

## ABSTRACT

PINILLA GALLEGO, MARIO SIMON. Transmission Dynamics of Bee Parasites (Under the direction of Dr. Rebecca Irwin).

Parasites are ubiquitous in nature, and emerging infectious diseases are a threat to biodiversity, domestic animals, crops and humans. However, we know little about the transmission dynamics of most parasites. Nonetheless, this information is essential to develop management strategies to control and mitigate the spread of parasites, and at the same time, can improve our understanding of disease ecology and evolution. Here, I used the model system of the gut parasite *Crithidia bombi* and its primary host, bumble bees (*Bombus* spp.), to explore the transmission dynamics of this parasite. Research also extended beyond *C. bombi* and bumble bees to include other parasite-host systems and alternative hosts. This work contributes to a growing effort to understanding what factors affect the spread of bee parasites, which could lead to developing ways to limit their spread. Specifically, I asked the following questions: 1) To what degree do floral traits and species identity predict transmission potential of *C. bombi* on flowers? 2) How does the initial prevalence of infection in the colony affect the within-colony transmission of *C. bombi* in *Bombus impatiens* colonies and *Nosema ceranae* in *Apis mellifera* colonies? and 3) How does infection of an alternative host, the alfalfa leaf-cutter bee (*Megachile rotundata*), affect the virulence of *C. bombi*?

To explore transmission of the parasite on flowers, I compared models that used either species identity or floral traits as predictors for three basic steps of parasite transmission on flowers: feces deposition on flowers, survival of the parasite on flowers, and acquisition of the parasite by a new host. I found that species identity better predicted deposition of feces and survival of *C. bombi* on flowers. However, trait-based models were better at predicting acquisition of the parasite on flowers. Floral shape was the trait that had the largest effect on

overall transmission potential, with flowers with smaller floral width and larger floral length promoting higher transmission. Identifying traits that can reduce parasite transmission on flowers would allow us to select plants that could slow down the spread of diseases to use in gardens and pollinator habitat.

To understand how the initial prevalence of infection impacts within-colony spread of *C. bombi* and *N. ceranae* on bumble bee and honey bee colonies, I infected either a low or high percentage of workers, and then followed the spread of the parasites for several weeks. In bumble bees, higher initial parasite prevalence increased both transmission and intensity of infection of *C. bombi*. In honey bees, higher initial prevalence increased the intensity of infection, but not the final prevalence of *N. ceranae*. These results suggest that social immunity of bee colonies, or other biological factors of social insects, could reduce within-colony transmission of parasites when they have low prevalence, but at high prevalence there could be rapid within-colony spread, especially in bumble bee colonies.

Finally, I conducted a serial passage experiment in which I passed *C. bombi* from leaf-cutter bee to leaf-cutter bee (alternative host). After each pass through the alternative host, I infected a group of bumble bees to test for changes in virulence in the primary host. I found that after serial passes through the alternative host, there was no significant change in virulence of the parasite on the alternative host. Interestingly, when the parasite was returned to the primary host after serial passes through the alternative host, both the probability and intensity of infection increased, potentially due to maladaptation after selection for new strains in the alternative host.

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# Transmission Dynamics of Bee Parasites

by  
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## **DEDICATION**

A mi familia, mis padres Susana Gallego y Gabriel Pinilla y mi hermana Gabriela Pinilla, quienes sin su apoyo nunca hubiera encontrado la fuerza para terminar este proyecto.

## **BIOGRAPHY**

I earned my B.S. in Biology at the Nueva Granada University in Bogotá, Colombia in 2013. After that, I worked at the Bee Research Laboratory of the National University of Colombia, studying the pollination service on passionfruit growing areas. In 2015 I started my Master's in Entomology at Michigan State University, working under the direction of Professor Rufus Isaacs. My Master's research focused on improving the management of mason bees in Michigan orchards. In the summer of 2017, I graduated with my Masters, and in the Fall started a PhD in Biology at North Carolina State University, working with Professor Rebecca Irwin on the transmission of bee parasites.

## ACKNOWLEDGMENTS

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## CHAPTER 1: Floral shape predicts bee-parasite transmission potential

### Abstract

The spread of parasites is one of the primary drivers of population decline of both managed and wild bees. Several bee parasites are horizontally transmitted by the shared use of flowers, turning floral resources into potential disease hotspots. However, we know little about how floral morphology and floral species identity affect different steps of the transmission process. Here, we used the gut parasite *Crithidia bombi* and its primary host, bumble bees (*Bombus* spp.), to examine whether floral traits or species identity better predict the three basic steps of parasite transmission on flowers: feces deposition on flowers, survival of the parasite on flowers, and acquisition by a new host. We also identified which traits and/or species were most strongly associated with each step in the transmission process. We found that both trait- and species-based models fit the data on deposition of feces and survival of *C. bombi* on flowers, but that species-based models provided a better fit than trait-based ones. However, trait-based models were better at predicting the acquisition of *C. bombi* on flowers. While different species tended to support higher fecal deposition or parasite survival, we found that floral shape provided explanatory power for each of the transmission steps. When we assessed overall transmission, floral shape had the largest explanatory effect on transmission potential, with narrower, longer flowers promoting higher transmission. Taken together, our results highlight the importance of species identity and floral traits in disease transmission dynamics, and floral shape as an important predictor of overall transmission potential. Identifying traits associated with transmission potential may help us create seed mix that presents lower risk for bees to be use in pollinator habitat.

## Introduction

Emerging infectious diseases (EIDs) are a threat to humans as well as domestic and wild animals (Daszak et al. 2000). EIDs can be driven by parasites that invade a new geographic area, or by parasites that increase in prevalence in their native range due to changes in external factors, such as alterations in environmental conditions that facilitate parasite transmission (Dobson and Foufopoulos 2001, Antonovics 2017). The decline of some wildlife species has been linked to the spread of EIDs (Lips et al. 2006, Craft 2015, Wani et al. 2018), which in most cases are microparasites that lack intermediate stages, have high transmission rates, and do not have vectors and so are spread directly (Dobson and Foufopoulos 2001). Bees (Hymenoptera: Apoidea) are hosts to a wide variety of micro- and macro-parasites (Shimanuki and Knox 2000, Hedtke et al. 2011, Graystock et al. 2016), and some of these parasites have been linked to the decline of both managed honey bees (VanEngelsdorp and Meixner 2010, Bianco et al. 2014) and wild bee populations (Potts et al. 2010, Meeus et al. 2011, Szabo et al. 2012). Given the important ecological and economic role of bees as pollinators (Losey and Vaughn 2006, Garibaldi et al. 2013, Schulp et al. 2014), understanding the transmission dynamics of bee parasites is an important step toward developing strategies to slow their spread (Bonsall 2004). Here, we investigated the role of floral morphology and floral species identity on several steps of the transmission process for the model host-parasite system of the bumble bee (*Bombus* spp.) and its gut parasite, *Crithidia bombi* (Lipa & Triggiani) (Trypanosomatida: Trypanosomatidae).

Parasites can exploit the use of shared resources by their hosts to infect new individuals. For example, avian mycoplasmosis spreads through bird populations via shared bird-feeders (Adelman et al. 2015), and supplemental feeding stations increase the transmission of bovine brucellosis among elk (Cotterill et al. 2018). In a similar way, many bee parasites spread to new

individuals via shared flowers, as parasites or fecal material can remain on the flowers and infect new individuals that visit those flowers (Durrer and Schmid-Hempel 1994, Graystock et al. 2015). Studies report that 10-30% of open flowers harbor at least one bee parasite (Figuerola et al. 2020, Graystock et al. 2020), and transmission of some bee parasites increases when flowers are added to flower-poor landscapes, due to the congregation of bees in flower-rich spots (amplification effect) (Piot et al. 2019). Parasite transmission on flowers can also occur in areas that use commercial pollinators in high densities, as commercial pollinators can act as a source or reservoir of parasites for wild populations (Murray et al. 2013, Fürst et al. 2014, Sachman-Ruiz et al. 2015). For example, wild bees were more likely to carry the microsporidian parasite *Nosema ceranae* in areas with *Nosema*-infected honey bee hives than in areas with healthy ones (Müller et al. 2019).

The transmission process of bee parasites via flowers can be broken down into three basic steps – deposition of the parasite on flowers, survival of the parasite on flowers until a new individual visits the flower, and acquisition of the parasite by the new individual (McArt et al. 2014, Figuerola et al. 2019). However, most studies only focus on one of the transmission steps when comparing plant species or traits (Durrer and Schmid-Hempel 1994, Graystock et al. 2015, Bodden et al. 2019), even though a given floral trait could have different or even opposing effects on each step of the transmission process. For example, composite flowers with large disk flowers could collect more bee feces (Bodden et al. 2019), but at the same time, UV radiation on these types of flowers could reduce parasite survival over time (Schmid-Hempel et al. 1999, Figuerola et al. 2019). Additionally, studies commonly evaluate only a small number of plant species for one or more steps in the transmission process (Durrer and Schmid-Hempel 1994, Graystock et al. 2015, Alger et al. 2019, Figuerola et al. 2019) or evaluate a number of species

but for only one transmission step (Adler et al. 2018), making it difficult to determine what species or floral traits are facilitating overall transmission and why. Due to those limitations, we need to increase the range of traits and the number of species tested for each step of the transmission process to determine the role of floral traits and species identity on parasite transmission dynamics and the mechanisms involved.

The goals of this study were to quantify the deposition, survival, acquisition, and overall transmission of a bee parasite on flowers, to assess the degree to which floral traits vs species identity better explained each parasite transmission step and overall transmission, and to identify plant species and/or floral traits that were most strongly associated with parasite transmission. We focused on the Trypanosomatid gut parasite *Crithidia bombi*, the host bumble bee *Bombus impatiens*, and 16 plant species commonly visited by bumble bees. This work builds on recent exemplar studies (Adler et al. 2018, Figueroa et al. 2019) by focusing on more floral species and across multiple transmission steps to understand the role of plant species identity and floral traits on transmission dynamics. Specifically, we asked whether species-based or trait-based models were better predictors of: i) the frequency of feces deposition on flowers (Experiment 1), ii) survival of *C. bombi* on flowers (Experiment 2), and iii) acquisition of *C. bombi* on flowers and subsequent intensity of infection of its host (Experiment 3). Because these transmission steps are multiplicative, we also used them to assess how floral traits affected overall parasite transmission. We predicted that both species and trait-based models would provide a reasonable fit to the data, but that trait-based models would be better at predicting each of the transmission steps, as trait-based models have proven to have more predictive power in previous studies, because they required fewer parameters to fit the data (Cronin et al. 2010, Adler et al. 2018,

Rowe et al. 2020). Identifying traits that facilitate transmission of bee parasites could allow us to select flower mixes that present a low risk of transmission to be used in pollinator habitat.

## Methods

### *Study system*

*Crithidia bombi* is a common intestinal parasite of bumble bees (Cordes et al. 2012). *Crithidia bombi* reproduces in the hindgut lumen of bumble bees, and new cells are released to the environment in feces 5–10 d after parasite ingestion (Schmid-Hempel and Schmid-Hempel 1993). The parasite is horizontally transmitted when individuals ingest contaminated material either on flowers or through contact with infected nest mates (Durrer and Schmid-Hempel 1994, Imhoof and Schmid-Hempel 1998, Smith 2012, Graystock et al. 2015). When infected with *C. bombi*, bumble bee colonies produce fewer workers, as well as fewer new queens at the end of the colony life cycle (Brown et al. 2003). Additionally, infected overwintering queens are less likely to successfully start a nest in the spring (Schmid-Hempel 2001, Brown et al. 2003). Although the *C. bombi* – bumble bee system has been used for many decades as a model system to study parasite-host interactions in insects (Schmid-Hempel and Schmid-Hempel 1993, Schmid-Hempel 2001, Baer and Schmid-Hempel 2003), only in the last few years have we begun to explore the role that flowers play in the transmission of this parasite (Durrer and Schmid-Hempel 1994, Adler et al. 2018, Graystock et al. 2020, Piot et al. 2020).

As a focal bumble bee species, we used the common eastern bumble bee, *Bombus impatiens* (Apidae). *Bombus impatiens* is a native eusocial bee species to eastern North America, is commercially reared for agricultural pollination, and is economically one of the most important pollinators identified in a worldwide meta-analysis (Kleijn et al. 2015). As a generalist

forager, *B. impatiens* visits a variety of plant species that span a diversity of floral traits. In this study, we used 16 plant species in total across eight plant families, but we could not test every plant species in every experiment, as availability was variable (Table 1). We chose plant species that were attractive to *Bombus* and that were locally available, and that had a wide range of variation in floral traits. We covered flowers with mesh bags before the flowers opened to ensure that no bees had deposited any parasites on the flowers.

### *Study site*

Experiments were carried out at the Honey Bee Lab (HBL) at the Lake Wheeler Road Field Laboratory of North Carolina State University (NCSU) (Raleigh, NC, USA; GPS coordinates: 35°43'23.5"N, 78°40'25.2"W). Source colonies and bees for the transmission experiments were kept at David Clark Labs (DCL) on NCSU North Campus. When transporting bees and inoculum from DCL to the HBL and back, we kept them chilled in a cooler with icepacks. Cages for all experiments were 60 x 60 x 60 cm with white mesh (680 µm aperture; MegaView Science Co, Taiwan). When running experiments, cages with bees were kept in the shade as much as possible. We kept plants outdoors at the HBL when not in use in experiments.

### *Floral traits*

For each plant species, we estimated floral size and shape and the number of reproductive structures (number of open flowers and flower buds) per inflorescence. We counted the number of reproductive structures on 20 inflorescences per species at peak bloom, each inflorescence coming from a different plant. We measured corolla length and width (Appendix B) using digital calipers to the nearest 0.01 mm on 20 flowers per species, with not more than five flowers

coming from the same individual plant. We then used corolla length and width in a principal component analysis using all plant species (Adler et al 2018) to generate a first component that reflected floral size (PC1:  $0.88 \times \text{corolla length} + 0.46 \times \text{corolla width}$ , accounting for 77% of total variance) and a second component that reflected floral shape (PC2:  $-0.46 \times \text{corolla length} + 0.88 \times \text{corolla width}$ , accounting for 22% of total variance). PC1 was positively correlated with corolla length and width, while PC2 was negatively correlated with corolla width and positively correlated with corolla length (Table S1.1). We present means, standard deviation and sample sizes for all predictor floral traits in Table S1.2.

### *Colony maintenance*

We used *B. impatiens* commercial colonies from Koppert Biological Systems (Howell, MI, USA), keeping them in a dark room at approx. 27 °C and 50% RH, and provided sugar-water (30% sucrose) and honey bee collected pollen *ad libitum*. Upon arrival, we screened colonies for *C. bombi* infection by taking a random sample of four workers per colony, collecting fecal samples, and examining them under a compound microscope at 400x for *C. bombi* cells. We always maintained 2-3 colonies infected with *C. bombi* isolated from *B. impatiens* collected in Raleigh, NC (GPS coordinates: 35°48'26.6"N 78°41'58.6"W), and used them to prepare inoculum for the survival and acquisition experiments, and as a source of infected bees for the deposition experiment. We also kept 2-3 uninfected colonies as sources of bees for the acquisition experiment.

### *Crithidia bombi* inoculum preparation and estimating infection intensity

*Crithidia bombi* inoculum was prepared fresh every day we ran a trial. We prepared inoculum according to a standard protocol (Richardson et al. 2015). Briefly, we dissected the guts of 5-10 workers from a source colony and homogenized each intestine in 300 µl of dH<sub>2</sub>O. We let the samples settle for 3-4 h to allow the *C. bombi* cells to swim up into the supernatant and the gut debris to sink to the bottom of the tube. We took a 10 µl sample from each tube and estimated the number of *C. bombi* cells per microliter in a Neubauer chamber with a compound microscope at 400x magnification. We took 200 µl of clean supernatant from the samples with the higher *C. bombi* concentration and mixed them together. Then we determined the new concentration of *C. bombi* cells, and if needed, we used dH<sub>2</sub>O to dilute the inoculum to 1200 cells/µl.

