Last we found that self-pollination increases fruit set and that autogamous self-pollination is relatively rare – this, suggests that insect visitors play an important role, even in self-pollinated plants.

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Appendix A

Supplemental Material for Chapter 1

Table A.1: Intertegular (IT) span of *A. murrayensis* and *B. impatiens* measured during this study.

	IT Span (mm)	Std. Dev	n
Amegilla murrayensis	2.915	0.252	20
Bombus impatiens	4.138	0.380	18

Table A.2: Mean and standard deviation of buzz-pollination and wing beat (flight) frequency for *A. murrayensis* and *B. impatiens*.

Bee	Behavior	Mean Freq.(Hz)	Std. Dev	n
Amegilla murrayensis	Pollination Buzz	346.60	23.56	22
Bombus impatiens	Pollination Buzz	239.99	37.62	53
Amegilla murrayensis	Flight	250.71	21.11	23
Bombus impatiens	Flight	161.78	12.73	53

Table A.3: Results of multiple linear regression model for buzz frequency. The regression equation is (Buzz Frequency (Hz))^2 $\sim$ Time of day + Temperature (°C) + Humidity (%) + Bee spp.

	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	176954.9442	36171.9677	4.89	0.0000
Time of day	-4524.8293	2666.5525	-1.70	0.0942
Temperature	-235.7993	722.7726	-0.33	0.7452
Relative humidity	9.4284	241.6511	0.04	0.9690
B. impatiens	-61912.6654	7325.3090	-8.45	0.0000

Table A.4: Results of multiple linear regression model for wing beat frequency. The regression equation is Sqrt(wingbeat Freq (Hz)) $\sim$ Time of day + Temperature (°C) + Humidity (%) + Bee spp.

	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	17.9392	1.1817	15.18	0.0000
Time of day	-0.0702	0.0875	-0.80	0.4253
Temperature	-0.0466	0.0236	-1.97	0.0525
Relative humidity	0.0021	0.0079	0.26	0.7945
B. impatiens	-3.2102	0.2401	-13.37	0.0000

Table A.5: Results of multiple linear regression model for buzz length. The regression equation is log(buzz length (sec)) $\sim$ Time of day + Temperature (°C) + Relative humidity (%) + Bee spp.

	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	-0.2316	1.3053	-0.18	0.8597
Time of day	0.0358	0.0962	0.37	0.7110
Temperature	0.0013	0.0261	0.05	0.9593
Relative humidity	-0.0063	0.0087	-0.72	0.4726
B. impatiens	0.2970	0.2643	1.12	0.2650

Table A.6: Results of multiple linear regression model for visit duration. The regression equation is log(time on flower (sec)) $\sim$ Time of day + Temperature (°C) + Relative humidity (%) + Bee spp.

	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	-0.6977	2.0448	-0.34	0.7343
Time of day	0.1377	0.1410	0.98	0.3332
Temperature	-0.0194	0.0374	-0.52	0.6062
Relative humidity	-0.0090	0.0116	-0.78	0.4417
B. impatiens	1.4200	0.3573	3.97	0.0002

Table A.7: Comparison of virgin and nonvirgin varieties of tomatoes. Buzz frequency and length (same as visit duration for *A. murrayensis*) for*A. murrayensis* on virgin flowers of *S. lycopersicum* 'Heirloom roma cherry' (n= 6), nonvirgin flowers of *S. lycopersicum* 'Heirloom roma cherry' (n = 8) and nonvirgin flowers of *S. lycopersicum* 'Tommy Toe' (n = 8). Individuals of *A. murrayensis* were never observed performing multiple buzzes with discernable breaks, as is commonly seen in *B. impatiens*.

	Heirloom-Virgin	Heirloom-Nonvirgin	Tommy Toe-Nonvirgin
Buzz Frequency (Hz)	357.84 ± 22	342.79 ± 24.81	341.97 ± 23.53
Buzz Length (s)	0.8 ± 0.28	1 ± 0.51	1.35 ± 0.69
Visit Duration (s)	0.61 ± 0.34	1.02 ± 0.45	1.31 ± 0.73

Table A.8: Results of multiple linear regression model to compare buzz frequency of *A. murrayensis* while pollinating on different tomato varieties. The regression equation is as follows: Avg buzz frequency (Hz) $\sim$ plant + virgin flower. (F(2, 19) = 0.9371, p-value = 0.4091).

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	341.9692	8.3535	40.94	0.0000
Tomato Variety - Heirloom Roma	0.8161	11.8136	0.07	0.9456
Virgin Flower	15.0583	12.7601	1.18	0.2525

Table A.9: Results of multiple linear regression model to compare buzz length of *A. murrayensis* while pollinating on different tomato varieties. The regression equation is as follows: log(avg buzz length (sec)) $\sim$ plant + virgin flower. (F(2, 19) = 1.515, p-value = 0.2452).

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.1997	0.1961	1.02	0.3213
Tomato Variety - Heirloom Roma	-0.3760	0.2773	-1.36	0.1910
Virgin Flower	-0.1017	0.2995	-0.34	0.7379

Appendix B

Supplemental Material for Chapter 2

Table B.1: Mean and SD of bee behaviors, stratified by the type of mark.

Behavior	Mark type	Mean Diff.	Std Dev
Sonication freq. (Hz)	Paint	-12.85	31.51
	Bee tag	-13.56	27.83
Sonication length (sec)	Paint	-0.01	1.06
	Bee tag	-0.35	0.75
Wingbeat freq. (Hz)	Paint	-1.53	7.28
	Bee tag	-5.44	9.69

Supplemental Resource 1. Cox Proportional Hazards Regression:

```
Call:
coxph(formula = survobj $\sim$ IT + Mark + IT:Mark, data = tagDF)

n= 177, number of events= 99
(34 observations deleted due to missingness)

coef exp(coef) se(coef)      z Pr(>|z|)
IT              0.5481    1.7300   0.2997  1.829   0.0674 .
MarkBee tag    -1.3678    0.2547   0.2291 -5.970 2.38e-09 ***
IT:MarkBee tag  1.0178    2.7670   0.4791  2.124   0.0336 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

exp(coef) exp(-coef) lower .95 upper .95
IT                 1.7300     0.5780    0.9614     3.113
MarkBee tag        0.2547     3.9266    0.1625     0.399
IT:MarkBee tag     2.7670     0.3614    1.0820     7.076

Concordance= 0.743  (se = 0.036 )
Rsquare= 0.284   (max possible= 0.995 )
Likelihood ratio test= 59.05  on 3 df,   p=9.364e-13
Wald test            = 46.56  on 3 df,   p=4.317e-10
Score (logrank) test = 57.92  on 3 df,   p=1.632e-12
```

Supplemental Resource 2. Manova for each covariate in the multivariate multiple regression.

```
Type II MANOVA Tests: Pillai test statistic
Df test stat approx F num Df den Df Pr(>F)
dot         1  0.045951   1.3967      3     87 0.2492
TagDayDiff  1  0.063389   1.9627      3     87 0.1255
```