To estimate the intensity of infection of experimental bees, we dissected the guts of individual bees and prepared them using the same protocol as for the inoculum preparation. We also collected the right forewing from each bee we dissected and measured the length of the radial cell as an estimate of bee size (Müller et al. 1996), using the software ImageJ (V 1.8).

### *Experiment 1: Deposition of feces on flowers*

We tested how frequently the feces of bumble bees infected with *C. bombi* fell on the flowers of seven plant species (Table 1). This experiment was carried out between June – October 2019 at the HBL. We fed bees with sucrose that contained a non-toxic fluorescent dye (Aurora Pink dye, product number ECO11, DayGlo Color Corp., Cleveland, OH, USA), which allowed us to find the feces droplets on the plants using a black light. The dyed sucrose was prepared by adding 0.25 g of the dye to 250 ml of 30% sucrose and mixing it until the dye was

completely dissolved (as in Figueroa et al. 2019). At least 24 h before each trial, we pulled workers from *C. bombi* infected colonies and transferred them to a container where we fed them *ad libitum* with the dyed sucrose. On the day of the trial, we transported bees to the HBL, and in each cage we placed 1-4 blooming plants of a particular focal species. We recorded the number of plants in the cage, number of blooming stalks, number of open flowers, and the total area that plants covered inside the cage. We placed 5-7 bees per cage, and allowed them to forage on the plants for 3 h. Halfway through the trials at 1.5 h, we checked each cage to make sure bees were foraging on the flowers and counted the number of visits to flowers in a 5 min period. If bees were not foraging, we put flowers in front of flying or stationary bees to encourage foraging. We noted the start and end time of each trial, and the experimental colony bees came from. When the trial was over, we collected bees from the cages, put them back in their containers, and noted if any of the bees died during the trial. We then took the cages with the plants to a dark room, being careful not to significantly disturb the plants. In a dark room, we used a black light (Ustellar 100 LED, 395nm) to find feces droplets. We counted how many flowers per cage had feces droplets on them, the total number of droplets inside and outside corollas, and on the calices, leaves and the floor of the cage. We defined droplets inside the corolla broadly, including droplets on the flower head of asters, inside the flower for snapdragon (*Antirrhinum majus*), and inside the corolla tube of tubular/closed flowers. Droplets outside the corolla included droplets on the ligules of asters and outside the corolla of all other flowers. After each trial, we thoroughly cleaned the cage with 70% ethanol and removed all plant parts that had feces on them.

We brought experimental bees back to the DCL after the cage experiment was over and provided them with sucrose *ad libitum*. The day following each cage trial, we dissected all bees and determined whether they were infected with *C. bombi* and the intensity of infection (cell/ $\mu$ l).

We only used data from trials where more than 50% of bees in the cage were infected, and three or more bees were alive at the end of the trial. After excluding these trials, we had 25-32 cage replicates per plant species, except for *A. majus*, for which we had 16 replicates (Table 1).

Statistical analyses: We performed all data analyses in R (v. 4.02) (R Core Team, 2018). Our analyses assessed whether the number of fecal droplets on several flower parts and the number of flowers with droplets was predicted by plant species identity and by floral traits, and which one was a better predictor.

Species-based models: To test whether plant species identity predicted the number of feces droplets on flowers and on different flower parts, we constructed generalized linear mixed models (GLMM) with negative binomial distribution using the package glmmTMB (Skaug et al. 2018). We ran separate models for five biologically meaningful response variables that could affect subsequent transmission probability: the total number of droplets on flowers, total number of droplets inside the corolla, total number of droplets outside the corolla, total number droplets on the calyx, and the number of flowers per cage that had droplets on them. The full model included the total number of droplets on the location of interest as a response variable, and as fixed effects plant species, time of day the trial started, initial number of bees per cage, number of alive bees at the end of each trial, average bee size, average intensity of infection of experimental bees, bee activity (number of flower visits in 5 min), and plant area. We included colony of origin of bumble bees as a random effect, and to account for the variable number of flowers per cage, we included it as an offset term in the models. We evaluated the significance of terms with a likelihood ratio chi-squared test, implemented via the ‘drop1’ function in R. We removed terms that were not significant ( $P > 0.05$ ) and compared the fit of the full and the reduced models using AIC values. We used Tukey’s HSD tests for post-hoc, pairwise

comparisons using the lsmeans package (Lenth 2018). To check model assumptions, we used QQ plots of residuals and residual vs. predicted plots using the package DHARMA (Hartig and Lohse 2020).

**Trait-based models:** For models using floral traits rather than species identity to predict the number of feces droplets on flowers, floral parts and the number of flowers with feces, we again used GLMM. As with the species-based models, we constructed separate sets of models for each floral part of interest. The full model had the same structure as the species-based models, but instead of including species identity as a fix effect, we included the floral size (PC1), floral shape (PC2) and reproductive structures per stalk (hereafter floral traits). We selected the best-fit model and conducted post hoc analysis in the same way as for the species-based models.

**Comparing species vs. traits models:** Here and in Experiment 2 and 3, we used AIC to compare whether the species or trait-based models provided the best predictive insight into response variables, selecting the model with the lowest AIC value by 2 units.

### *Experiment 2: Survival of C. bombi on flowers*

We measured the survival of *C. bombi* on flowers of 14 plant species (Table 1). This experiment was carried out between June – October 2018 at the HBL. We assessed *C. bombi* survival after 30 min, 1 and 3 h of being placed on flowers, and for most species we also tested differences in survival between two locations on the flowers (Table 1, Appendix B). Inside cages, we placed four (if testing one flower part) or eight (if testing two flower parts) flower stalks in florist water tubes to keep flowers fresh. On each flower, we placed one 10 µl drop of inoculum, made with no sucrose, using a micropipette. We used 10 µl because it is within the natural range for a single *B. impatiens* fecal event ( $7 \pm 5$  µl, mean  $\pm$  SD) (W.H. Ng, unpublished

data), and we did not add sucrose because *B. impatiens* feces typically does not contain sugar (Figuerola et al. 2019). When testing one flower part (e.g., petal), each flower received one drop of inoculum at the location of interest. When testing two flower parts (e.g., ligules and flower head), four flowers (each on separate stalks) received the inoculum at one location, and another four flowers (again each on separate stalks) received the inoculum at the second location. At each time interval (0 min, 30 min, 1 and 3 h), we collected one drop of inoculum per location using 10  $\mu$ l microcapillary tubes, and measured the height of the liquid column to later estimate the volume of the recovered droplet. Care was taken to remove the entire liquid droplet from the specified location. If the inoculum droplet had evaporated, we placed 10  $\mu$ l of distilled water on the same place the inoculum was, to try to recover any remaining *C. bombi* cells. We placed the recovered droplet on a hemacytometer and estimated the number of alive *C. bombi* per  $\mu$ l in the inoculum. We considered cells alive if they were ‘swimming’ by flagellum movement (Figuerola et al. 2019). We calculated the total number of alive *C. bombi* cells per droplet as cells/ $\mu$ l \* volume of the droplet. Each time we collected a droplet from flowers, we measured temperature and relative humidity (RH) (AcuRite Digital Humidity & Temperature Monitor) and cloud cover (by eye, in 25% intervals). We conducted 16-22 trials per plant species (Table 1).

Statistical analyses: We conducted hazard ratio analyses using Cox proportional hazard models via the Survival package (Terry 2020) in R. This analysis evaluated *C. bombi* survival (whether there were any alive cells in the droplet) by time elapsed when the flower was inspected. In some cases, it was not possible to estimate mean survival time due to right censoring.

Species-based models: These Cox models included plant species and time elapsed between inoculum preparation to the beginning of the trial as fixed effects. We did not include

weather variables in the final models because they were correlated with the date of the trials, and the effect was confounded with plant species, as we tested different plant species on different days and weeks based on their natural phenology. To determine the significance of the terms in the models, we conducted a likelihood ratio test comparing the full model with a model that excluded plant species as an explanatory variable. Differences in survival across plant species were determined post hoc with Tukey's HSD tests using the `lsmeans` package.

**Trait-based models:** These models had the same structure as the species-based models, but instead of species identity, they included floral traits as fix effects. To determine significance of each floral trait, we conducted a likelihood ratio test comparing the full model with reduce models where we removed one of the floral traits at a time.

**Effect of droplet location:** As an additional analysis, we tested the effect of droplet location on the survival of *C. bombi* separately for each plant species, because for some species we only tested one location due to the small size of the flowers. We did not include *Plectranthus* sp. for this analysis due to high censoring (80%) for this species. For the rest of species for which we measured survival at two locations on the flower (Table 1), we ran a proportional hazard model testing *C. bombi* survival by time elapsed when the flower was inspected. The model included location of the droplet as a fix effect. As for the species-based and trait-based models, we determined the significance of the fixed effect (droplet location) by conducting a likelihood ratio test comparing the full model with a model that excluded droplet location as an explanatory variable.

### *Experiment 3: Acquisition of C. bombi from flowers*

We evaluated the probability of acquisition of *C. bombi* on flowers after a single visit on five plant species (Table 1), with trials carried out between June – October 2019 at the HBL. We prepared fresh inoculum each day, and to ensure it was infective, we inoculated a control group of 10-12 bees from the same uninfected colony where we took bees from for the trials (inoculations as in Richardson et al. 2015). Given that we were trying to simulate transmission via a fecal-oral route, we did not add sucrose to the inoculum used in transmission trials, as there is no detectable sugar in bumble bee feces (Figueroa et al. 2019). This methodology departs from Adler et al. (2018) which did add sugar to inoculum prior to putting it on flowers. However, for control bees used to determine inoculum infectivity, we prepared the inoculum with 25% sucrose to encourage consumption. Inoculum for both controls and trials had 1200 cells/ $\mu$ l. For the control bees, we starved them for 4 hours and inoculated them with 10  $\mu$ l of inoculum (12,000 cells/bee). We made sure bees drank the entire inoculum droplet and placed them in individual containers (12 x 7 x 5 cm) with sucrose and pollen *ad libitum*. Seven days after inoculation, we dissected the guts of individual bees and estimated the number of *C. bombi* cells per  $\mu$ l using the same methods as for inoculum preparation.

For acquisition trials, we placed 4-6 uninfected bees per cage and allowed them to acclimate to the cage for 5-10 min. Then, we placed one inflorescence in the cage. In the case of Asteraceae, it was one flower head per cage. In the case of all other plants, it was one inflorescence with multiple flowers. Then we applied 10  $\mu$ l of inoculum (1,200 cells/ $\mu$ l and 0% sucrose) to the flowers in the form of 3-4 small droplets. For inflorescences with multiple flowers, the inoculum droplets were applied to 3-4 flowers, while for asters the inoculum droplets were spread across the flower. We placed the droplets simulating where we most often

observed feces falling in the deposition experiment, or where we considered feces would likely fall when a bee was visiting a flower of that species. We never placed the inoculum on the nectaries or inside the corolla because we typically did not find feces in these locations in the deposition trials (see Results). If bees did not visit flowers after 2 min, we “presented” the stalks to bees by raising them in front of the bees to encourage foraging. When a bee started foraging, we allowed it to visit the flowers and captured it in a vial when the bout was over. If the bout lasted more than 10 min or if the bee stopped probing flowers but was still on the stalk, we considered the bout over and captured the bee. Each bee captured was considered a replicate, and we used fresh stalks for each new trial. For each bee captured, we recorded the number of flowers per stalk (in the case of asters it was considered one), length of the bout (in sec), and elapsed time between inoculum preparation and trial (in min).

We returned bees to the lab and placed them in individual containers with sucrose and pollen *ad libitum*. Seven days after each trial, we dissected out the guts of individual bees and estimated the number of *C. bombi* cells per  $\mu\text{l}$ . We only used data from days when more than 50% of the control bees were infected with *C. bombi*. After removing bees from days that did not meet this criterion, we had between 33-82 replicate bees per plant species (Table 1).

Statistical analyses: We analyzed ‘incidence’ (presence/absence of *C. bombi* infection) and ‘intensity’ (*C. bombi* counts of infected bees) as separate components of *C. bombi* acquisition. We used GLMM to analyze both components and to explore the effect of plant species identity and floral traits on *C. bombi* transmission.

Incidence analysis: We modelled pathogen incidence using logistic regression with the package glmmTMB. The response variable was the binary outcome of whether a bee got infected or not. For the species-based models, we included as fixed effects plant species, length of the

bout, time elapsed since inoculum preparation, number of flowers on the stalk used in the trial and bee size. We tested experimental bees' source colony and *C. bombi* source colony as potential random effects using the “gam” function of the mgcv package (Wood 2020), as it reports statistical significance ( $P$  value) for the random effects (Adler et al. 2018). Neither of them had a significant effect, and therefore we did not include them in the full model. To determine the significance of the fixed effects, we conducted a likelihood ratio test comparing the full model with a model that excluded each of the fixed effects as an explanatory variable. We removed terms that were not significant and selected the model with the lowest AIC value. We evaluated model assumptions by generating QQ plots of residual vs. predicted with the DHARMA package. For trait-based models, we used the same approach, but instead of using plant species as a fixed effect in the models, we included floral traits.

Intensity analysis: We modelled intensity of *C. bombi* infection using only data from bees that were infected. We used the cell count per 0.02  $\mu$ l gut sample as the response variable. We followed the same approach as with the incidence models. First, we tested potential random effects with linear mixed regression models using the “gam” function. Then, we used GLMM with a truncated negative binomial distribution to account for the lack of zeros in the dataset, using the package glmmTMB. For species-based models, we included plant species, length of the bout, time elapsed since inoculum preparation, number of flowers on the stalk used in the trial, and bee size as fixed effects. For trait-based models, we included the same fixed effect structure, but instead of plant species, we included floral traits. We determined the significance of the fixed effects, checked for model assumptions, and compared species-based and trait-based models as in the incidence analyses.

### *Combining results to assess overall transmission*

In a simple bipartite fecal-oral transmission model, the contribution of an individual plant to the basic reproduction number ( $R_0$ ) is estimated to be the total number of fecal droplets shed on its flowers by an infected bee over its infectious lifetime, multiplied by the number of susceptible bees one such fecal droplet can infect before losing infectivity. The second factor can be further decomposed into the rate of infection by one droplet, multiplied by the lifetime of the droplet. This suggests that the results of the three experiments on deposition, survival, and acquisition can be combined multiplicatively to provide a relative comparison of how much different plants or traits might contribute to overall transmission: the relative number of droplets per infected bee from the deposition experiment, the relative droplet lifetime from the survival experiment, and the relative infection rates per droplet from the acquisition experiment.

Because the same plant species were not used across all three experiments, we compared overall transmission based on floral traits, i.e. we assessed how the contribution to overall transmission varied within the flower trait space. First, to avoid overextrapolation, we restricted each trait to the intersection between the ranges of values used in the three experiments. Next, for every set of trait values, we generated predictions of the relevant variables from the best trait-based models of the three experiments, and then multiplied the results together (see Appendix C for details on how the Cox proportional hazards were used to assess relative droplet lifetimes). Finally, we rescaled the overall transmission values so that the maximum value within the trait space was 1. We did not utilize information about the intensity of infection from the acquisition experiments. This is because while bees with higher parasite loads might shed fecal droplets with more parasite cells, and while droplets with more cells are expected to have higher infectivity, we did not know exactly how level of infection and droplet infectivity were related.

## Results

### *Experiment 1: Deposition of feces on flowers*

Across all plant species, most of the fecal droplets we observed were on the floor of the cage (mean percentage range across all species and trials: 63-90% of droplets on the cage floor) (Fig. S1.1A), followed by plant leaves (mean range: 1.4-19.9% on leaves) (Fig. S1.1B), and only a small percentage of the droplets were on flowers (mean range: 1.9-16.6% on flowers) (Fig. S1.1C).

Species-based models: For all variables (number of droplets on flowers, number of droplets inside and outside the corolla and on the calix, and the number of flowers with droplets), species identity was a significant predictor ( $P < 0.0001$  in all cases; Table 1.2). We observed differences between species (Table S1.3-S1.7), with *Rudbeckia hirta* having the most droplets per flowers (Fig. 1.1A), the most flowers with droplets (Fig. 1.1B) and the most droplets inside the corolla (Fig. S1.2A) relative to all other plant species (Tables S1.3-S1.7). *Rudbeckia hirta* was also the species with the most droplets outside the corolla (Fig. S1.2B) and on the calix (Fig. S1.2C) in most comparisons with other species (Table S1.5, S1.6). Initial number of bees in the cage was a significant predictor in the species-based model for all response variables except number of droplets inside the corolla (Table 1.2). The total number of droplets on flowers and outside the corolla decreased as the number of bees in the cage increased, while the number of droplets on the calix and the number of flowers with droplets increased with the number of bees in the cage.

Trait-based models: Floral shape was a significant predictor for all response variables evaluated (Table 1.2). The number of droplets on flowers, inside and outside the corolla decreased as flowers became longer and narrower. But the number of droplets on the calix and

the number of flowers with droplets showed the opposite pattern (Fig. S1.3). The number of reproductive structures per inflorescence was also a significant predictor for the number of droplets on flowers, number of droplets outside the corolla, and number of flowers with droplets per cage (Table 1.2), with all variables decreasing as the number of flowers per inflorescence increased. Length of trial was a significant predictor for the number of droplets outside the corolla (Table 1.2), with more droplets being deposited as the length of the trial increased. Bee size was a significant predictor of the number of droplets on the calix and the number of flowers with droplets per cage (Table 1.2), with more droplets deposited on the calix and more flowers per cage having droplets as the average size of bees increased. The intensity of the infection of the bees used in the trial was a significant predictor of the number of flowers with droplets (Table 1.2), with less flowers having droplets as the average intensity of infection increased. Initial number of bees in the cage was a significant predictor of the number of droplets on flowers, with less droplets landing on flowers as the initial number of bees increased (Table 1.2).

Comparing species- vs trait-based models: Species-based models were better predictors than trait-based models for the total number of droplets on flowers, on all flower parts (inside corolla, outside corolla, calix) and the number of flowers with droplets per cage ( $\Delta$  AIC > 22; Table 1.2).

#### *Experiment 2: Survival of C. bombi on flowers*

In 69% of trials across all plant species, all *C. bombi* cells became immobile after 3 h. However, for three plant species (*Antirrhinum majus*, *Phlox paniculate* and *Plectranthus* sp.), *C. bombi* survived longer than 3 h in more than 85% of trials (Table S1.8). The estimated mean survival time across all species ranged from 117-180 minutes.

Species-based models: Species identity was a significant predictor of *C. bombi* survival on flowers ( $X^2_{12} = 117.48$ ,  $P < 0.0001$ ) (Fig. S1.4). *Echinacea purpurea* represented the highest hazard ratio, and thus lowest survival of *C. bombi*, which was 34% higher than the reference species, *Agostache foeniculum* (*A. foeniculum* was used as a reference because it had average levels of *C. bombi* survival). Species which represented lower hazard ratios, and therefore higher survival of *C. bombi*, were *A. majus*, *P. paniculate* and *Plectranthus* sp. (80%, 88%, and 90% lower, respectively) ( $P < 0.001$  in all cases from pairwise comparisons; Table S1.9, Fig. 1.2). All other species showed intermediate levels of *C. bombi* survival that did not differ significantly from one another (Table S1.9).

Trait-based models: Floral size and shape were significant predictors of the survival of *C. bombi* on flowers ( $X^2_1 = 21.1$ ,  $P < 0.0001$ ;  $X^2_1 = 144.9$ ,  $P < 0.0001$ , respectively). Larger flowers supported higher parasite survival (or a lower hazard rate; 0.97), whereas an increase in flower shape (smaller corolla width, longer corolla length) was associated with a reduction in parasite survival (higher hazard rate: 1.14).

Comparing species- vs. trait-based models: The species-based model was a better predictor of the survival of *C. bombi* on flowers than the trait-based model ( $\Delta AIC = 43.2$ ).

Location of the droplets on flowers: Location of the inoculum droplet on flowers only had a significant effect for *E. purpurea* (Table S1.10, Fig S1.5). The mean survival time was 1.5-times higher on the flower head than on the ligules (mean survival time  $\pm$  SE:  $146 \pm 8.3$  min (flower head) and  $93 \pm 8.8$  min (ligules);  $X^2_1 = 22.1$ ,  $P < 0.0001$ ).

### *Experiment 3: Acquisition of C. bombi on flowers*

Across all plant species and trials, *C. bombi* acquisition rates ranged between 4-19% (Fig. 1.3). Given this low transmission rate, there were few infected bees that we could use in our analyses of infection intensity (range: 3-7 infected bees per plant species tested).

Species-based models: Species identity was the only factor retained in the final model for incidence, but it was not a significant predictor ( $X^2_4 = 7.83$ ,  $P = 0.098$ ). For the intensity of infection, species identity, length of bout and time elapsed since inoculum preparation were retained in the final model, but again, none of these factors were significant predictors ( $X^2_4 = 8.48$ ,  $P = 0.075$ ;  $X^2_1 = 2.22$ ,  $P = 0.13$ ;  $X^2_1 = 3.10$ ,  $P = 0.078$ , respectively; Fig. S1.6).

Trait-based models: Floral shape was a significant predictor for incidence ( $X^2_1 = 6.27$ ,  $P = 0.012$ ), where the probability of acquisition increased with floral shape (narrower, longer corollas; Fig 1.4). Floral shape was not a significant predictor for the intensity of infection ( $X^2_1 = 2.62$ ,  $P = 0.105$ ), while time elapsed since inoculum preparation was a significant predictor ( $X^2_1 = 3.95$ ,  $P = 0.047$ ), with higher intensity of infections as time elapsed increased.

Comparing species- vs. trait-based models: For incidence of infection, the trait-based model was a better fit ( $\Delta AIC = 8.5$ ), while for the intensity of infection, both species-based and trait-based models produced similar fits ( $\Delta AIC = 0.6$ ). However, the best trait-based required fewer parameters than the best species-based model.

### *Overall effect of floral traits on transmission of C. bombi*

We assessed how floral traits affected the overall transmission potential by combining the best-fit trait-based models from the three experiments multiplicatively. The overall transmission depended on each of the three traits, when marginalized across the other two (Fig. 1.5). While we

found each trait to be statistically significant in at least one of the three models, the effect sizes of floral size and number of reproductive structures per inflorescence were small. In contrast, floral shape had a large effect on transmission potential, with large values (smaller corolla width, longer corolla length) leading to higher transmission (Fig 1.5B). Looking at the individual models, a large value for floral shape reduced the droplet survival lifetime, but this was compensated for by the higher deposition and acquisition rates. The results were similar when we looked at the simultaneous dependence on floral size and shape, evaluated at three different values for the number of structures per inflorescence (Fig. 1.5D-F).

## **Discussion**

One approach commonly used to improve habitat for bee pollinators is wildflower strips on private and public lands or near agricultural settings (Goulson 2009, Hatfield et al. 2012), but in most cases, this approach primarily focuses on maximizing forage for pollinators (Blaauw and Isaacs 2014, Landis 2017). However, flower plantings could turn into transmission hotspots for bee parasites, as increased density of bees foraging in those areas can lead to higher transmission rates (Theodorou et al. 2016, Bailes et al. 2020). Plant community composition is also likely to mediate bee-pathogen transmission dynamics (Adler et al. 2018, Figueroa et al. 2019).

Therefore, understanding the transmission processes of bee parasites on flowers is essential for developing measures to control their spread. In recent years, we have begun to explore the role of floral morphology on the transmission of bee parasites (Reviewed in McArt et al. 2017, Adler et al. 2020). Here, we expand our understanding of the transmission dynamics on flowers of a bumble bee parasite, and we present the first combined analysis of the effect of floral traits on the three main steps of floral parasite transmission. In general, both species-based and trait-based

models explained parasite deposition, survival, and/or acquisition; species-based models provided better fits than trait-based models for parasite deposition and survival, whereas trait-based models performed equally or better for parasite acquisition. Floral shape was a consistent trait associated with parasite deposition, survival, and acquisition, and had a strong overall effect on transmission potential, with longer and narrower flowers having higher transmission.

#### *Deposition of feces on flowers*

Our results for the percentage of total droplets that end up on flowers (1.9-16.6 %) and the average number of droplets per flower (0-1.5 droplets/flower) were congruent with values reported in Figueroa et al. (2019) for cage deposition values on three plant species (25-55 % of droplets falling on flowers and leaves and 0-1.5 droplets/flower). This similarity indicates that at least in cage settings, the chances of feces falling on flowers are consistently low. Although Figueroa et al. (2019) did not find differences between plant species, we found that *R. hirta*, an aster with platform-like flowers, was the species most likely to collect feces droplets. Another study also found that composite flowers, especially those with a large area of disk flowers, were more likely to collect feces than tubular flowers (Bodden et al. 2019).

In the case of floral traits, higher values of floral shape (long and narrow corollas) were associated with a decrease in the percentage of the total droplets that end up on flowers, but a higher proportion of the flowers in the cage having droplets on them. This pattern suggests that as flowers get longer and narrower, the probability of droplets of falling on flowers decreases, and those droplets that do fall on flowers are spread across a larger number of flowers, rather than congregated on a small number of flowers. Thus, a higher density of flowers with this type of morphology could cause a higher proportion of flowers to be contaminated but with a smaller

number of feces droplets. To understand the implications of this patterning of fecal deposition for infection dynamics, information on the minimum parasite dose required for infection and the dose-response curve is warranted.

We also found that an increase in the number of reproductive structures per inflorescence was associated with a decrease in the proportion of total number of droplets that land on flowers, outside the corolla, and the number of flowers with droplets per cage. One possibility is that as the number of flowers per inflorescence increases, changes in the architecture of the inflorescence could influence the likelihood of droplets landing on flowers. Another possibility is that an increase in the number of reproductive structures results in a dilution effect, spreading the risk of flowers acquiring feces across more floral units. In a similar vein, a field study found that parasite prevalence on flowers declined when floral abundance was higher (Graystock et al. 2020). These results imply that increased numbers of reproductive structures on a per plant or per site basis could reduce the likelihood of a host coming into contact with a feces-contaminated flower.

#### *Parasite survival on flowers*

Earlier studies indicated that *C. bombi* is susceptible to desiccation and exposure to UV light, showing reduced survival and transmission as the time the parasite spent on the flower increased (Schmid-Hempel et al. 1999, Figueroa et al. 2019). We found that in most trials, *C. bombi* cells died after 3 h on the flower, in many cases we presume due to the evaporation of the simulated fecal droplet the parasite was in (Fig. S1.7). These results also match the maximum survival times reported by Figueroa et al. (2019) in Massachusetts, USA. However, in three plant species (*A. majus*, *P. paniculate* and *Plectranthus* sp.), we found that in only 20% of trials

did all *C. bombi* cells die after 3 h. This indicates that the maximum infectivity time of fecal droplets could be much longer than 3 h depending on the plant species and potentially environmental variables such as temperature, relative humidity, and direct sunlight exposure.

We found that *E. purpurea* presented a higher hazard for *C. bombi*, while *A. majus*, *P. paniculata* and *Plenctactus* represented a lower hazard. Although lower survival on *E. purpurea* could be due to high exposure to UV light due to the disk-shape of the flowers, other asters with disk-shaped flowers showed higher survival for *C. bombi*, suggesting that these results could be due to other traits not accounted for in this study. We did not find differences in the survival of *C. bombi* when placed inside vs. outside the corolla for most species; this result was surprising as we assumed that the parasite would survive longer when placed inside the corolla due to shielding from environmental conditions. Moreover, Figueroa et al. (2019) found increased survival inside of the corolla and bracts compared to outside for three species. Both floral size and shape were also significant predictors of *C. bombi* survival on flowers, but floral shape had a larger effect on *C. bombi* survival, with longer and narrower corollas reducing survival on flowers. It is possible than when feces fall on a narrow corolla, it could eventually fall off the flower when there are disturbances caused by wind or other mechanical stimulus, due to the small surface area of this type of flowers. This is something that we observed when placing inoculum on flowers for the survival and acquisitions experiments, and is probably facilitated by the epicuticular wax layer that covers the corolla of many flowers (Heredia and Dominguez 2009).

## Parasite acquisition on flowers

The acquisition of *C. bombi* on flowers has been studied in a diverse set of plant species. Reports of acquisition rates on flowers are highly variable, ranging from 20-40% on *Rubus caesius* and *Echium vulgare* (Durrer and Schmid-Hempel 1994), and between 40-80% on a set of 14 plant species (Adler et al. 2018). However, both studies used a sugar solution as a medium for the inoculum placed on flowers. This is unrealistic, as *C. bombi* is rarely found in the nectar of wild plants (Cisarovsky and Schmid-Hempel 2014), and bumble bee feces contains little to no sugar (Figuerola et al. 2019). It also can inflate acquisition rates as the presence of sugar can encourage consumption of inoculum. In our experiment, we did not add sugar to the inoculum, finding that *C. bombi* acquisition rates were substantially lower, between 4-19 %, which we suspect is closer to the natural acquisition rate. We note, however, that although prior studies likely report unnaturally high parasite acquisition rates, they may still provide comparative insight into which species result in higher transmission risk. For example, plant species that supported the highest parasite acquisition in Adler et al. (2018) also were those that supported higher (micro)colony parasite infection over a two-week period (Adler et al. 2020).

We found that larger values of floral shape (narrower and longer) were associated with higher acquisition of *C. bombi*. It is possible that due to the narrow corolla, a bee would be more likely to have contact with a fecal droplet than in a wide corolla, or that this type of morphology could encourage a behavior or body positioning on the flower that would increase the chances of coming into contact with the parasite. For example, in flowers like *Chelone glabra* and *Gentiana andrewsii*, the entrance to access the nectaries is blocked by overlapping petals, so bumble bees have to position themselves in a way that allows them to pull apart the petals and insert their tongues into the tubular corolla (Lavery 1994). In contrast, Adler et al. (2018) did not find floral

size or shape to be significant predictors of *C. bombi* acquisition on flowers. This discrepancy of results could be due to the high acquisition rates that Adler et al. (2018) reports, which could have obscured any effect of floral morphology on the acquisition rate. The number of flowers per inflorescence and the arrangement of the flowers on the inflorescence have also been reported to influence parasite acquisition rates (Durrer and Schmid-Hempel 1994, Adler et al. 2018). However, we did not find the number of flowers per inflorescence to be a significant predictor of acquisition of *C. bombi*. This seems to indicate that there are a variety of floral traits that are affecting acquisition of *C. bombi* and the likelihood of detecting patterns may be a function of where in trait space the species reside, interactions among traits, as well as experimental conditions.

### *Overall transmission potential*

To the best of our knowledge, this is the first analysis that combines the three steps of parasite transmission on flowers to assess the overall effect of floral traits on parasite transmission. We found that floral shape had the largest effect on transmission potential, with narrow and long corollas leading to higher transmission. In the analysis of each transmission step, increases in floral shape led to a lower survival of *C. bombi* on flowers; however, it also led to a higher number of flowers with feces droplets and a higher acquisition rate. This shows that a single floral trait can affect each transmission step in different ways, and to be able to determine whether a particular floral trait is going to have an overall positive effect on parasite transmission, we need to assess its effect on multiple transmission steps and not only on the acquisition of parasites on flowers. Although our analysis indicates that flowers with long and narrow corollas are more likely to contribute to parasite transmission, our conclusions are limited

to the trait range of the species we tested. Before making general recommendations about what type of flowers could be used to slowdown parasite spread in managed landscapes, we need to assess a wider span of floral traits, to confirm that the pattern we observed is consistent as we increase species and trait variation.