Supplemental Resource 3. Overall evaluation of multivariate multiple regression (likelihood ratio test).

```
Analysis of Variance Table

Model 1: cbind(buzzDiffTag, wingDiff.tag, buzzLengthDiffTag)
                                                    $\sim$ dot + TagDayDiff
Model 2: cbind(buzzDiffTag, wingDiff.tag, buzzLengthDiffTag) $\sim$ 1
Res.Df Df Gen.var.  Pillai approx F num Df den Df  Pr(>F)
1     89      36.513
2     91  2   37.442 0.13321   2.0932      6    176 0.05625 .
```

Supplemental Resource 4. Each of the multiple regressions

```
Response buzzDiffTag :

Call:
lm(formula = buzzDiffTag $\sim$ dot + TagDayDiff, data = df1)

Coefficients:
Estimate Std. Error t value Pr(>|t|)
(Intercept)  -11.381      4.668  -2.438   0.0168 *
dotTRUE        1.006      7.357   0.137   0.8915
TagDayDiff    -1.481      1.493  -0.992   0.3238

Residual standard error: 31.93 on 89 degrees of freedom
Multiple R-squared:  0.01111, Adjusted R-squared:  -0.01111
F-statistic: 0.5001 on 2 and 89 DF,  p-value: 0.6082


Response wingDiff.tag :

Call:
lm(formula = wingDiff.tag $\sim$ dot + TagDayDiff, data = df1)

Coefficients:
Estimate Std. Error t value Pr(>|t|)
(Intercept)   0.5539     1.1504   0.481   0.6314
dotTRUE      -3.2025     1.8129  -1.766   0.0807 .
TagDayDiff   -0.8975     0.3679  -2.439   0.0167 *

Residual standard error: 7.87 on 89 degrees of freedom
Multiple R-squared:  0.1201, Adjusted R-squared:  0.1003
F-statistic: 6.072 on 2 and 89 DF,  p-value: 0.003372


Response buzzLengthDiffTag :

Call:
lm(formula = buzzLengthDiffTag $\sim$ dot + TagDayDiff, data = df1)

Coefficients:
Estimate Std. Error t value Pr(>|t|)
(Intercept) -0.0003685  0.1447563  -0.003    0.998
dotTRUE     -0.2428648  0.2281246  -1.065    0.290
TagDayDiff  -0.0167412  0.0462953  -0.362    0.718

Residual standard error: 0.9902 on 89 degrees of freedom
Multiple R-squared:  0.01735, Adjusted R-squared:  -0.004727
F-statistic: 0.7859 on 2 and 89 DF,  p-value: 0.4588
```

Supplementary Resource 5. Results for proportion test of dead bees (for only bees that were not observed sonicating).

```
2-sample test for equality of proportions with continuity correction

data:  deads out of totals
X-squared = 0.82725, df = 1, p-value = 0.3631
alternative hypothesis: two.sided
95 percent confidence interval:
-0.3708130  0.1031362
sample estimates:
prop 1    prop 2
0.2272727 0.3611111
```

Appendix C

Supplemental Material for Chapter 4

Processing audio recordings

We first listened to the recordings to identify the longest sonication sound, and determined its length (duration). We focused on the longest sonication, because we observed that bees sometimes performed shorter, higher-frequency sonications on the petals of the flowers, and we wanted to exclude these from analysis, so our results had only one type of sonication. We classified a bout of buzzing as a single sonication if there was no audible break for > 0.1 seconds. After selecting the sequence for analysis, we used the "spec" function from the seewave package to calculate power spectral density, using a hanning window of 2048 points (Sueur et al. 2008). To determine the sonication frequency (the dominant frequency at which the bee was vibrating), we selected the highest peak on the spectrum between 195 Hz and 400 Hz (a reasonable range for sonication frequency, based on previous studies (Switzer et al. 2016) and preliminary experiments with commercial colonies.

To check the accuracy of the frequency obtained by this method, we generated a sine wave at the frequency identified as the highest peak, and compared it aurally to the audio recording of sonication. If the frequency returned from the spectrum was noticeably higher or lower in pitch from the audio recording (which can occur due to background noise), we used Audacity (Audacity Development Team 2015) to obtain the correct sonication frequency. Within Audacity, we selected the sonication portion of the audio recording, and plotted the spectrum (hanning window, 2048 points). We then generated sine waves at each of the frequencies corresponding to the peaks in the spectrum. We compared each of these sine

waves to the recording, aurally, and chose the peak that corresponded most closely to the audio recording of the sonication.

Recordings collected in the greenhouse contained significantly more background noise (often due to other bees flying nearby the microphone) than those collected in the field. We used the same technique as above to analyze greenhouse recordings – first using R to calculate the dominant frequency and checking the result aurally; however, we also double-checked all of the greenhouse recordings using Audacity.

Recordings of irritation buzzes collected in the field were analyzed using only Audacity, as the frequency of irritation buzzes tended to be highly variable, unlike the relatively consistent sonication sounds produced during pollen-collection. Since irritation buzzes typically reached higher frequencies than pollen-collection buzzes and we were interested in determining how much bees can change their buzzing frequency, we listened to each recording of irritation buzzing and selected the three sections with the highest-frequency buzzes. We plotted the spectrum as above and listened to sine waves generated from the frequency peaks in the spectrum to identify the frequency of irritation buzzing. We then averaged the dominant frequencies from the three highest irritation buzz segments.

Model selection for field studies

We analyzed sonication length and sonication frequency in separate regressions (rather than using multivariate multiple regression), because the two dependent variables, sonication length and sonication frequency, were not highly correlated.

Before conducting regressions, we centered the independent variables (subtracted the overall mean from each of the observations), including bee IT span, bee mass, time, date, relative humidity, and temperature. We did this to reduce collinearity of quadratic terms in the model and to make interpretation easier. We then checked for multicollinearity using variance inflation factor with the R package, car (Fox and Weisberg 2010). At the Concord Field Station, we found that plant and date were highly collinear (largely because R. multiflora blooms in the early summer and the other two plants bloomed later in the

summer), so we dropped the date from our analysis at the Concord Field Station. At the Arboretum we found that IT span and mass were highly collinear, so we dropped mass from that analysis. We chose to drop mass because we expected mass to be more variable within an individual than IT span, since wild bumblebees carry variable amounts of nectar (up to their entire body weight; see Allen et al. 1978).For each location and dependent variable we built three initial models – one model with main effects only, one model with all interactions and quadratic terms, and one minimal model with only plant as an independent variable.

We used Bayesian Information Criterion (BIC) as a method to select the best model, because we wanted to penalize complexity and select a relatively simple model (Hastie et al. 2005). We conducted forward, backward, and multidirectional stepwise procedures to explore a large subspace of possible models, using the "stepAIC" function from the R package, MASS (Venables and Ripley 2002). Though the function is called "stepAIC", we used BIC by changing the penalty term. After running the three different procedures we selected the model that had the lowest BIC and added main effects if the best model was not hierarchical (e.g., we added day as a covariate if day2 was a significant covariate).

We evaluated the assumptions of homoscedasticity and normally distributed residuals from the multiple regressions by examining normal q-q plots and residual vs. fitted plots. We also checked for outliers using Cook's Distance.

Model selection for greenhouse study

For both sonication frequency and sonication length, we started with a model including multiple covariates – flower species, bumblebee colony, amount of time the flower had been in the cage, time of day, and whether or not the bee was one of the 18 individuals who foraged on all three plants. We included date and bee ID as random intercept terms. We did not include interactions in this model because we had no a priori reason to do so. We checked to see if modeling each individual with a random slope significantly improved the model, using a likelihood ratio test, and found no evidence that the random slopes model was better than the random intercepts model. We did not test the significance of the

other random effects in the model, because those were not of interest. We used a series of likelihood ratio tests to remove covariates that did not contribute significantly to the model.

We checked the homoscedasticity and normality assumptions by visually inspecting fitted vs. residual plots and normal q-q plots for the main effects and both of the random effects. We found that the first day of the experiment was potentially an outlier (we had guessed that this might be the case, because bees may require some time to learn how to sonicate on the three different plants). We dropped this day and re-ran the analysis, but this produced no significant changes in the results, so we report the final model including the first day of the experiment. We also re-ran the analysis only on the subset of bees that sonicated on all three plants. We found the trends to be similar to the model with all bees included, but the smaller sample size resulted in larger standard errors and therefore higher p-values (Tables C.10, C.11,C.12, C.13). Because we found the same trend (similar coefficient estimates for both sonication frequency and sonication length), and because we found that the subset of bees that sonicated on all three plants were not significantly different from the rest of the bees in their sonication behaviors on the three plant species, we report our final models using all bees that foraged on at least one of the three *Solanum* spp.

We found the best models after conducting a series of likelihood ratio tests, removing terms that did not improve the model (at the α=0.05 level).

Significance level adjustments

We adjusted significance levels for the field studies. To account for multiple comparisons, we used more conservative significance levels (i.e. lower than α=0.05). We adjusted the significance levels in each of the studies, using the Bonferroni adjustment, which is very conservative when tests are not independent (Bland and Altman 1995). For field studies in both locations, we performed two regressions (one for sonication length and one for frequency), so we used a significance level of α=0.025 (0.05/2). This means that if the overall p-value for the regression was > 0.025, we considered it non-significant.

If the regressions had a significant overall p-value, we then conducted post-hoc tests to

evaluate all pairwise differences among plants in sonication frequency or log sonication length, at each location. We used simultaneous pairwise tests for general linear hypotheses to test for differences among plants, while accounting for other covariates, using the "glht" function from the R package, multcomp (Hothorn et al. 2008). At the Arnold Arboretum, we investigated five plant species, so we performed ten post-hoc tests for each regression. We adjusted the significance level for post-hoc pairwise tests for both sonication length and sonication frequency to α=0.005 (0.05/10). We used no adjustment for the paired t-test for sonication frequency vs. irritation buzz frequency. At the Concord Field Station, we investigated three plant species, so we conducted three post-hoc, pairwise tests. We used α=0.017 (0.05/3) as a significance level for these post-hoc tests.

We also adjusted significance levels for the greenhouse study. For the final models, we used α=0.025 as the significance level, since we conducted two regressions in this study. After finding significant models, we conducted three post-hoc tests to compare all pairwise combinations of plants for differences in sonication frequency and sonication length using the glht function from the R package, multcomp (Hothorn et al. 2008). We adjusted the significance level for the three post-hoc pairwise tests for sonication length and sonication frequency to α=0.017 (0.05/3).

Plant species sonicated by wild *B. impatiens*

In the Arnold Arboretum, we observed bees sonicating on flowers from the following plants: *Callicarpa cathayana* (Lamiaceae), *Callicarpa japonica* (Lamiaceae), *Hypericum* 'Hidcote' (Hypericaceae), *Rosa* 'Bucbi' (Rosaceae), and *Rubus odoratus* (Rosaceae). With the exception of *R. odoratus*, which grows wild at the Arnold Arboretum, these plants were part of the living collection at the Arnold Arboretum, and are not representative of native plants in the region. All plants at the Arnold Arboretum had yellow anthers.

The two *Callicarpa* spp. are shrubs with clusters of small (5-10 mm diameter), purple flowers. These plants are native to eastern Asia (Zheng-Yi and Raven 1994). Each flower has four petals, four stamen extending beyond the petals, and a pistil extending with the stamen.

We examined the anthers of these and other *Callicarpa* spp. from the living collection, and found that the anthers had longitudinal separations, but the largest opening was at the distal portion of the anther. These plants appeared to produce little or no nectar – we never observed bumblebee extending its proboscis on these flowers.

Hypericum 'Hidcote' is a cultivar of St. John's Wort, a shrub with large (∼7 cm diameter), yellow flowers ([MBG] n.d.). The flowers have many long filaments with longitudinally dehiscent anthers at their tips. We observed some bees extending their proboscis and collecting nectar from these flowers.

Rosa 'Bucbi' is a cultivar of a rose, a shrub with large (∼10 cm diameter), pink flowers with semi-double petals. Near the petal bases, there are many stamen, with filaments that are shorter than the petals and longitudinally dehiscent anthers. The flower contains multiple pistils (Buck 1978). Roses do not produce significant amounts of nectar (Lovell 1910).

Rubus odoratus is a shrub that is native to northeastern United States, with magenta flowers (∼3-5 cm diameter) and five petals ([MBG] n.d.). The flower has many stamens and pistils at its center, similar to *Rosa* 'Bucbi'. The anthers we observed with a microscope had longitudinal separations, but these may not open completely – i.e. one end of the locule was separated more widely than the rest of the longitudinal split. *R. odoratus*, like other roses, does not produce significant amounts of nectar (Lovell 1910).

At the Concord Field Station, we observed bees sonicating on flowers of the following plants: *Coronilla varia* (Fabaceae), *Physalis philadelphica* (Solanaceae), and *Rosa multiflora* (Rosaceae).

Coronilla varia is a low-growing vine that is not native to North America, but is found throughout the United States. This plant has clusters of small (∼1 cm long), bilaterally symmetric flowers with five pink petals. It has papilionaceous (pea-like) flowers with ten stamen and one style enclosed in the keel. This plant produces small amounts of nectar (Gucker 2009). We were uncertain if the bees were sonicating to release pollen or to extend the proboscis deeper into the flower.

Physalis philadelphica is an annual, cultivated tomatillo plant with small (∼1-3 cm diameter), yellow flowers and five fused petals ([GB] 2016). It is native to North America. The five anthers protrude from the flower center and are blue or greenish blue. The pistil extends among the anthers, which are longitudinally dehiscent but curl inward at the tip. *P. philadelphica* produces nectar, though we could not tell if bees were extending their proboscis while foraging, because the petals often occluded the bees' head (Prigge and Gibson 2016).

Rosa multiflora is a large shrub or climbing vine that produces medium (4 cm diameter), white flowers with five white or light pink petals. Like the other roses, it does not produce significant amounts of nectar. The center of the flower has a central column of styles ringed by many bright yellow anthers. This species is not native to the United States, and blooms in the late spring or early summer ([IWF] n.d.).