### *Comparing species-based vs. trait-based models*

We found that species-based models provided a better fit for the deposition of feces on flowers and survival of *C. bombi* on flowers, while trait-based models provided a better fit for the acquisition of the parasite. Adler et al. (2018) found that species-based and trait-based models had similar predictive power for parasite acquisition, but trait-based models were preferred based on parsimony given that those models required fewer parameters in their study. Trait-based models have several advantages over species-based models. Because they consider within-species variation, results can be generalized across communities due to taxonomic independence, and they can simplify data analysis by reducing the number of parameters needed (Dobson 2004, Truitt et al. 2019). That species-based models were a better fit for two out of three transmission steps in our study could be due to limited trait variation in the plants we tested. Experiments that use a wider range of taxonomic and trait variation could shed light on whether trait- or species-based models better predict transmission of bee parasites on flowers. Moreover, it is also possible that the traits that we measured were not the most relevant traits for each of the transmission steps, or that there are additional traits that should be included to improve the fit of the trait-based models. One indication for this is that some studies report conflicting effects of the same trait. For example, Bodden et al. (2019) report that large disk flowers are more likely to have bee feces than cup and tubular flowers, while Graystock et al.

(2015) found that deposition of *C. bombi* was more likely on a bell-shaped flower than on flat platform-like flower.

It is important to note that our study focused on how species identity and floral traits affected various transmission steps, but did not take into account bee visitation rates to flowers or bee behavior on flowers. There is a rich body of literature documenting how both floral species identity and floral traits affect pollinator attractiveness to flowers and visitation rates (Rollings and Goulson 2019, Dibble et al. 2020). Floral traits such as floral area, flower height, color and scent can influence visitation rates (Gumbert 2000, Rowe et al. 2020). In addition, bee behavior could also interact with floral traits to determine the probability of parasite deposition and acquisition. For example, bees that forage for pollen pick up more microbes from flowers than bees that forage for nectar (Russell et al. 2019), and flowers that receive longer visits by honey bees are more likely to become contaminated with viruses (Alger et al. 2019). Additionally, bumble bees can recognize and avoid flowers that are contaminated with *C. bombi* (Fouks and Lattorff 2011), which would ultimately reduce the risk of transmission on flowers. Models that include traits that influence both transmission as well as visitation and behavior will be important extensions of this research and applying the results to natural or managed communities of bees, parasites, and flowers.

## *Conclusions*

Recent concerns over bee health and the possibility of flower plantings acting as disease-transmission hotspots (Piot et al. 2019) have sparked interest in understanding the role that flowers play in the transmission dynamics of bee parasites. Parasite transmission is a complex process that can be influenced by many factors, including the floral traits affecting several steps

of the transmission process (Adler et al. 2021). We are just beginning to explore these complex relationships, and this study integrates the effects of floral traits on the three main steps of the transmission of parasites via flowers: deposition, survival and acquisition. The scope of our results is limited to the plant species and floral traits that we tested, but our results imply that species identity is a better predictor of deposition of feces and survival of *C. bombi* on flowers, while floral traits better predicted acquisition on flowers. Although several floral traits were significant predictors of some transmission steps, floral shape was the trait that best predicted overall transmission of *C. bombi*, with narrow and long flowers promoting higher transmission of the parasite. Identifying floral traits that promote parasite transmission could help us select flower mixes that reduce the risk of parasite transmission in flower plantings while providing floral resources for bees.

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Table 1.1. The 16 plant species used in this study and the experiments (Deposition, Survival, and Acquisition) that they were used in. In the survival experiment, we also note the location where inoculum droplets were tested. Numbers indicate sample size; in the survival experiment, the sample size indicate the number of replicates for each flower part tested. Blank cells indicate species that were not tested in particular experiments.

Family	Species	Deposition	Survival	Acquisition
Apocynaceae	<i>Asclepia tuberosa</i>		Anthers & petals (42)	
Asteraceae	<i>Coreopsis verticillata</i>	30	Center & petal (38)	33
	<i>Solidago nemoralis</i>		Center (22)	
	<i>Echinacea purpurea</i>		Center & petal (40)	
	<i>Kalimeris integrifolia</i>		Center & petal (44)	
	<i>Rudbeckia hirta</i>	32		37
Lamiaceae	<i>Agastache foeniculum</i>		Lower & upper petal (42)	
	<i>Vitex agnus-castus</i>	25	Lower & upper petal (40)	54
	<i>Plectranthus</i> sp.*		Center & petal (32)	
	<i>Caryopteris clandonensis</i>	29		
Phytolaccaceae	<i>Phytolacca americana</i>		Center (20)	
Plantaginaceae	<i>Angelonia Angustifolia</i>		Center & upper petal (32)	
	<i>Antirrhinum majus</i>	16	Center & petal (34)	
Polemoniaceae	<i>Phlox paniculata</i>		Center & petal (42)	
Rubiaceae	<i>Pentas lanceolata</i>	32		82
Verbenaceae	<i>Lantana camara</i>	29	Center & petal (42)	71

\* Hybrid of *P. saccatus* and *P. hilliardiae*

Table 1.2. Experiment 1, Deposition: Summary of the species-based and trait-based models for the deposition of bumble bee feces on flowers.

Response variable	Random effects	Fixed effects terms left in the model	X2	DF	P	AIC
Species models						
Number of droplets on flowers/cage	-	Species ID	293.31	6	<0.0001	1254.4
		# Bees in the cage	5.89	1	0.0152	
Number of droplets inside the corolla/cage	-	Species ID	66.21	6	<0.0001	627.7
Number of droplets outside the corolla/cage	-	Species ID	121.73	6	<0.0001	878.47
		# Bees in the cage	5.33	1	0.0209	
Number of droplets on the calix/cage	-	Species ID	81.757	6	<0.0001	308.7
		# Bees in the cage	4.2031	1	0.0404	
Number of flowers with droplets in the cage	-	Species ID	357.41	6	<0.0001	1017.1
		# Bees in the cage	5.3065	1	0.0212	
Trait-based models						
Number of droplets on flowers/cage	Colony	Floral shape	48.410	1	<0.0001	1296.8
		Flowers/inflorescence	9.867	1	0.00168	
		# Bees in the cage	5.616	1	0.0177	
Number of droplets inside the corolla/cage	Colony	Floral shape	62.197	1	<0.0001	665.7
Number of droplets outside the corolla/cage	-	Floral shape	9.8337	1	0.00171	1025.8
		Flowers/inflorescence	40.015	1	<0.0001	
		Length of trial	15.249	1	<0.0001	
Number of droplets on the calix/cage	-	Floral shape	102.30	1	<0.0001	331
		Bee size	4.5353	1	0.033	
Number of flowers with droplets in the cage	-	Floral shape	19.52	1	<0.0001	1046.9
		Flowers/inflorescence	20.12	1	<0.0001	
		Bee size	4.35	1	0.0369	
		Intensity of infection	4.22	1	0.0390	

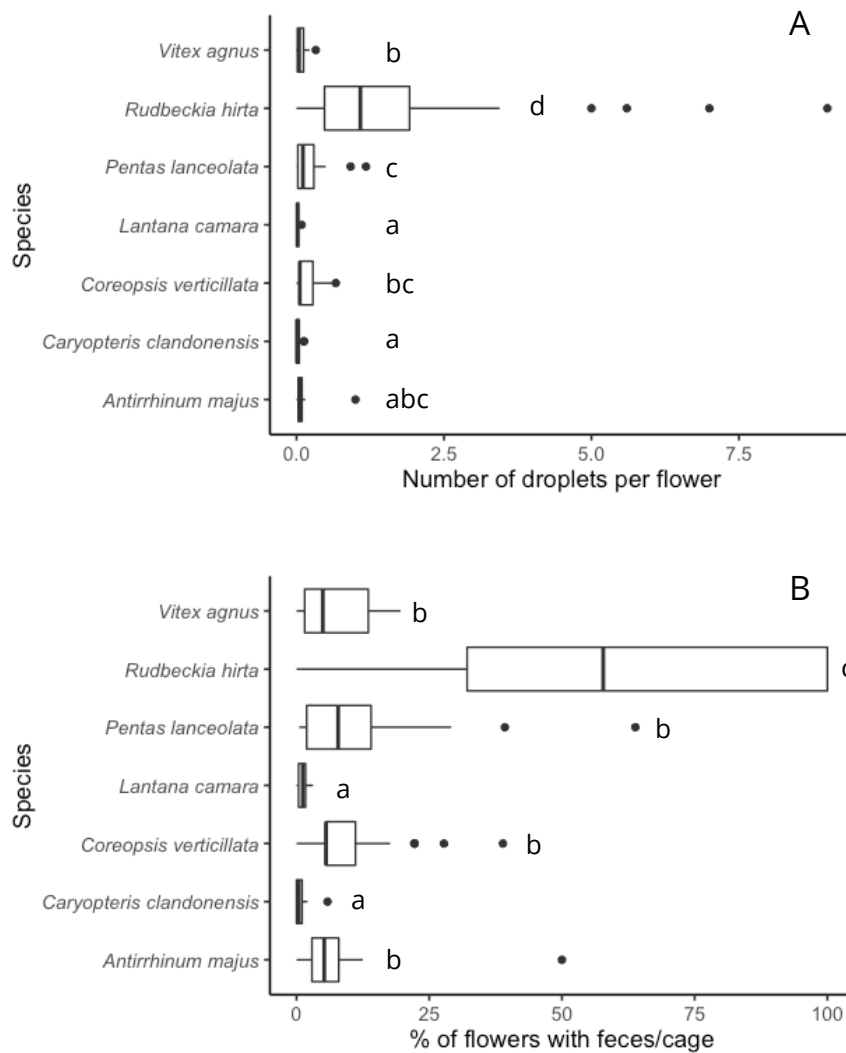


Figure 1.1. Species identity had a significant effect on (A) the number feces droplets received per flower (number of feces droplets on flowers in the cage/total number of flowers in the cage) and (B) the percentage of flowers per cage that received feces droplets. Letters to the left of species names indicate significant differences among species in deposition response variables.

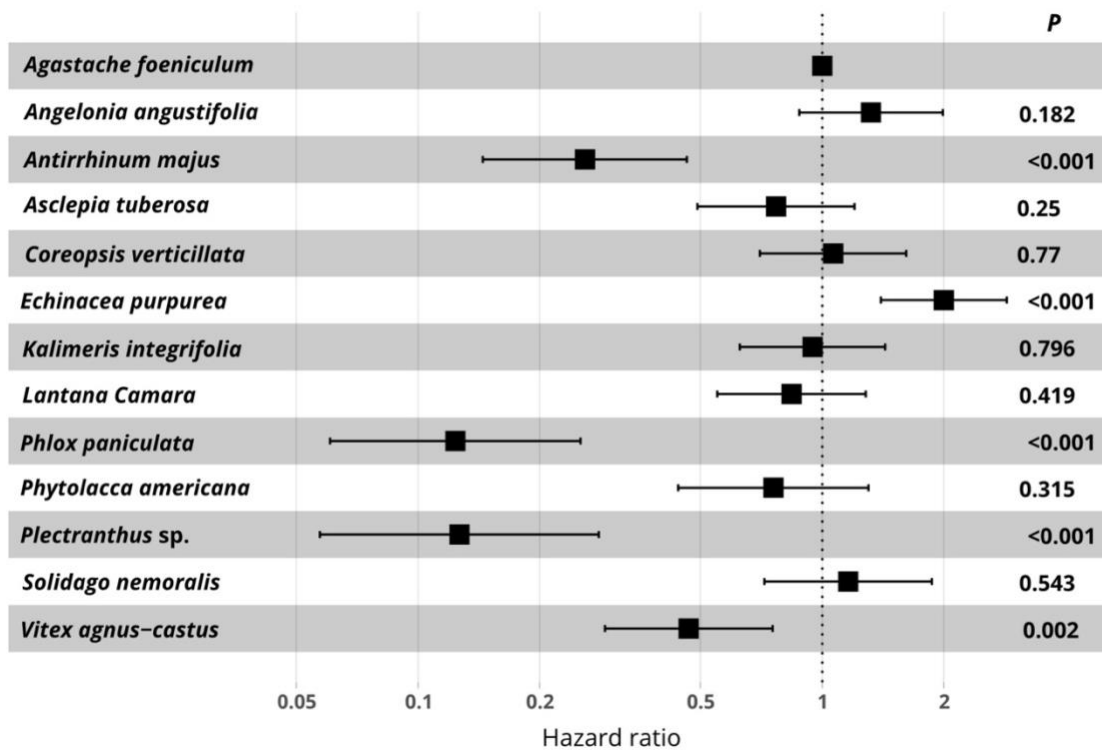


Figure 1.2. Hazard ratio plot of *C. bombi* on different plants, using *Agastache foeniculum* as a reference species. Values at the right of the dotted line represent higher risk for *C. bombi*, and therefore lower survival. Values at the left of the dotted line represent lower hazard for *C. bombi*, and therefore higher survival. *P* values are from comparing the hazard ratio of each plant species to the reference species, *A. foeniculum*.

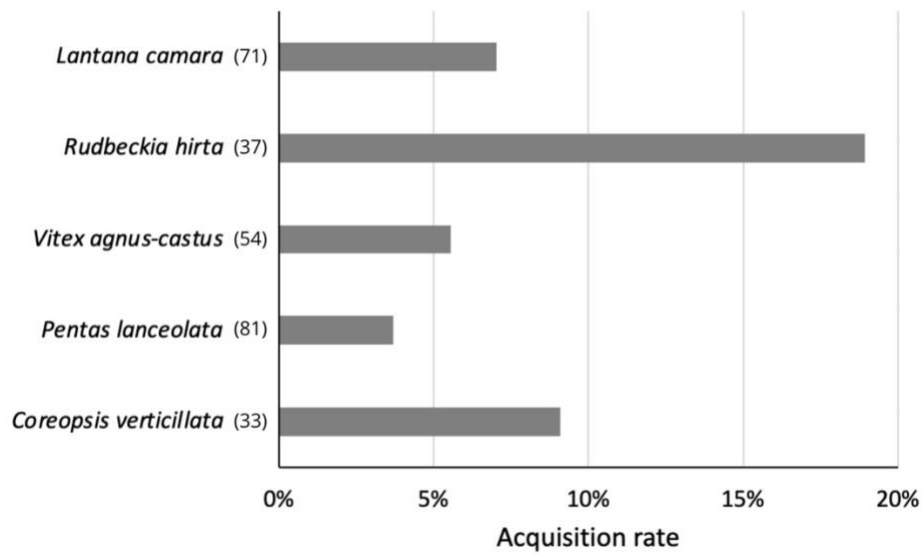


Figure 1.3. Acquisition rate of *C. bombi* on five flower species (Experiment 3). Species identity was not a significant predictor in the species-based model. The number in parenthesis represents the number of individuals tested.