Plants species sonicated by commercial *B. impatiens*

In the greenhouse study, we used three plants from the genus *Solanum*. None of these species produce nectar, so all foragers were collecting only pollen. We chose these species for several reasons. First, they could all be placed in a common environment with similar water and fertilization schemes. They survived well in pots and could easily be transferred between bee cages – most of the plants that bee sonicated in the field portion of this study could not be easily potted and transferred. Second, they are common plants in North America, with poricidal anthers that are frequently sonicated by bumblebees. Third, these species represent a variety of morphological characteristics – petal color, flower size, anther size, and anther structure.

Solanum lycopersicum "Cherry Roma" is an annual, cultivated, cherry tomato plant. It has typical "solanoid" flowers. They are small (∼1 cm diameter) flowers with five yellow petals and a yellow, central, anther cone, consisting of five fused anthers. The style does not extend beyond the anther cone. This variety produces a fairly large number of flowers, when compared to varieties that produce larger fruits. We observed that this plant appeared to release relatively little pollen, however, compared to S. dulcamara. The *S. lycopersicum*

plants used in this experiment were grown from seeds (`Burpee.com`) in a greenhouse.

Solanum dulcamara is another "solanoid" flower. It is a perennial vine that is invasive in the Untied States ([IPANE] 2016). It has small ($\sim$1 diameter) flowers with five purple petals and a yellow, central anther cone ([IPANE] 2016). The style extends beyond the anther cone ([IPANE] 2016). We observed that this plant seems to release more pollen than the other two species. The S. dulcamara plants used in this experiment were grown from seeds collected in Cambridge, Massachusetts (42.3805, -71.1143).

Solanum carolinense is a perennial plant that is native to the warmer parts of the United States. It has larger flowers ($\sim$ 3 cm diameter) than *S. lycopersicum* and *S. dulcamara.* The flowers have five white or pale purple petals. The five yellow anthers are not fused into a central cone. The anthers are longer than the filaments and dehisce through pores, and the style sometimes extends beyond the anthers ([CBS] 2015). The *S. carolinense* used in the experiment was collected at the Arnold Arboretum before flowering, and replanted in the same size pot as the other plants.

Supplemental Figures

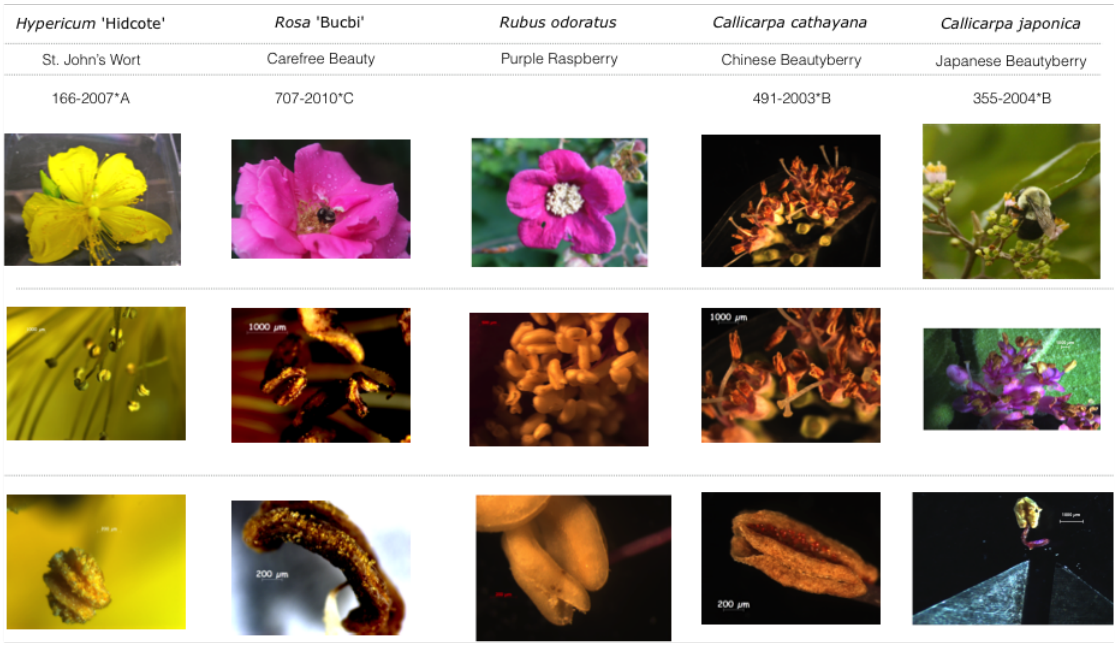


Figure C.1: Photos of the five plants on which wild bees were observed at the Arnold Arboretum, visualized at increasing magnification from top to bottom. Text in columns includes Latin name, common name, and accession number (if applicable) for the Arnold Arboreum. All photos by C. Switzer.

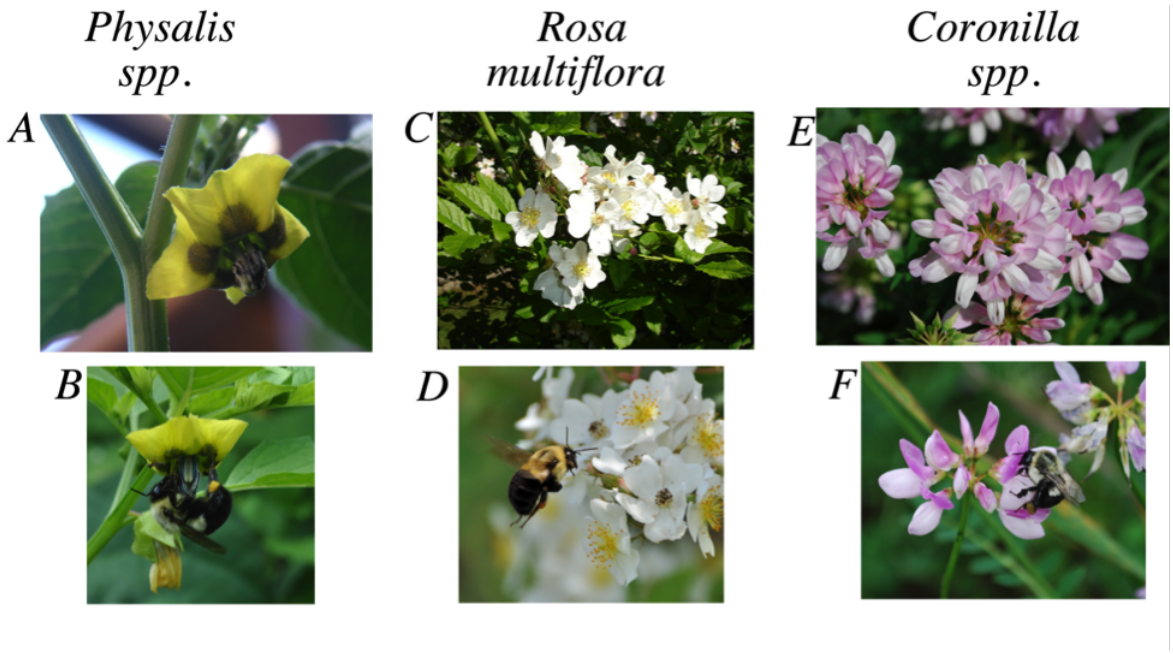


Figure C.2: Photos of the plants on which wild bees were observed at the Concord Field Station. All photos are under a creative commons license (`http://creativecommons.org/licenses/by-sa/3.0` unless otherwise noted). Photo atrributions (A) B. Carstor (B) B. Carlson (C) U. Eliasson (D) Putneypics (E-F) J. Mayer

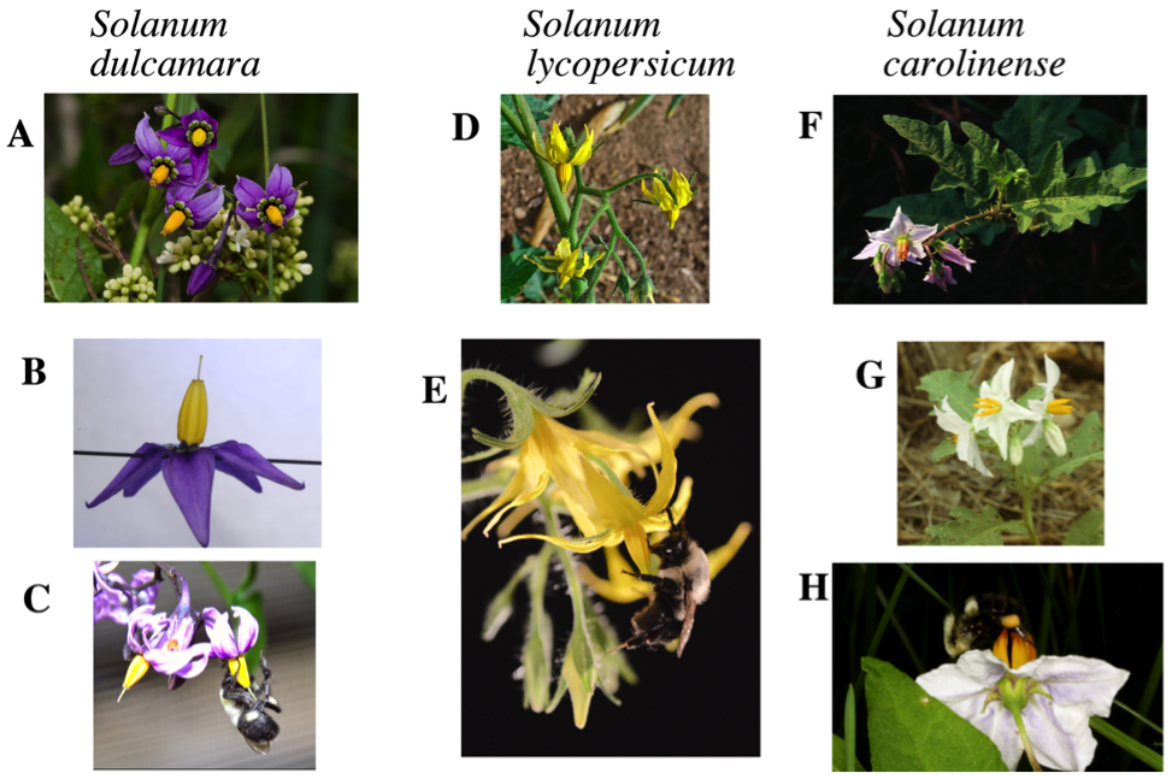


Figure C.3: Photos of *Solanum* plants used in the experiment. All photos are under a creative commons license (`http://creativecommons.org/licenses/by-sa/3.0` unless otherwise noted). Photo attribution: (A) S. van der Molen (B-C) C. Switzer (D) H. Zell (E) C. Switzer (F) T. Bodner (G) G.A. Cooper (H) M. Tulig.

Supplemental Tables

Table C.1: Paired t-test to compare individual bees' max irritation buzz frequency and sonication frequency (Hz). Significance level is 0.05. (n = 399).

	Estimate	df	t value	95% CI	Pr(>|t|)
Irritation buzz - pollination sonication	91.41	398	44.469	87.37, 95.46	$< 2 \times 10^{-16}$

Table C.2: Summary of best model for sonication frequency (Hz) for the field investigation at the Concord Field Station (F(4, 85) = 4.022, p = 0.0049). The adjusted significance level is 0.025 for overall model. Sample sizes are as follows: n*C.varia*=31, n*P.phil*=31, n*R.multi*=28.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	314.1954	6.9820	45.00	0.0000
Intertegular span (mm)	-44.6265	12.6317	-3.53	0.0007
Bee mass (g)	570.9755	181.3821	3.15	0.0023
plant: *Coronilla varia*	-23.2347	9.4784	-2.45	0.0163
plant: *Physalis philadelphica*	-30.6691	9.9928	-3.07	0.0029

Table C.3: Summary of best model for log sonication length (sec) for field investigation at the Concord Field Station (F(3, 86) = 6.367, p = 0.000599). The adjusted significance level is 0.025 for overall model. Sample sizes are the same as for Table C.2

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.1818	0.0922	-1.97	0.0519
plant: *Coronilla varia*	-0.3005	0.1241	-2.42	0.0176
plant: *Physalis philadelphica*	-0.0731	0.1341	-0.55	0.5870
Relative humidity	0.0110	0.0035	3.13	0.0024

Table C.4: Post-hoc tests for pairwise differences in sonication frequency (Hz) on flowers from the field investigation at the Concord Field Station, while accounting for other variables (bee mass and intertegular span). The significance level for this test is 0.017.

	Estimate	Std. Error	t value	Pr(>|t|)
Coronilla varia - Rosa multiflora	-23.23	9.48	-2.45	0.016
Physalis philadelphica - Rosa multiflora	-30.67	9.99	-3.07	0.003
Physalis philadelphica - Coronilla varia	-7.43	8.72	-0.85	0.396

Table C.5: Post-hoc tests for pairwise differences in log sonication length (sec) for field investigation on flowers at the Concord Field Station, while accounting relative humidity. The significance level for this test is 0.017.

	Estimate	Std. Error	t value	Pr(>|t|)
Coronilla varia - Rosa multiflora	-0.30	0.12	-2.42	0.0176
Physalis philadelphica - Rosa multiflora	-0.07	0.13	-0.55	0.5870
Physalis philadelphica - Coronilla varia	0.23	0.13	1.79	0.0770

Table C.6: Summary of best (lowest BIC) model for sonication frequency (Hz) in the Arnold Arboretum field investigation (F(6, 349) = 10.98, p = 3.241 x 10-11). Adjusted significance level is 0.025 for overall model. Sample sizes are as follows: n(*C.cathayana*)=173, n(*C.japonica*)=21, n(H.Hidcote)=33, n(*R.Bucbi*)=103, n(*R.odoratus*)=26.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	263.5574	2.7282	96.61	0.0000
plant: *Callicarpa japonica*	57.0985	10.6376	5.37	1.46×10^{-7}
plant: *Hypericum* 'Hidcote'	28.7047	8.5934	3.34	0.0009
plant: *Rosa* 'Bucbi'	5.9437	3.9653	1.50	0.1348
plant: *Rubus odoratus*	18.8768	7.4833	2.52	0.0121
Relative humidity (%)	0.5718	0.1313	4.36	1.74×10^{-5}
Date	0.4757	0.1327	3.58	0.0004