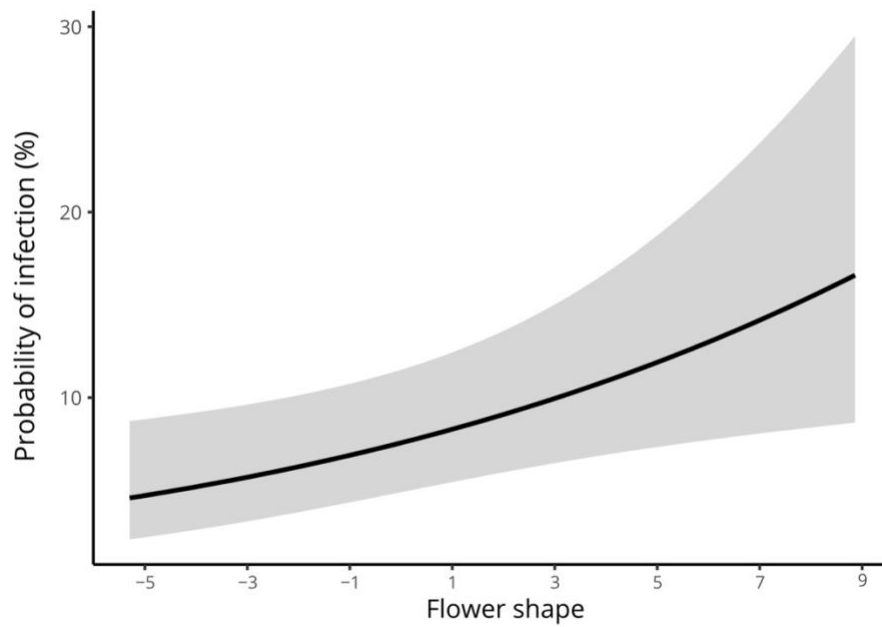


Figure 1.4. Effect of flower shape on the predicted probability of infection of *B. impatiens* when visiting flowers that were inoculated with *C. bombi*. Narrower and longer flowers increase the risk of acquiring the parasite. Flower shape was a significant predictor of acquisition of *C. bombi* in the trait-based model.

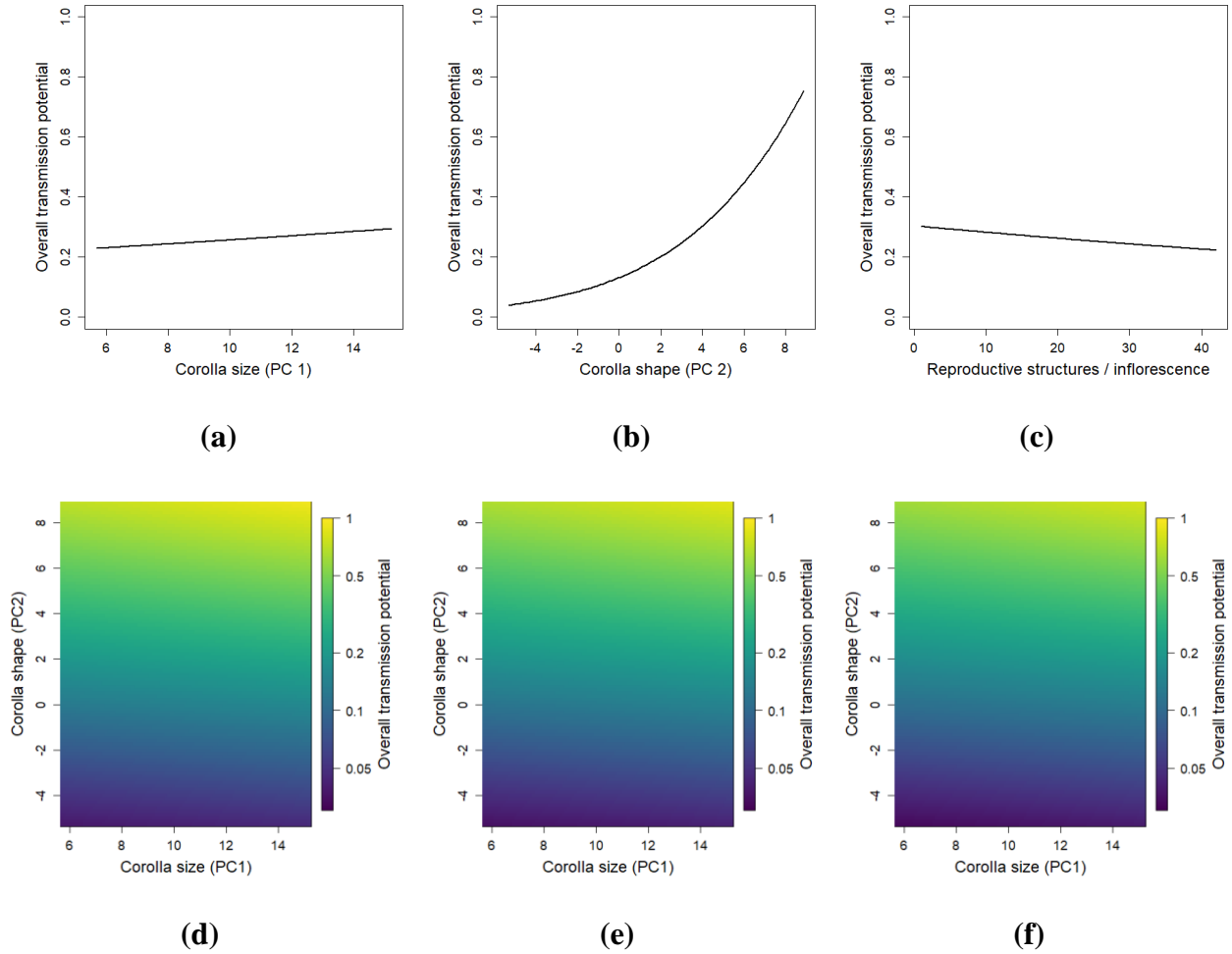


Figure 1.5. Overall transmission potential varies with floral traits. Transmission potential was assessed by combining the best-fit trait-based models from the three experiments multiplicatively, and then rescaled to a maximum of 1 for the range of trait values being considered. (a), (b) and (c) show the dependence on corolla size, shape, and number of reproductive structures per inflorescence respectively, each marginalized across the other two trait values. (d), (e) and (f) show the simultaneous dependence on corolla size and shape, with the number of structures per inflorescence set at the 25%, 50% and 75% quantiles of the range, respectively.

## CHAPTER 2: Within-Colony Transmission of Microsporidian and Trypanosomatid Parasites in Honey Bee and Bumble Bee Colonies

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### Abstract

Parasites are commonly cited as one of the causes of population declines for both managed and wild bees. Epidemiological models sometimes assume that increasing the proportion of infected individuals in a group should increase transmission. However, social insects exhibit behaviors and traits which can dampen the link between parasite pressure and disease spread. Understanding patterns of parasite transmission within colonies of social bees has important implications for how to control diseases within those colonies, and potentially the broader pollinator community. We used bumble bees (*Bombus impatiens* Cresson) (Hymenoptera: Apidae) and western honey bees (*Apis mellifera* L.) (Hymenoptera: Apidae) infected with the gut parasites *Crithidia bombi* (Lipa & Triggiani) (Trypanosomatida: Trypanosomatidae) and *Nosema ceranae* (Fries et al.) (Dissociodihaplophasida: Nosematidae), respectively, to understand how the initial proportion of infected individuals impacts within-colony spread and intensity of infection of the parasites. In bumble bees, we found that higher initial parasite prevalence increased both the final prevalence and intensity of infection of *C. bombi*. In honey bees, higher initial prevalence increased the intensity of infection in individual bees, but not the final prevalence of *N. ceranae*. Measures that reduce the probability of workers bringing parasites back to the nest may have implications for how to control transmission and/or

severity of infection and disease outbreaks, which could also have important consequences for controlling disease spread back into the broader bee community.

## **Introduction**

Parasites and pathogens are considered main drivers of host ecology and evolution (Ebert et al. 2000, Miura et al. 2006). They can have short-term effects on their hosts, including changes in host behavior (Moore 2002, Gegear et al. 2005) and reduced fitness (Brown et al. 2003b, Bonsall 2004), as well as long-term effects, including changes in host population cycles, genetic diversity, and life history (Kirzhner et al. 1999, Brown et al. 2003b, Bonsall 2004). One challenge for the evolution of sociality in animals is that living in close proximity with genetically related individuals increases the transmission probability of parasites and the risk of epidemic outbreaks (Schmid- Hempel 1995, Kurvers et al. 2014). However, many factors influence the outcome of the infection at the group level (Godfrey 2013, Liu et al. 2019). Transmission dynamics within social groups is of special interest for controlling emerging diseases in humans, domestic animals and wildlife populations (Hajek and Shapiro-Ilan 2018), especially ones that perform critical ecosystem services (Potts et al. 2016). Here, we studied how initial within-colony parasite prevalence influences transmission and the outcome of infection at the colony level in two eusocial bee species.

Conventional epidemiological models generally assume that interactions in social groups are random, and therefore, increasing the proportion of infected individuals in a group should expand transmission probability to susceptible hosts by increasing the interactions between infected and uninfected individuals (Anderson and May 1991, Kappeler et al. 2015). However, members within social groups often do not interact randomly, but based on a network structure,

such that the potential of an individual to transmit parasites depends on their location in the social network (Naug and Camazine 2002, Godfrey 2013, Kurvers et al. 2014). For example, the social structuring of students into classes and grades strongly affected transmission of the influenza virus in an elementary school (Cauchemez et al. 2011). Although behaviors of infected and uninfected individuals (such as changes in movement patterns, avoidance of infected individuals, and mate choice) can also affect the probability of parasite spread in social groups (Loehle 1995), understanding how parasite prevalence affects transmission in social groups has important implications for how to control disease spread in social animals and reduce the risk of epidemics.

Social Hymenoptera (ants, social wasps, and social bees) provide unique opportunities to explore the dynamics of pathogen transmission in groups (Schmid-Hempel and Schmid-Hempel 1993, Schmid-Hempel 1999). They exhibit a range of network structures (from high to low compartmentalization; Jandt and Dornhaus 2009, Baracchi and Cini 2014) and individual behaviors that can reduce the spread of parasites within the colony. For example, due to division of labor in honey bee colonies, individuals interact more with nestmates performing the same task, creating compartmentalization in the interaction network (Liu et al. 2019), which is associated with reduced parasite transmission at the colony level (Naug 2008). Other behaviors that may reduce parasite spread within the colony include sanitary behaviors, avoidance of infected individuals, self-medication, and self-exclusion of infected individuals (Cremer et al. 2007, Richardson et al. 2015, Liu et al. 2019). In these cases, transmission between colonies may be necessary to sustain epidemics at the population level (White et al. 1996). Empirical and theoretical studies of directly transmitted parasites show that when the transmission rate of the

parasite is low, an infection can fade out of the host population unless there is constant immigration of infected individuals (Hudson et al. 2002).

We studied within-colony parasite transmission in the eusocial western honey bee (*Apis mellifera* L.) (Hymenoptera: Apidae) and common eastern bumble bee (*Bombus impatiens* Cresson) (Hymenoptera: Apidae), which have high ecological and economic value due to the pollination services they provide for agricultural production (Losey and Vaughn 2006, Garibaldi et al. 2013, Schulp et al. 2014). Parasites that have been linked to the population decline of bees include the honey bee parasite *Nosema ceranae* Fries et al. (Microsporidia), and the bumble bee parasite *Crithidia bombi* Lipa & Triggiani (Trypanosomatida) (VanEngelsdorp and Meixner 2010, Meeus et al. 2011, Bianco et al. 2014). Both are gut parasites, horizontally transmitted when a host ingests parasite cells from flowers where infected bees have defecated (Smith 2012, Graystock et al. 2015). They can also be transmitted within the colony by food exchange with infected individuals, ingestion of contaminated food, or contact with feces (Graystock et al. 2015, Goblirsch 2018). In this context, social immunity (e.g., social network structure and host behaviors) can influence parasite transmission, but whether this is enough to dampen the link between initial parasite pressure and transmission dynamics are unknown. Honey bees have colonies that are several orders of magnitude bigger than bumble bee colonies (>20,000 workers) and have a perennial life cycle (Winston 1987), which means that they could harbor more parasites (Hudson et al. 2002). At the same time, honey bees have a more structured division of labor than bumble bees, which can help control parasite spread in the colony by creating compartmentalization within the interaction network (Winston 1987, Naug and Camazine 2002, Goulson 2010).

We manipulated a key epidemiological parameter, the initial percentage of infected individuals (prevalence), and measured subsequent within-colony transmission rates, severity of infection (parasite loads), and colony performance. We hypothesized that with higher initial prevalence, parasites would spread faster within the colony, suggesting that there is a limit to how much social immunity and other unknown aspects of bee biology can reduce within-colony transmission. We note that by using two different parasites, one each in bumble bees and honey bees, any differences in transmission dynamics observed between the two could be ascribed not only to differences between the two bee species but also the biology of two different parasites. Therefore, we did not make any statistical comparison between bee species in this study, though we do discuss qualitative differences in results between the host–parasite systems. For each bee species, we asked the following questions: 1) How does the probability of an individual being infected vary with the initial prevalence of the parasite and over time? 2) Does initial parasite prevalence affect the intensity of infection per individual bee? And 3) to what degree does initial parasite prevalence affect colony growth rate and final colony productivity? Studying the dynamics of parasite spread within colonies is an important step toward a better understanding of and controlling outbreaks in commercial colonies and spillover to wild populations.

## **Methods**

### *Study System*

The common eastern bumble bee (*B. impatiens*) and western honey bee (*A. mellifera*) are both managed, generalist pollinators (Crane 1984, Mader et al. 2010). *Bombus impatiens* is a native, primitively eusocial species ranging from southern Canada through Florida (Colla et al. 2011). Colonies are annual and founded in the spring by single, mated queens (Goulson 2010).

Because of this annual life cycle, parasites typically overwinter with infected daughter queens. Honey bees are native to Europe, Asia, and Africa. Colonies have a perennial lifecycle that allows parasites to stay within the colony year-round (Winston 1987).

We studied the parasites *Crithidia bombi* (Trypanosomatida) and *Nosema ceranae* (Microsporidia), which commonly infect bumble bees and honey bees, respectively (Cordes et al. 2012, Martín-Hernández et al. 2018), and are ecologically and economically relevant for their host (Brown et al. 2003b, Goblirsch 2018). *Crithidia bombi* usually shows a prevalence of about 30%, but in some areas, as many as 80% of flying bumble bee workers can be infected (Gillespie 2010, Cordes et al. 2012). *Crithidia bombi* reproduces in the gut lumen, and new infective cells are released in feces 5–10 d after inoculation (Schmid-Hempel and Schmid-Hempel 1993, Brown et al. 2003a). *Crithidia bombi* can reduce the number of workers, drones and new queens produced per colony (Brown et al. 2003b), impair learning of foragers (Gegeer et al. 2006), and decrease probability of infected overwintering queens to successfully start a nest in the spring (Schmid-Hempel 2001, Brown et al. 2003b). The parasite is horizontally transmitted within and among colonies when individuals ingest contaminated material either on flowers or through contact with infected nest mates or their nest mates' feces (Durrer and Schmid-Hempel 1994, Imhoof and Schmid-Hempel 1998, Smith 2012, Graystock et al. 2015). Estimates of *C. bombi* transmission rates on artificial and/or natural flowers range from 20 to 80% (Durrer and Schmid-Hempel 1994, Adler et al. 2018), suggesting variation in the number of exposed foragers returning to the colony. Within small experimental colonies (first brood workers), *C. bombi* spreads faster in colonies with dense social networks (more contacts among individuals), and workers have a higher risk of getting infected as the rate of contact with infected individuals

increases (Otterstatter and Thomson 2007). Whether these transmission dynamics hold in mature colonies that are an order of magnitude larger is unknown.

The microsporidian *N. ceranae* is an obligate intracellular parasite (VanEngelsdorp and Meixner 2010, Bianco et al. 2014). Spores infect and replicate inside midgut epithelial cells and are later released to the environment in feces (Higes et al. 2007). *Nosema ceranae* was originally described in the Asian honey bee, *Apis cerana* (Fries et al. 1996) but can also infect *A. mellifera*, and it is now the most prevalent *Nosema* species in honey bees in the United States (Higes et al. 2006, Chen et al. 2008). *Nosema ceranae* can reduce the life span of workers, decrease colony size, and cause sudden collapse of the colony (Malone et al. 1995, Higes et al. 2008). Adult bees come into contact with *N. ceranae* spores when workers rob food from an infected colony or visit contaminated flowers; within-colony transmission occurs when workers exchange food with infected workers or when they clean fecal material (oral-oral and fecal-oral route; Smith 2012, Goblirsch 2018). Larvae and the queen can also become infected when they are fed contaminated food (Rutrecht and Brown 2008, Higes et al. 2009), and an infected queen can transmit the parasite to workers when they start a new hive (Fries and Scott 2001, Higes et al. 2009). Honey bee colonies can survive *N. ceranae* infection for more than a year, but when the prevalence in nurse bees is greater than 40%, it is often followed by a sudden collapse of the colony (Higes et al. 2008). The outcome of *N. ceranae* infections can also be influenced by interactions with other stressors, such as insecticides and other pathogens (Retschnig et al. 2014, Doublet et al. 2015).