Table C.7: Summary of best (lowest BIC) model for log sonication length (sec) in Arnold Arboretum field investigation (F(7, 348) = 7.32, p-value = 3.33 x 10-8). Adjusted significance level is 0.025 for overall model. Sample sizes are the same as for Table 6.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.1083	0.0445	2.43	0.0155
plant: *Callicarpa japonica*	-0.3710	0.2980	-1.24	0.2141
plant: *Hypericum* 'Hidcote'	-0.5210	0.1797	-2.90	0.0040
plant: *Rosa* 'Bucbi'	0.1631	0.0605	2.70	0.0074
plant: *Rubus odoratus*	-0.1320	0.1163	-1.13	0.2572
Relative humidity	0.0077	0.0020	3.89	0.0001
Date^2	0.0002	0.0001	2.24	0.0258
Date	0.0009	0.0024	0.37	0.7130

Table C.8: Post-hoc tests for pairwise differences in sonication frequency (Hz) on flowers at the Arnold Arboretum, while accounting for other variables (relative humidity and date), with no adjusted p-values. The adjusted significance level is 0.005.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	263.5574	2.7282	96.61	0.0000
plant: *Callicarpa japonica*	57.0985	10.6376	5.37	1.46×10^{-7}
plant: *Hypericum* 'Hidcote'	28.7047	8.5934	3.34	0.0009
plant: *Rosa* 'Bucbi'	5.9437	3.9653	1.50	0.1348
plant: *Rubus odoratus*	18.8768	7.4833	2.52	0.0121
Relative humidity (%)	0.5718	0.1313	4.36	1.74×10^{-5}
Date	0.4757	0.1327	3.58	0.0004

Table C.9: Post-hoc tests for pairwise differences in log sonication length (sec) on flowers at the Arnold Arboretum, while accounting for other variables (relative humidity and date, and date$\hat{2}$), with no adjusted p-values. The adjusted significance level is 0.005.

	Estimate	Std. Error	t value	Pr(>|t|)
Callicarpa japonica - *Callicarpa cathayana*	-0.37	0.30	-1.24	0.2141
Hypericum 'Hidcote' - *Callicarpa cathayana*	-0.52	0.18	-2.90	0.0040
Rosa 'Bucbi' - *Callicarpa cathayana*	0.16	0.06	2.70	0.0074
Rubus odoratus - *Callicarpa cathayana*	-0.13	0.12	-1.13	0.2572
Hypericum 'Hidcote' - *Callicarpa japonica*	-0.15	0.18	-0.82	0.4101
Rosa 'Bucbi' - *Callicarpa japonica*	0.53	0.31	1.70	0.0907
Rubus odoratus - *Callicarpa japonica*	0.24	0.26	0.92	0.3601
Rosa 'Bucbi' - *Hypericum* 'Hidcote'	0.68	0.20	3.50	0.0005
Rubus odoratus - *Hypericum* 'Hidcote'	0.39	0.15	2.51	0.0124
Rubus odoratus - *Rosa* 'Bucbi'	-0.30	0.13	-2.32	0.0210

Table C.10: Mixed-effects model for sonication frequency for the subset of bees that sonicated on all three different plants in the greenhouse experiment. Note that the coefficients are very similar to those in the model with all bees (Table C.15), but standard errors are larger.

Fixed effects	Estimate	Std. Error	t value
(Intercept)	286.471	5.384	53.21
Solanum carolinense	12.365	4.432	2.79
Solanum dulcamara	4.241	4.730	0.90
Random effects	**Variance**		
Bee ID (Intercept)	260.16		
Date (Intercept)	20.01		
Residual	461.51		

Number of obs: 139, groups: Bee ID, 18; Date, 6

Table C.11: Final model for the length of sonication for the subset of bees that sonicated on all three different plants in the greenhouse experiment. Note that the coefficients are very similar to those in the model with all bees (Table C.17), but standard errors are larger.

Fixed effects	Estimate	Std. Error	t value
(Intercept)	-0.22561	0.09997	-2.257
Solanum carolinense	0.02218	0.09660	0.230
Solanum dulcamara	0.23471	0.10249	2.290
Colony B	0.27513	0.11877	2.316
Random effects	**Variance**		
Bee ID (Intercept)	0.026126		
Date (Intercept)	0.005319		
Residual	0.223561		

Number of obs: 140, groups: Bee ID, 18; Date, 6

Table C.12: Post-hoc tests to see which plants were sonicated at different frequencies (Hz) in the greenhouse experiment, including only individuals that sonicated on all three plants – See bottom of Table C.11 for sample sizes.

	Estimate	Std. Error	z value	Pr(>|z|)
Solanum carolinense - Solanum lycopersicum	12.36	4.43	2.79	0.00528
Solanum dulcamara - Solanum lycopersicum	4.24	4.73	0.90	0.36997
Solanum dulcamara - Solanum carolinense	-8.12	4.63	-1.76	0.07923

Table C.13: Post-hoc tests to see which plants were sonicated for different lengths in the greenhouse experiment, including only individuals that sonicated on all three plants.

	Estimate	Std. Error	z value	Pr(>|z|)
Solanum carolinense - *Solanum lycopersicum*	0.02	0.10	0.23	0.8184
Solanum dulcamara - *Solanum lycopersicum*	0.23	0.10	2.29	0.0220
Solanum dulcamara - *Solanum carolinense*	0.21	0.10	2.12	0.0343

Table C.14: Likelihood ratio test to see if plant significantly improved the model for sonication frequency (Hz) in the greenhouse experiment. Adjusted significance level is 0.025.

Model	Model df	Deviance	Chisq	Chi df	Pr(>Chisq)
Frequency ~ (1|Date) + (1|ID)	4	2209.1			
Frequency ~ plant + (1|Date) + (1|ID)	6	2190.3	18.788	2	8.324×10^{-5}

Table C.15: Mixed-effects model for sonication frequency (Hz) of bumblebees on different plants in the greenhouse experiment.

Fixed effects	Estimate	Std. Error	t value
(Intercept)	283.08	4.35	65.01
Solanum carolinense	14.32	3.49	4.11
Solanum dulcamara	2.96	3.72	0.80
Random effects	**Variance**		
Bee ID (Intercept)	400.9		
Date (Intercept)	27.4		
Residual	419.8		

Number of obs: 237, groups: Bee ID, 64; Date, 6

Table C.16: Likelihood ratio test to see if plant was a significant predictor of log sonication length (sec) in the greenhouse, while accounting for differences in bee colonies. Adjusted significance level is 0.025.

Model	Model df	Deviance	Chisq	Chi df	Pr(>Chisq)
log(Sonication length) ~ bee colony + (1|Date) + (1|ID)	6	354.05			
log(Sonication length) ~ plant + bee colony + (1|Date) + (1|ID)	7	344.83	9.2162	2	0.009971

Table C.17: Mixed-effects model for log sonication length (sec) for experiment in greenhouse with different *Solanum* plants. This model accounts for differences in the colonies.