## *Within-Colony Transmission of C. bombi in B. impatiens*

### Colony Origin and Maintenance

We used 12 *B. impatiens* commercial colonies from Koppert Biological Systems (Howell, MI). We kept colonies in a dark room at approx. 27°C and 50% RH, and provided sugar-water (30% sucrose) and honey bee collected pollen *ad libitum*. Upon arrival, we screened colonies for *C. bombi* infection by taking a random sample of four workers per colony, collecting fecal samples and examining the samples under a compound microscope at 400×. All 12 colonies were free of *C. bombi*. Because the colonies initially had variable numbers of workers, ranging from 30 to 80, we removed workers from each colony so that each colony had  $30 \pm 5$  workers at the start of the experiment.

*Crithidia bombi* can be transmitted by contact with feces (Shykoff and Schmid-Hempel 1991), and when bees are confined in the colony, feces can build up quickly. To avoid unnatural levels of parasite transmission within the colony due to excessive contact with fecal material, each colony was connected by a plastic tube to an additional container that had cat litter (PetCentral), so bees could use this container as a ‘latrine’, as cat litter absorbed the feces. We replaced the cat litter approx. every other week. The access to the sucrose solution was also in this additional container to encourage bees to forage for nectar outside of the colony box. Pollen was provided directly to the main colony box.

### Inoculum Preparation

To prepare the inoculum, we dissected the intestines of five *B. impatiens* workers from a source colony infected with *C. bombi* isolated from Stone Soup Farm, Hadley, MA (GPS coordinates: 42.363911 N, -72.567747 W). We prepared inoculum according to a standard

protocol (Richardson et al. 2015). Briefly, we homogenized each intestine in 300 µl of dH<sub>2</sub>O. We let the samples settle for 4 h to allow the *C. bombi* cells to swim up into the supernatant and the gut debris to sink to the bottom of the tube. We removed 200 µl of clean supernatant from each tube and mixed them together. To determine the concentration of *C. bombi* cells, we took a 10-µl sample and estimated the number of *C. bombi* cells per microliter in a Neubauer chamber with a compound microscope at 400x magnification. Based on this *C. bombi* cell count, we mixed the solution with 50% sucrose to make an approx. 25% sucrose solution that contained 1,200 *C. bombi* cells per microliter. This solution was used to inoculate bees in the experimental colonies.

#### Experiment Set-up

To assess the effect of initial parasite prevalence on within-colony spread of *C. bombi*, we infected either 1 or 30% of the workers in the colonies (1 and 9 workers, respectively), with the 30% treatment representing a high, but realistic, percentage of workers in the colony foraging (Cartar 1992) and potentially coming into contact with *C. bombi* cells on flowers. Six colonies were randomly assigned to each treatment. On 20 February 2018, we haphazardly removed the desired number of workers from each colony and starved them for 4 h. We then gave each bee 10 µl of the inoculum (12,000 cells per bee), making sure bees drank the entire inoculum droplet. This *C. bombi* dose is within the range that bees are exposed to when foraging in nature (Schmid-Hempel and Schmid-Hempel 1993, Otterstatter and Thomson 2006). We marked inoculated bees with a dot of indelible paint on the dorsal side of the thorax. After the paint was dry, we returned bees to their respective colonies to serve as the source of infection.

## Parasite Sampling

Sampling started on 26 February 2018, 1 wk after we inoculated the colonies, given that it takes ~1 wk for *C. bombi* in *B. impatiens* to develop patent infections (Otterstatter and Thomson 2006). We collected bees from each colony twice per week for 7 wk, when colonies started to produce drones and new daughter queens (13 April 2018). On each sampling day, we haphazardly removed 10% of the workers in each colony (up to 10 workers per colony), avoiding workers marked with the paint dot and callow bees, as they would not have developed infection yet (Otterstatter and Thomson 2006). We dissected the intestines and ground each one in 500  $\mu$ l of dH<sub>2</sub>O. We allowed samples to sit for ~4 h, after which we used a Neubauer chamber to estimate the number of cells per microliter using a 10- $\mu$ l subsample of the supernatant. As bee size can influence the intensity of *C. bombi* infection (Otterstatter and Thomson 2006), we used the length of the radial cell of the right forewing as a proxy for bee size (Müller et al. 1996). We taped the wings to microscope slides and scanned the wings. The length of the radial cell was then measured using the software ImageJ (V 1.8; Schneider et al. 2012).

## Measures of Colony Growth

We recorded the initial and final weight of the colony and calculated the change in weight. Once per week, we also recorded the approximate number of workers, drones, dead workers, and new queens in each colony by visual inspection. When the experiment ended at the end of week 7 (because colonies were starting to produce sexuals), we froze the colonies at –20°C. Once colonies were frozen for at least 48 h, we dissected each colony to record the final number of adult workers, drones, daughter queens, worker pupae, queen pupae, larvae and eggs, as well as the total weight for each of those categories (to the nearest 0.01 g).

## *Within-Colony Transmission of Nosema ceranae in Apis mellifera*

### Colony Origin and Maintenance

Fifteen colonies with replicate honey bee queens were created by grafting from the genetic line MP56 in June 2017. The MP line originated from a local northeastern U.S. beekeeper. Virgin queens were openly mated in three-frame nucleus colonies at the Dyce Lab for Honey Bee Studies, Ithaca, NY, and checked after 4 wk. On 10 July 2017, we screened all 15 colonies for *N. ceranae* by collecting 30 workers from the entrance of each colony. Bees were surface sterilized with 70% ethanol and 0.5% bleach solution. We then dissected and homogenized the intestines of bees from the same hive in 1 ml of phosphate buffered saline (PBS). After vortexing the samples, we took a 10  $\mu$ l subsample, and using a Neubauer chamber with a compound microscope at 400 $\times$  magnification, we estimated the number of spores per bee (Fries et al. 2013). It is important to note that spores of *N. ceranae* are released only after completing the intracellular cycle (3–7 d) (Higes et al. 2007), so our method could identify only sporulating infections. All but two colonies were already infected with *N. ceranae*; therefore, we used the nine colonies with the lowest *Nosema* levels (0–20,500 spores per bee) for the experiment, and the two colonies with the highest levels (58,000–74,000 spores per bee) were used as inoculum sources (Table S2.1). Treatments (control, 1% and 10% of workers inoculated with *N. ceranae*) were blocked by initial infection intensity of the colonies such that mean initial infection intensity was approximately equal for each treatment. Given that the foraging force of a honey bee colony can be 10–20% of the total colony population (Danka et al. 1986, Winston 1987), the 10% treatment represents a realistic percentage of foragers who could come into contact with *N. ceranae* while foraging.

After selecting the experimental colonies, we removed one frame containing workers, brood, food and the queen from each colony and placed it in an insulated five-frame nuc box, to create nine, one-frame colonies containing ~1,000 adult workers. Each colony was placed inside a  $1.8 \times 1.8 \times 3.0$ -m tent that contained a feeder with 30% sucrose and an artificial pollen source filled with BeePro (Mann Lake, Ltd., Hackensack, MN). We allowed colonies to acclimate in the tents for 1 wk before starting the experiment.

### Inoculum Preparation

To prepare the inoculum, we collected 100 workers from the source colonies with the highest *N. ceranae* levels. We dissected and homogenized the intestines in 2,500  $\mu$ l of phosphate buffered saline. The spore solution was then purified using the Percol triangulation method (Fries et al. 2013). We estimated the concentration of the purified spores via a hemocytometer as described above. This solution was combined with 1,500  $\mu$ l of 30% sucrose to make an inoculum containing  $4.6 \times 10^4$  spores in each 5  $\mu$ l aliquot that was administered to the bees.

### Experiment Set-up

After the 1-wk acclimation in the tents (22–28 July 2017), we assigned three colonies to each treatment, consisting of control (0 workers), 1% (10 workers), or 10% (100 workers) of bees in the colony inoculated with *N. ceranae*. Bees were starved for 1 h, and then given 5  $\mu$ l per bee of inoculum ( $4.6 \times 10^4$  spores per bee), making sure that bees drank the entire inoculum droplet. In the control colonies, the 100 workers were mock inoculated with 30% sucrose. In the 1% treatment colonies, 10 workers received the inoculum and 90 workers received only sucrose. In the 10% treatment colonies, all 100 workers received the inoculum.

## Parasite Sampling

Sampling started on 29 July 2017, 1 wk after we inoculated the colonies, as *N. ceranae* start producing new spores ~6 d after inoculation (Forsgren and Fries 2010). We sampled colonies once per week for 5 wk, haphazardly collecting 20 workers from each colony each week by opening each hive and collecting workers directly from the frames. The sampled individuals were immediately frozen and stored at  $-20^{\circ}\text{C}$  until dissection and spore quantification. For dissection and quantification, individual guts from 10 bees per colony per week (approx. 1% of the colony population) were processed individually. We prepared each sample by homogenizing the gut in 500- $\mu\text{l}$  PBS and vortexed the sample for 30 s. Then, we used a Neubauer chamber to estimate the number of spores per microliter using a 10- $\mu\text{l}$  subsample from the bottom of the tube. We counted the number of spores per sample three times, vortexing the samples between counts. Bees were considered ‘infected’ if they had an average spore count  $\geq 1$ . To estimate the size of workers in each colony, we measured the radial cell of the right forewing for the remaining 10 workers sampled from each colony. We measured them in the same way as for bumble bees (see above).

## Measures of Colony Growth

At the end of the experiment, we took a picture of both sides of the frames in each colony, and later used those photos to count the number of workers in each colony. We calculated the change in the number of workers compared to the initial 1,000 workers per colony.

## Data Analysis

All statistical analyses were conducted using R version 3.5.2 (R Core Team 2018), with separate analyses for each bee-parasite combination.

1. How does the probability of an individual being infected vary with the initial prevalence of the parasite and over time?

To test how the initial parasite prevalence treatment (proportion of infected workers) affected the probability of an individual being infected, we performed logistic regression using the package ‘glmmTMB’. The full models included the initial prevalence (treatment), time (week of sampling), and their interaction as fixed effects. We also included bee size (length of the radial cell) and the number of workers in the colony (only for *B. impatiens*) as covariates, and colony ID was included as a random effect. We evaluated the significance of terms with a likelihood ratio chi-squared test, implemented via the ‘drop1()’ function in R. We removed terms that were not significant ( $P > 0.05$ ) and compared the fit of the reduced and full models using the AIC values and a  $\chi^2$ -test with the analysis of variance (‘anova’) function. We assessed the significance of predictor variables with likelihood ratio via the ‘Anova’ function from package ‘car’. We used Tukey’s HSD tests for post hoc pairwise comparisons using the ‘lsmeans’ package. We did not include data from the first week of sampling for either parasite because of the time required to develop and pass the infection to susceptible hosts (Schmid-Hempel and Schmid-Hempel 1993, Higes et al. 2007).

## 2. Does initial parasite prevalence affect the intensity of infection per individual bee?

To test how the initial parasite prevalence treatment (proportion of infected workers) affected *C. bombi* and *N. ceranae* infection intensity (*C. bombi* cells per microliter and *N. ceranae* spores per microliter), we used a generalized linear mixed model with negative binomial error distribution, because the data showed overdispersion, using the package ‘glmmTMB’. For this analysis we only included data from bees that were infected (cell count  $\geq 1$  for *C. bombi*, and average spore count  $\geq 1$  for *N. ceranae*). Initial prevalence (treatment), time (week of sampling), and their interaction were included as fixed effects; bee size and number of workers in the colony (only for *B. impatiens*) were included as covariates, and individual bee ID nested in each experimental colony was included as a random effect. We evaluated the significance of terms and compared full and reduced models as we did in the analyses of probability of infection. Because we only included infected bees in this analysis, for both parasites, we only included data from the last three weeks of sampling, because earlier weeks resulted in too few infected bees for analysis, especially in the low initial prevalence treatments.

## 3. To what degree does initial parasite prevalence affect colony growth rate and final colony productivity?

To compare the effect of initial parasite prevalence on colony performance of *B. impatiens* (change in total colony weight, total number of workers produced, final number of drones, daughter queens, worker pupae, queen pupae, larvae and eggs, and the total weight for each group), we used t-tests with the ‘t.test’ function. To compare the effect of treatments on *A. mellifera* colony performance (change in number of workers in the colony), we used ANOVA with the ‘aov’ function.

## Results

*How does the probability of an individual being infected vary with the initial prevalence of the parasite and over time?*

*Bombus impatiens* infection with *C. bombi*

The final best-fit model included initial parasite treatment, time (week), and their interaction, with colony ID as a random effect (Fig. 2.1A). The initial prevalence of the parasite had a significant effect on the probability of bees being infected ( $\chi^2_1 = 35.5$ ,  $P < 0.001$ ). By the end of the seventh week, the prevalence of *C. bombi* in the high treatment was on average 3.3-fold higher than in the low treatment ( $t = 4.18$ ,  $P < 0.0001$ ; Fig. 2.1A). There was also a significant effect of time on probability of infection ( $\chi^2_5 = 131.4$ ,  $P < 0.001$ ). In the high treatment, the probability of workers becoming infected grew markedly between weeks one and three, from zero to nearly 0.75, with a slower rate of transmission from week three to seven. In the low treatment, parasite prevalence hovered near zero until week five, reaching a maximum of 0.25 at week seven (Fig. S2.1A). Moreover, the maximum as well as average infection rate was higher in the high compared with low initial prevalence treatments; for example, the probability of infection increased on average 14% per week for the high treatment but only 4.5% per week for the low treatment (Fig. S2.1A). Finally, there was no significant interaction between initial prevalence treatment and time ( $\chi^2_5 = 9.4$ ,  $P = 0.095$ ), suggesting that week did not alter how initial parasite prevalence affected the probability of infection.

*Apis mellifera* Infection with *N. ceranae*

The final best-fit model included only the main effect of time and colony ID as a random effect. Parasite treatment was not a significant term and was dropped from the model ( $\chi^2_2 = 4.66$ ,

$P = 0.097$ ). Time (week) had a significant effect on the probability of a bee being infected ( $\chi^2_4 = 19.45$ ,  $P < 0.001$ ; Fig 2.1B). Although there were no significant differences from 1 wk to the next in the proportion of infected bees ( $P > 0.05$  for all comparisons), at the end of the experiment, the average *N. ceranae* prevalence across all treatments had increased 2.2-fold compared with the beginning of the experiment. Additionally, although treatment was not a significant term in the model, the average prevalence of *N. ceranae* in the high-treatment colonies by the end of the experiment was 5.5-fold higher than in the control colonies (Fig. S2.1B).

*Does initial parasite prevalence affect the intensity of infection per individual bee?*

*Bombus impatiens* infection with *C. bombi*

The final best-fit model included only the main effect of treatment and colony ID as a random effect. Considering only bees that were infected, treatment had a significant effect on intensity of *C. bombi* per bee ( $\chi^2_1 = 8.79$ ,  $P = 0.003$ ). By the end of the experiment, the intensity of *C. bombi* infection was 1.8-fold higher in bees in the high compared with the low treatment (Fig. 2.2A). The lack of effect of time (week) is probably due to the fact that we only included data from the last 3 wks of the experiment (see Methods and Fig. 2.2A). However, in all weeks except week 5, the intensity of infection was higher in the high treatment compared with the low treatment (Fig. 2.2A).

*Apis mellifera* infection with *N. ceranae*

The final best-fit model was the full model. Treatment did not have a significant effect on the intensity of *N. ceranae* ( $\chi^2_2 = 4.33$ ,  $P = 0.11$ ). However, time and the interaction between

treatment and time had significant effects on the intensity of infection (time:  $\chi^2_2 = 27.55$ ,  $P < 0.0001$ ; interaction:  $X^2_5 = 17.44$ ,  $P < 0.001$ ). Week of the experiment altered how the treatment affected the intensity of *N. ceranae* infection (Fig. 2.2B), but by the end of the experiment, the average *N. ceranae* concentration per bee was 1.9-fold higher in the high treatment compared with the low treatment, and it was 17-fold higher in the high treatment compared with the control (Fig. 2.2B). Finally, bee size had a significant effect on the intensity of infection ( $X^2_1 = 5.80$ ,  $P = 0.016$ ), with bigger bees having lower spore counts.

*To what degree does initial parasite prevalence affect colony growth rate and final colony productivity?*

For bumble bees, there was no effect of the treatment on any of the colony performance metrics: total number of workers, drones, queens, worker pupae, queen pupae, larvae and eggs, as well as the total weight ( $P > 0.05$  in all cases; Table S2.2). For honey bees, there was no effect of the treatment on the change in number of workers ( $F_{2,6} = 0.237$ ,  $P = 0.79$ ).

## Discussion

Conventional epidemiological models generally assume that increasing the proportion of infected individuals in groups should increase transmission probability to susceptible hosts. We tested this assumption in bumble bees infected with the trypanosomatid *C. bombi* and honey bees infected with the microsporidian *N. ceranae*. For *C. bombi*, we found support for our initial prediction, with higher initial *C. bombi* prevalence resulting in a faster spread of the parasite and higher intensity of infection. For *A. mellifera*, results were mixed; we found no effect of the

initial parasite prevalence treatment on the spread of the parasite, but a higher initial prevalence increased the intensity of infection in infected bees.

Our results should be interpreted within the context of our controlled, laboratory experimental design. The transmission rates likely represent extreme scenarios, given the confined environment bees were in. Nonetheless, we were able to detect an effect of treatment, so the potentially high transmission rates did not obscure treatment effects. The confined laboratory nature of our experiments also means that colonies were not exposed to environmental factors that can have synergistic negative effects on colony fitness (e.g., pesticides; Wu et al. 2012, Goulson et al. 2015), or continuous exposure to parasites from the environment. However, our approach provides valuable information as it allowed us to explore within-colony transmission of the parasites without the noise of multiple infection events or influence of external factors.

#### *Bombus impatiens* infected with *Crithidia bombi*

In the case of the trypanosomatid *C. bombi*, it is important to note that in the high treatment, the prevalence of *C. bombi* increased from 25 to 75% from the second to the third week of the experiment. In the low treatment, the prevalence at the end of the experiment was 25%. We think it is unlikely that we would have observed a dramatic increase as in the high treatment if we had let the experiment run for more weeks because a colony is more prone to an epidemic during the growing phase, when the number of susceptible individuals is high (Naug and Camazine 2002). Our results suggest that if only one infection event occurs, or a small number of workers bring *C. bombi* back to a mature colony, within-colony parasite prevalence may never reach high levels.

Parasite prevalence at the end of the colony life cycle is also important in transmission to the next generation. New queens, produced at the end of the colony life cycle, are the only individuals that overwinter, so *C. bombi* must infect them in order to stay in its host population (Shykoff and Schmid-Hempel 1991). A lower within colony prevalence at the time of queen emergence can decrease transmission to new queens (Buechel and Schmid-Hempel 2016). Thus, we could expect that a lower parasite pressure early in the colony cycle would result in lower within-colony spread, and a smaller proportion of new queens infected with *C. bombi* in their natal nest near the end of colony life. Lower prevalence in new queens could have a positive impact on bumble bee populations, because infected queens are less likely to start a colony in the spring (Schmid-Hempel 2001, Brown et al. 2003b).

Some studies indicate that *C. bombi* can impact a bumble bee queen's fitness by reducing colony-founding success and colony size, as well as reducing the number of workers, males and new queens produced by a colony (Brown et al. 2003b). In this study, we did not find any effect of initial parasite prevalence on the different measures of colony performance. This lack of effect is something that we might expect given that colonies were kept in laboratory conditions with no exposure to other stressors or food limitation and has been reported in other studies (Shykoff and Schmid-Hempel 1991). However, in field conditions, we note that fitness differences at the colony level have been observed with *C. bombi* infection, as the parasite can have negative synergetic effects with stressors like pesticides and malnutrition (Fauser- Misslin et al. 2014, Goulson et al. 2015).

*Apis mellifera* infection with *Nosema ceranae*

Two caveats are important in the interpretation of our honey bee results. First, we imposed our treatments upon colonies with underlying *N. ceranae* infection. Although this may have obscured our ability to detect an effect of our treatments, it is rather representative of what a beekeeper would find in field conditions, with a high proportion of colonies infected with *N. ceranae* (44–100%) at low intensity levels (Traver and Fell 2011, Fernández et al. 2012, Szalanski et al. 2013). Second, we used one-frame colonies with ~1,000 workers and one queen, which is similar to a young, recently started colony. It would be important to determine whether *N. ceranae* transmission in mature colonies is any different from what we observed here, given that parasites that are transmitted by direct contact can increase in prevalence and intensity as the host group size increases (Naug and Camazine 2002). Furthermore, as the colony grows and changes in social organization occur (Michener 1998), it is possible that parasite transmission dynamics would be affected (Naug and Camazine 2002). Studies that manipulate honey bee colony size and assess transmission rates would yield additional epidemiological insight. It is also important to note that we did not homogenize the age of the workers that were inoculated at the beginning of the experiment due to practical reasons of the experimental design. The dose of *N. ceranae* we used to infect workers (46,000 spores) was at least 4–10 times higher than the ID50 for all ages of honey bee workers (Huang et al. 2015), so we suspect the lack of age homogenization minimally impacted our results. However, it is possible that not controlling for age increased variability within treatments.

We did not find an effect of initial parasite pressure on the within-colony prevalence of *N. ceranae*. The lack of an effect could be due to multiple nonmutually exclusive mechanisms, two of which we describe here. First, the lack of an effect could be due to the short, 5-wk

duration of the experiment, which might not be enough time to detect an effect, as changes in *N. ceranae* prevalence are usually observed over seasons or years (Higes et al. 2008; Gisder et al. 2010, 2017). Second, the slow within-colony spread could be due to honey bee social network structure and behaviors such as allogrooming (Konrad et al. 2012, Guzman-novoa and Morfin 2019). Additional network and behavioral studies would be required, however, to understand how these factors affect the link between parasite pressure and within-colony prevalence.

We did find that higher initial parasite pressure increased the parasite loads per individual bee. *Nosema ceranae* reaches the highest number of spores in the gut ~15-d postinfection (Paxton et al. 2007). We started observing higher spore counts after the third week of the experiment (Fig. 2.2B), so it is possible that *N. ceranae* only started spreading in the colony after the group of originally inoculated bees developed high spore counts, and we observed the increase in spore counts of the secondary infections in the colony. Higher per-bee parasite loads in the colony could also facilitate the spread of *N. ceranae* during the winter, when bees remain in the colony and prevalence tends to increase (Gisder et al. 2017), possibly leading to higher chances of colony collapse (Higes et al. 2008).

#### Parasite Transmission and Future Directions

Taken together, our results suggest that initial parasite pressure is an important factor that can determine the outcome of an infection at the colony level in honey bees and bumble bees. These results also seem to be consistent with the theory that social immunity helps to control the spread of parasites in honey bees up to certain point (Godfrey 2013, Liu et al. 2019). This pattern still needs to be tested in more natural conditions, where colonies are exposed to other environmental factors that can influence the host–parasite interaction, including pesticides and

other parasites. In the case of honey bees, following the within-colony spread of the parasite for a longer time period would be important to fully understand the transmission dynamics and effect of parasite pressure.

Our results also indicate that measures that reduce the acquisition of parasites in the environment would help reduce within-colony transmission by reducing parasite pressure. This is also likely to have a positive feedback effect given that slower within-colony transmission would reduce the number of infected individuals that could spread parasites in the environment. The use of flowers with low parasite transmission probability could be an option to achieve lower parasite pressure of bees returning to colonies (Adler et al. 2018), as well as better monitoring and control of parasites in commercial pollinators, in order to reduce the spillover of pathogens to wild bees (Goulson and Hughes 2015). As studies find that interspecific transmission and prevalence of bee parasites can be higher than what was once thought (Gisder and Genersch 2017, Müller et al. 2019), understanding transmission dynamics and factors that affect it are critical for developing strategies that can slow the spread of parasites and diseases in commercial and wild pollinators.

#### Data Availability Statement

Data from this study are available from the Dryad Digital Repository:  
<https://doi.org/10.5061/dryad.qfttdz0f4> (Pinilla-Gallego et al. 2021).

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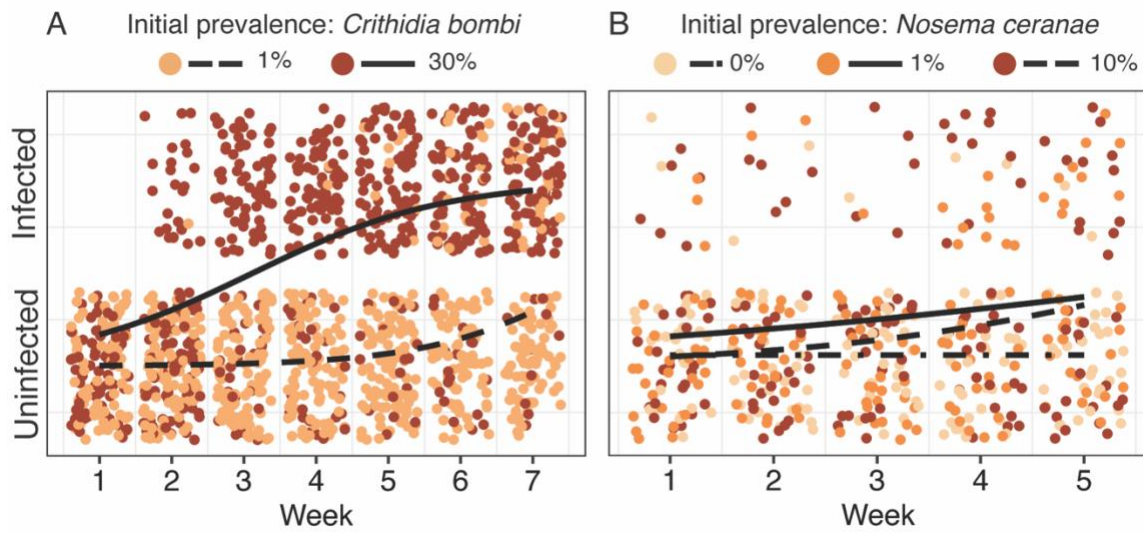


Figure 2.1. Distribution of infected and uninfected individuals in (A) bumble bee (*Bombus impatiens*) colonies where 1 or 30% of initial workers were experimentally infected with *Crithidia bombi* (six colonies per treatment) and (B) honey bee (*Apis mellifera*) colonies where none (control), 1 or 10% of workers were experimentally infected with *Nosema ceranae* (three colonies per treatment).

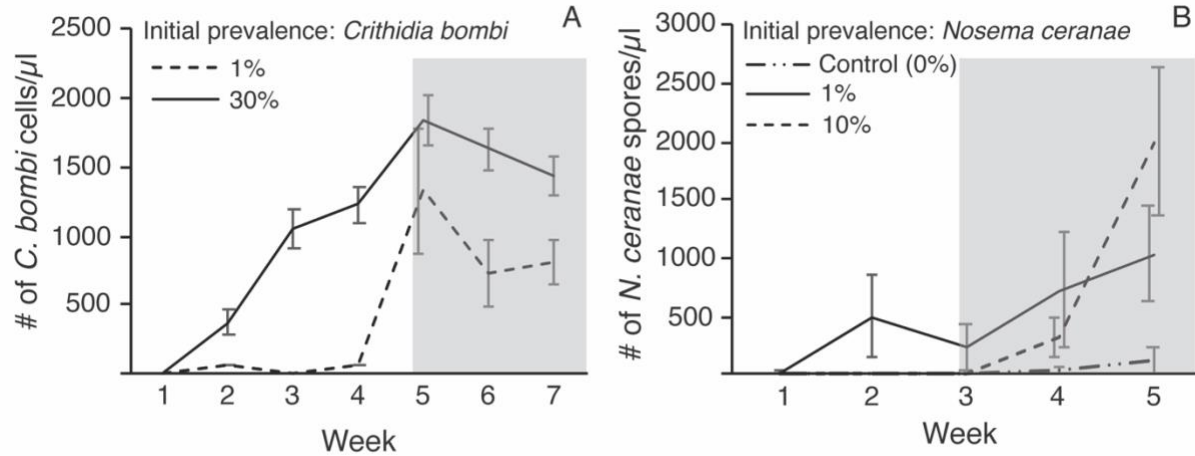


Figure 2.2. Mean ( $\pm$ SE) intensity of infection for (A) *Crithidia bombi* in bumble bee (*Bombus impatiens*) colonies where 1 or 30% of initial workers were experimentally infected (six colonies per treatment) and (B) *Nosema ceranae* in honey bee (*Apis mellifera*) colonies where none (control), 1 or 10% of workers were experimentally infected (three colonies per treatment). Shaded area represents the weeks that were included in the statistical models (see Methods). Error bars were jittered to improve clarity.

### CHAPTER 3: Effects of an alternative host on the virulence of a bumble bee parasite

#### Abstract

Parasites are ubiquitous in nature, with most parasites being able to infect multiple host species. Encounter rate, host quality and transmission patterns are some of the factors that can affect the evolution of parasite virulence. The worldwide decline of bee populations has been linked to the spread of parasites and their synergetic interaction with other stressors. Many parasites of bees spread on flowers, where there is potential for encountering multiple host species. Here, we use the trypanosomatid parasite *Crithidia bombi* and its primary host, bumble bees (*Bombus* spp.), to explore the effect of infecting an alternative host, the alfalfa leaf-cutter bee (*Megachile rotundata*), on parasite virulence. We conducted a serial passage experiment in which we passed *C. bombi* from primary host to primary host and from alternative host to alternative host. To test the effect of infecting multiple host species on the parasite virulence, we used parasite cells from the serial passages on the alternative host to inoculate the primary host. We found that after serial passage through the primary host, the probability of infection of *C. bombi* decreased, while the intensity of the infection remained constant. After serial passage through the alternative host, both the probability and intensity of infection remained unchanged in the alternative host. Interestingly, when the parasite was returned to the primary host after serial passage through the alternative host, both the probability and intensity of infection in the primary host increased. This increase in virulence could be due to maladaptation after selection of newly recombined strains has occurred in the alternative host. Given that *M. rotundata* is a lower quality host for *C. bombi*, and because they are less likely to pass the parasite to bumble queens, we consider that the presence of leaf-cutter bees as an alternative host would have little impact on the adaptation of *C. bombi* to bumble bees.

## Introduction

Host–parasite interactions are ubiquitous in nature (Poulin and Morand 2004) and can have a profound effect on host ecology, including community structure, host population cycles and abundance, genetic diversity, and trophic relationships (Kirzhner et al. 1999, Marcogliese 2002, Mouritsen and Poulin 2002, Bonsall 2004, Thompson et al. 2005). Most parasites have multiple host species, and most hosts are usually attacked by multiple parasite species, with important ecological and evolutionary implications for both hosts and parasites (Rigaud et al. 2010). Theoretical studies suggest that when a multi-host parasite is likely to encounter multiple host species that differ in quality, the population can take one of two evolutionary paths – it could split into two populations, each one with an optimal virulence for each host species, or the population could remain generalist with a suboptimal virulence for both host species (Regoes et al. 2000). In a situation where host species differ in quality for the parasite, parasites should evolve towards optimal virulence in their primary host, and suboptimal virulence in other host species (Gandon 2004). However, these predictions have rarely been tested in field or laboratory experiments, making it difficult to predict the evolutionary trajectory of parasites, which has implications for management of human and wildlife emerging diseases (Rigaud et al. 2010, but see Guidot et al. 2014, Benesh and Kalbe 2016). Here, we used the model system of the trypanosomatid parasite *Crithidia bombi* and its primary hosts, bumble bees (*Bombus* spp.), to investigate the effect of the presence of an alternative host on the evolution of parasite virulence.