Fixed effects	Estimate	Std. Error	t value
(Intercept)	-0.29010	0.07385	-3.928
Solanum carolinense	0.04932	0.07785	0.633
Solanum dulcamara	0.23381	0.08176	2.860
Colony B	0.24963	0.08232	3.032
Random effects	**Variance**		
Bee ID (Intercept)	0.024800		
Date (Intercept)	0.001367		
Residual	0.232639		

Number of obs: 238, groups: Bee ID, 64; Date, 6

Table C.18: Post-hoc tests to see which plants were sonicated at different frequencies (Hz) in the greenhouse experiment. Adjusted significance level is 0.017.

	Estimate	Std. Error	z value	Pr(>|z|)
Solanum carolinense - Solanum lycopersicum	14.32	3.49	4.11	4.03×10^{-5}
Solanum dulcamara - Solanum lycopersicum	2.96	3.72	0.80	0.4254
Solanum dulcamara - Solanum carolinense	-11.35	3.51	-3.23	0.0012

Table C.19: Post-hoc tests to see which plants were sonicated at different lengths (sec) in the greenhouse experiment, while accounting for differences in colonies. Adjusted significance level is 0.017.

	Estimate	Std. Error	z value	Pr(>|z|)
Solanum carolinense - Solanum lycopersicum	0.05	0.08	0.63	0.52643
Solanum dulcamara - Solanum lycopersicum	0.23	0.08	2.86	0.00424
Solanum dulcamara - Solanum carolinense	0.18	0.08	2.37	0.01768

Appendix D

Supplemental Material for Chapter 5

Accession numbers of *Kalmia latifolia* plants used in this experiment from the Arnold Arboretum

The accession numbers are as follows: 1129-74*A, 1129-74*B, 1129-74*C, 1129-74*E, 1129-74*F, 12-2006*A, 128-96*MASS, 132- 98*MASS, 150-58*A, 232-46*A, 35-40*C, 39-60*A, 425-74*D, 440-66*A, 46-18*A, 572-64*MASS, 624-64*B, 673-69*B, 667-66*MASS, 685-70*A, 721-79*B.

Notes on code

All code can be found online in the GitHub repository: `https://github.com/callinSwitzer/KalmiaCatapult`. In addition to the files described in this supplement, we have also made our code for statistical analysis available. The statistical analysis and making of the figures can be found in the following files on GitHub:

`3_1_KalmiaInsectVisitorsAndBehaviorAnalysis_Submission.RMD`

`3_1_KalmiaInsectVisitorsAndBehaviorAnalysis_Submission.html`

`4_2_KalmiaFruitSizeAnalysis.RMD`

`4_2_KalmiaFruitSizeAnalysis.html`

`The code for segmenting images to count fruits and measure`

`their size is in this file:`

`4_1_KalmiaFruitImageSegmentation_Submission.ipynb`

Smoothing of the digitized *Kalmia latifolia* pollen trajectories

The goal of smoothing the digitized paths of pollen is to reduce digitization errors, thereby making the estimates of maximum velocity and acceleration more accurate. In general, the process of smoothing goes like this:

1. Randomly split the path pollen into 10 groups (each time step gets put into 1 of 10 groups)

2. For each group (or "fold") in 10-fold validation, we smooth the trajectory on 9/10 of the data, and then we calculate the out-of-sample error for the group that was left out. We average the out-of-sample error after repeating the process on all 10 groups. The goal is to minimize the out-of-sample error. The parameter that we tuned is the smoothing parameter (spar), from the function, smooth.spline, in R (R Core Team (2016), `https://www.R-project.org/`).

3. The out-of-sample error was calculated as the Euclidian distance from the predicted values to the actual values that were withheld when calculating the smoothing spline.

4. We calculated a best smoothing parameter for every trial. However, we chose to apply the same smoothing parameter to all trials, for consistency. We selected the median best smoothing parameter (0.29) and used that for all trials.

Below, we show some examples. Figure D.1 shows an example of how the smoothing parameter affects the out-of-sample error. This example suggests that the best smoothing parameter is 0.26. However, when we viewed the distribution of the best smoothing parameters for every trial, we found that a better choice for an average smoothing parameter was the median of all best smoothing parameters, which was 0.29.

Figure D.2 shows how the smoothing parameter affects the predicted path of pollen. As the smoothing parameter increases, the path becomes smoother. Additionally, the plot shows that the difference between unsmoothed (spar = 0) and the smoothed data with the smoothing parameter = 0.29 is small. The R code that was used for selecting a smoothing

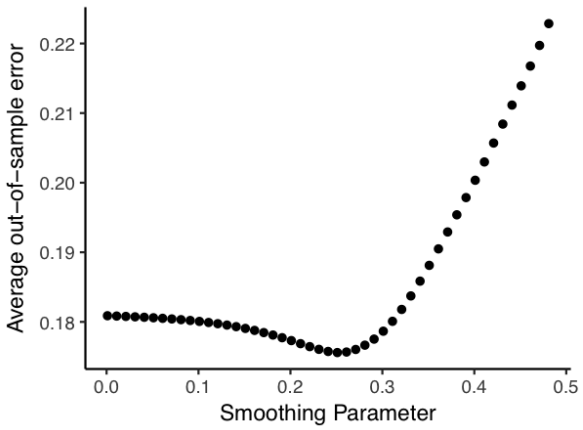


Figure D.1: Example plot of the smoothing parameter vs. average out-of-sample error. In this example, the out-of-sample error is minimized by selecting a smoothing parameter of ~ 0.26.

parameter can be found on GitHub. The R code is in the file, 1_1_KalmiaPollenKinematics_CV_Submission.RMD, and an HTML document with the R code and the output is in the file, 1_1_KalmiaPollenKinematics_CV_Submission.html.

Calculations for velocity and linear acceleration

After smoothing the digitized paths of pollen in two dimensions, we calculated speed and linear acceleration. In each of our videos, we digitized a calibration object that allowed us to convert between pixels and meters. In some of the videos, the calibration object was the head of an insect pin, and in other videos, the object was a small piece of paper with a square grid printed on it.

After converting position from pixels to meters, we calculated the velocity separately for the x and y dimensions (Equations 1-2). To convert position data to speed, we used the numerical derivative, which is calculated as the change in position divided by the change in time. The units for position are m, and the units for speed and velocity are m s-1. That is, for each time step, i, in t total time steps, we calculated the instantaneous velocity for both the x and y dimensions. We start at time step 2, because we're computing a numerical

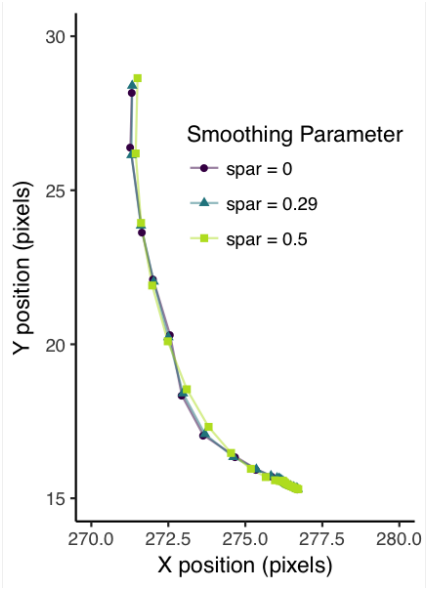


Figure D.2: Example plot showing how the smoothing parameter affects the path in two dimensions. As the smoothing parameter increases, the path becomes more smooth.

derivative, which can only be calculated with t-1 time points.

$$\begin{aligned} \text{velocity}_{x,i} &= \frac{\Delta \text{ position}_{x,i}}{\Delta \text{ time}_i} \\ &= \begin{cases} \left(\text{position}_{x,i} - \text{position}_{x,i-1}\right) \cdot 5000 & \text{if } i \in [2,t] \\ N/A & \text{otherwise} \end{cases} \end{aligned} \tag{1}$$

$$\begin{aligned} \text{velocity}_{y,i} &= \frac{\Delta \text{ position}_{y,i}}{\Delta \text{ time}_i} \\ &= \begin{cases} \left(\text{position}_{y,i} - \text{position}_{y,i-1}\right) \cdot 5000 & \text{if } i \in [2,t] \\ N/A & \text{otherwise} \end{cases} \end{aligned} \tag{2}$$

To get the speed for each time step, i, we used the formula in Equation 3.

$$\text{speed}_i = \begin{cases} \sqrt{(\text{velocity}_{y,i})^2 + (\text{velocity}_{x,i})^2} & \text{if } i \in [2,t] \\ N/A & \text{otherwise} \end{cases} \tag{3}$$

We then used the speed to calculate the acceleration for every time step. There are two possible quantities for acceleration that could be relevant – tangential acceleration

and normal acceleration. Linear (or tangential) acceleration is a measure of the change in speed, whereas normal acceleration is a measure of the change in direction. If an object was traveling in a circle at a constant speed, then it would have 0 linear acceleration, and a nonzero normal acceleration. If an object is traveling along a straight path, but increasing it's speed, then it would have 0 normal acceleration, but a nonzero linear acceleration. We chose to report linear acceleration in our analysis (though normal acceleration may be important for releasing pollen from the anthers).

To calculate linear acceleration at time step i, we used Equation 4. The units for linear acceleration are m s-2.

$$\begin{aligned} \text{linear acceleration}_i &= \frac{\Delta \text{ speed}_i}{\Delta \text{ time}_i} \\ &= \begin{cases} (\text{speed}_i) - (\text{speed}_{i-1}) \cdot 5000 & \text{if } i \in [3, t] \\ N/A & \text{otherwise} \end{cases} \end{aligned} \tag{4}$$

The code used for calculations can be found on GitHub. The file, 1_2_KalmiaPollenKinematics_Submission.RMD is the R code only, and the file, 1_2_KalmiaPollenKinematics_Submission.html, shows the R code and output from the code after it was run.

In-depth description for how pollen paths were digitized, converted to images, and aligned.

The process of converting s of *Kalmia latifolia* flowers launching pollen into a 3D heat map is composed of five steps:

1. Video processing (MATLAB)
2. Manual outlining of pollen paths in polygons (MATLAB)
3. Rotating and scaling polygons, and then saving as images (R)
4. Converting polygon images into 3D representations (Python)
5. Resampling 3D representations to make a heat map (Python)



Figure D.3: The final frame of an example vido after processing with MATLAB. Everything that is white moved during the course of the video.

Video processing

We used MATLAB (The MathWorks Inc. 2014) to process each of the videos of *K. latifolia* releasing pollen. The basic procedure involved loading a video, calculating a median value for each pixel over the course of the movie, and then changed the pixels that changed significantly from their median values – this allowed us to remove all parts of the image that did not change over the course of the movie. Figure D.3 shows the final frame of the black and white video that was processed in this way. The code for processing the videos can be found on GitHub, with filename, 2_1_KalmiaVideoProcessing_showPollenTrajectories_Submission.m. We created Movie 4 by overlaying the pollen path over the original video, using custom-written code in Python (Python Software Foundation n.d.). The code for creating that movie can be found in the same GitHub repository, with filename, 2_5_StackVideoFrames_Submission.ipynb.

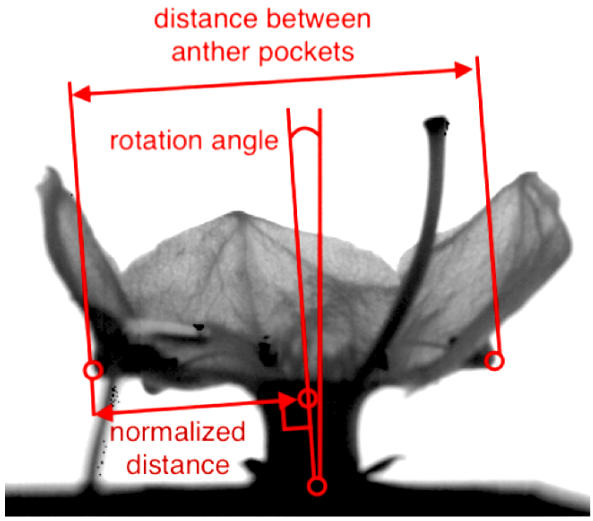


Figure D.4: Four additional points, shown in open circles, that were digitized for *Kalmia latifolia* experiments in 2016. We digitized the two outer anther pockets, the middle of the bottom of the flower, and the middle of the flower, where the petals spread away from each other. We used these points to rotate all flowers so that they were oriented with the flower facing parallel with the vertical axis. We scaled each flower so that the "normalized distance" from the figure above was 1 unit.

Manual outlining of pollen paths in polygons

Next, we loaded in the final frame of our processed videos into MATLAB (Ex. Figure D.3), and manually outlined the space that pollen occupied. We re-watched the original video, and we included the anther inside the outline, if it contained pollen. From MATLAB, we exported the locations of all of the vertices of the polygon as a text file. In outlining the polygon, we digitized several other points from the original videos. Figure D.4 shows the four additional points that we digitized on the original video. The code for outlining the pollen paths manually and digitizing additional points can be found in the same GitHub repository as above, with filename, 2_2_KalmiaPolygonSelect_Submission.m.

Rotating and scaling polygons, and then saving as images

We imported the text files that outlined the pollen's space in two dimensions into R. We rotated each of the digitized points, according to Figure D.3, so that the flower was facing parallel with the vertical axis, and we scaled the points so that the "normalized distance"

from Figure D.3 was 1 unit. This allowed us to align all of our images, regardless of the original flower size and orientation. We moved all of the points so that they were all aligned – the new coordinate system had the upper point in the center of the flower at the origin. Last, we plotted all of the vertices of the polygon, and filled in the polygon (making the pixels non-white). We exported these polygon shapes as PNG images (See Figure 5.1C for an example). The code used for this step of the process can be found on the GitHub repository above, under the filenames, 2_3_ImportDigitizedPolygonsAndSaveAsImages_Submission.Rmd or 2_3_ImportDigitizedPolygonsAndSaveAsImages_Submission.html.

Extruding and resampling 3D representations to make a heat map

The last step of the process is described in the methods section. Using Python, we resampled these polygons, extruded them into 3D space, resampled them, and randomly rotated them in 3D space to represent the 10 anthers of the *K. latifolia* flower. Figure 5.3 shows the final result. The code for the extruding, rotating, and resampling can be found in the GitHub repository above, under the filename, 2_4_KalmiaPolygon3DVis_Submission.ipynb.