Bees (Hymenoptera: Apoidea) host a wide variety of parasites, from internal microparasites like viruses and bacteria, to external macroparasites like mites (Shimanuki and Knox 2000, Hedtke et al. 2011, Goulson and Hughes 2015, Graystock et al. 2016), and several of these parasites have been linked to the population decline of managed and wild bees

(VanEngelsdorp and Meixner 2010, Bianco et al. 2014). Many of these parasites were once thought to have a small host range, but recent assays are finding that some of these parasites can actually attack host species in other genera or even families than the primary host (e.g., Ngor et al. 2020). For example, the Microsporidian *Nosema ceranae* was originally found in the Asian honey bee (*Apis ceranae*, Apidae), and now it has been reported to infect and replicate in the European honey bee (*Apis mellifera*, Apidae) (Higes et al. 2006), mason bees (*Osmia bicornis*, Megachilidae) (Müller et al. 2019) and bumble bees (*Bombus terrestris*, Apidae) (Graystock et al. 2013).

A common route of horizontal transmission of bee parasites is by the shared use of flower resources, as infected individuals deposit parasites on flowers that can be picked up by other bees (Durrer and Schmid-Hempel 1994, Graystock et al. 2015, Alger et al. 2019). Additionally, habitat degradation and limited floral resources can cause managed and wild bees to congregate on patches of flowers, increasing the risk of parasite transmission (amplification effect) (Becker et al. 2015, Piot et al. 2019). Recent studies find that 10-30% of flowers in a field can have at least one bee parasite (Graystock et al. 2020, Piot et al. 2020), so it would be expected that parasites commonly encounter several host species that can differ in quality, as variation in host size and immune level can result in changes in the amount of parasite propagules produced by a host (Baer and Schmid-Hempel 2003, Brown, Schmid-Hempel, et al. 2003, Sinpoo et al. 2018). This heterogeneity in host quality, as well as different encounter rates with different host species, could drive the evolution of virulence of parasites (Gandon 2004, Wilber et al. 2020).

Due to the risk of emerging infectious diseases that can affect humans, domestic animals and wildlife (Daszak et al. 2000, Dobson and Foufopoulos 2001), it is important to understand the processes that drive virulence evolution of parasites in multi-host communities, which can

have important conservation implications for managing disease impacts (Wilber et al. 2020). Here, we use the parasite *Crithidia bombi* (Trypanosomatida: Trypanosomatidae) and its primary host, bumble bees (Hymenoptera: Apidae: *Bombus impatiens*), to explore the effect of the presence of an alternative host on parasite virulence (production of new parasite cells and ability to infect the host). Specifically, we addressed the following questions. 1) Does infecting an alternative host and the number of passes through the alternative host influence the parasite's ability to infect the primary and/or alternative host? And 2) does the number of passes through the alternative host increase parasite intensity of infection in the primary and/or alternative host? We predicted that both the ability to infect a host and intensity of infection would increase after serial passes through any host, primary or alternative, because any trade-off between transmission and virulence is removed in serial passage experiments (Alizon et al. 2013). We also predicted that virulence in the primary host would be suboptimal after the parasite infected the alternative host, assuming that the parasite becomes more adapted to the alternative host (Yañez et al. 2020). Taken together, this work can help us understand the role of alternative hosts on the evolution of virulence of multi-host parasites, which is relevant for the management of infectious diseases of humans, domestic animals and wildlife (Lloyd-Smith et al. 2005, Paull et al. 2012, Wilber et al. 2020).

## Methods

### *Study system*

*Crithidia bombi* is an intestinal parasite of bumble bees (*Bombus* spp.) that reproduces in the hindgut lumen with new cells released to the environment in bee feces (Schmid-Hempel and Schmid-Hempel 1993, Imhoof and Schmid-Hempel 1999). Although it is typically considered a

benign parasite (Yourth and Schmid-Hempel 2006), it can reduce the number of new queens and males a wild bumble bee colony produces (Goulson et al. 2018) and the success rate of infected overwintering queens when starting a nest in the spring, potentially limiting the abundance of its host (Schmid-Hempel 2001, Brown, Schmid-Hempel, et al. 2003). *Crithidia bombi* reproduces clonally, but there can be genetic exchange, and new strains are produced in 7-16% of infections (Schmid-Hempel et al. 2011, Tognazzo et al. 2012). This genetic variability is also reflected in a strong genotype-genotype interaction between the parasite and its host (Shykoff and Schmid-Hempel 1991, Imhoof and Schmid-Hempel 1998).

As a bumble bee host, we used the common eastern bumble bee (*Bombus impatiens*) which is a native eusocial bee species to eastern North America, is commercially reared for agricultural pollination, and is economically important for the pollination of many crops (Kleijn et al. 2015). As a generalist forager, *B. impatiens* visits a variety of plant species that span a diversity of floral traits (Mader et al. 2010).

Recently, Ngor et al. (2020) found that *C. bombi* can also infect and actively replicate in the alfalfa leaf-cutter bee (ALCB) *Megachile rotundata* (Megachilidae), a commercial solitary bee originally from Europe, that is now widely distributed in North America (Mader et al. 2010). Adults emerge in late spring and fly for a period of approximately one month. In cooler climates, pre-pupae will enter diapause and finish development the following spring, but in warmer climates, pupa can finish development the same year and have a second generation that will overwinter until the following spring (Pitts-Singer and Cane 2011). Given the overlapping flying period of *B. impatiens* and *M. rotundata* during summer months and their ability to visit similar flowers (Scott-Dupree et al. 2009, Mader et al. 2010), there is the potential for the sharing of

parasites on flowers between these two species. Hereafter, *B. impatiens* is referred to as the primary host, and ALCB as the alternative host.

#### *Bee sources and maintenance*

**Bumble bees:** We used *B. impatiens* commercial colonies from Koppert Biological Systems (Howell, MI, USA), kept in a dark room at approx. 27 °C and 50 % RH, and provided sugar-water (30% sucrose) and honey bee collected pollen (CC High Desert Pollen, Phoenix, AZ, USA) *ad libitum*. Upon arrival, we screened colonies for *C. bombi* infection by taking a random sample of four workers per colony, collecting fecal samples, and examining them under a compound microscope at 400x. We always maintained 2-3 uninfected colonies as a source of experimental bees, and 1-2 colonies infected with *C. bombi* isolated from *B. impatiens* collected at Stone Soup Farm, Hadley, MA (GPS coordinates: 42.363911 N, -72.567747 W) as sources of *C. bombi* (Figueroa et al. 2019).

**Alfalfa leaf-cutter bees:** We obtained cocoons of ALCBs from JWM Leafcutters, Inc. (Nampa, ID, USA). We stored cocoons at 4 °C, and then we incubated them at 30 °C until emergence (approx. 3 weeks). Once bees emerged, we transferred them to a small container with access to sucrose solution *ad libitum*. Bees used in the experiments were 1-3 days old.

#### *Inoculum preparation*

To prepare inoculum to start each replicate, we followed a standard protocol (Richardson et al. 2015). Briefly, we collected 8-10 workers from a *C. bombi* infected bumble bee colony. We dissected the guts of each individual bee and homogenized each intestine in 300 µl of dH<sub>2</sub>O. We let the samples settle for 3-4 h to allow the gut debris to sink to the bottom of the tube. We took

200 µl of clean supernatant from each sample and mixed them together, and used a 10 µl aliquot to estimate the number of *C. bombi* cells per microliter in a Neubauer chamber with a compound microscope at 400x magnification. Then we used dH<sub>2</sub>O and 50% sucrose to dilute the mix to be 25% sucrose with 1200 cells/µl.

To prepare inoculum from experimental bees, we dissected the guts of each individual bee. In the case of bumble bees, we homogenized the guts in 300 µl of dH<sub>2</sub>O; in the case of ALCBs, we homogenized the guts in 50 µl of dH<sub>2</sub>O, as these bees are much smaller than bumble bees (intertegular distance 2.3-2.8 mm for ALCB). We let the samples settle for 3-4 h, and then we took a 10 µl sample and estimated the number of *C. bombi* cells in the sample, as described above. We mixed the supernatant of samples with positive counts within each group, and took a new sample to determine the *C. bombi* concentration. Then we mixed equal parts of the *C. bombi* solution with 50% sucrose to make an inoculum that was 25% sucrose. Because the number of infected bees and the level of infection of experimental bees was variable, and because of the small volume obtained from ALCBs, the concentration of the inoculum made from experimental bees was variable each time we prepared it. Inoculum from ALCBs was made with guts from 7-23 bees, and it ranged from 275 – 1200 cells/µl with a mean of 820 cells/µl. Inoculum from experimental bumble bees was made with guts from 2-7 bees and it ranged from 25 - 1200 cells/µl with a mean of 680 cells/µl.

We collected the right forewing from each experimental bee we dissected and measured the length of the radial cell as an estimate of bee size (bumble bees: Müller et al. 1996; ALCBs: Fig. S3.1), using the software ImageJ (V 1.8).

### *Experiment set-up*

To test the effect of host switching on *C. bombi* virulence, we set up a serial passage experiment (Fig. 3.1). In the control group, we passed *C. bombi* from bumble bee to bumble bee, simulating a situation in which the parasite transmits between primary hosts. In the treatment group, we passed *C. bombi* from ALCB to ALCB (AA treatment). To test the effect of the number of passes through the alternative host on the ability of the parasite to infect the primary host, after each pass on ALCBs, we infected a group of bumble bees with *C. bombi* from the treatment group (AB treatment).

To start each replicate, we made inoculum from a bumble bee source colony (see *Inoculum preparation*), and used it to inoculate 10-12 bumble bees, and 25-35 ALCBs. Each bumble bee received 10 µl of inoculum while each ALCB received 5 µl, as they are smaller than bumble bees. Both species were kept in 15 ml vials with sucrose and pollen *ad libitum*, refreshed every other day. After 7 days, the parasites reach a representative level in bumble bees (Logan et al. 2005) and so we dissected each individual bumble bee and ALCB and determined whether they were infected and the intensity of infection (as in *Inoculum preparation*). Guts from bumble bee controls were used to make inoculum for another passage through bumble bees. ALCB guts were used to make inoculum for another passage through ALCBs and passage back into 10-12 bumble bees (Fig 3.1). We repeated this process 2-5 times. The number of passes for each replicate varied based on the availability of the number of newly emerged bees of ALCB's and *B. impatiens* on the inoculation date. Bumble bees for each replicate came from the same source colony.

### *Statistical analysis*

We performed all data analyses using R (v. 4.02) (R Core Team, 2018). We analyzed ‘incidence’ (presence/absence of *C. bombi* infection) and ‘intensity’ (*C. bombi* counts from infected bees) as separate components of *C. bombi* virulence, using generalized linear mixed models (GLMM).

**Incidence analysis:** We modelled parasite incidence using logistic regression with the package glmmTMB (Skaug et al. 2018). The response variable was the binary outcome of whether a bee was infected or not. The full model included treatment (control, AA or AB treatments), the number of passes, an interaction term between those two factors, and bee size (radial cell). We included bee species as a random effect. To determine the significance of the fixed effects, we conducted a likelihood ratio test comparing the full model with a model that excluded each of the fix effects as an explanatory variable. We removed terms that were not significant and selected the model with the lowest AIC value. We evaluated model assumptions by generating QQ plots of residual vs. predicted with the DHARMA package (Hartig and Lohse 2020). To explore the effect of interactions, we calculated the odds ratio and performed pairwise comparisons of the slope of each treatment with the “emtrends” function of the emmeans package (Russell et al. 2021).

**Intensity analysis:** To model intensity of *C. bombi* infection, we only used data from infected bees (positive counts). We used the cell count per 0.02 µl gut sample as the response variable. These data were highly right-skewed, so we log-transformed the cell counts, and used a GLMM with Gaussian distribution. We used the same fixed and random effect terms as in the incidence analysis, and performed model selection, tested assumptions and explored interactions of the models in the same way as in the incidence analysis.

## Results

Incidence of infection: Neither treatment ( $X^2_2 = 4.73$ ,  $P = 0.093$ ) nor the number of passes ( $X^2_1 = 0.89$ ,  $P < 0.345$ ) were significant predictors of incidence of infection. However, there was a significant interaction between treatment and the number of passes ( $X^2_2 = 13.44$ ,  $P < 0.0012$ ; Fig. 3.2). While each additional pass through the bumble bee control reduced the probability of infection by 36%, each pass through the alternative host (AA treatment) and back to the primary host (AB treatment), increased the probability of infection by 2.3% and 24%, respectively (Table S3.1). Pairwise comparisons revealed that while there were not differences between the slopes of the AA and AB treatments ( $t_{926} = 1.04$ ,  $P = 0.55$ ), both treatments were significantly different from the control ( $t_{926} = -3.16$ ,  $P = 0.004$ ;  $t_{926} = -3.22$ ,  $P = 0.003$ , respectively).

Intensity of infection: Treatment had a marginally significant effect ( $X^2_2 = 5.78$ ,  $P < 0.057$ ), while the number of passes was not a significant predictor for the intensity of the infection ( $X^2_1 = 1.57$ ,  $P = 0.29$ ). But similar to the incidence analysis, there was a significant interaction between treatment and the number of passes ( $X^2_2 = 20.15$ ,  $P < 0.0001$ ; Fig. 3). While each additional pass from bumble bee to bumble bee (control) and from ALCB to ALCB host (AA treatment) had little effect on the intensity of infection (8% increase and 3% decrease, respectively), each pass through ALCB increased the intensity of infection on bumble bees (AB treatment) by 78% (Fig. 3.3, Table S3.2). The pairwise comparison confirmed that the slope of the AB treatment was significantly different from the control and AA treatment ( $t_{619} = -3.39$ ,  $P = 0.0021$ ;  $t_{619} = 4.48$ ,  $P < 0.001$ , respectively), but there were no differences between the slopes of the control and AA treatment ( $t_{619} = 1.08$ ,  $P = 0.525$ ).

## Discussion

Bee parasites are geographically wide-spread and many have a wide host range within the Anthophila both within and across genera and families (Singh et al. 2010, D'Alvise et al. 2019, Bartolomé et al. 2020, Graystock et al. 2020, Yañez et al. 2020). Multi-host parasites have the advantage of surviving in alternative hosts when the primary host is less abundant (Dunn et al. 2009), but at the same time, it is more difficult for multi-host parasites to evolve an optimal virulence level for any particular host (Leggett et al. 2013). Thus, it has been argued that increasing host community diversity should reduce parasite transmission and virulence due to an "encounter reduction" effect (Keesing et al. 2006, Johnson et al. 2009). Here, we tested the effect of infecting an alternative host on the virulence of *C. bombi* on both the original and alternative host. Although we did not find an increase in virulence on the alternative host after serial passages, there was an increase in incidence and intensity of infection on the original host after the parasite had infected the alternative host. Testing the predictions of mathematical models for the evolution of virulence of multi-host parasites is essential to manage emerging infectious diseases of humans and wildlife (Perlman and Jaenike 2003), for example, by identifying host maintenance potential in multi-host parasite communities (Wilber et al. 2020).

When natural selection of parasites operates at the transmission level, it should select for virulence levels that maximize transmission (Anderson and May 1982). In serial passage experiments, selection for transmission is removed, and so are any trade-offs between transmission and virulence (Alizon et al. 2013). In this situation, within-host competition between strains usually selects for higher parasite growth rates (Ebert 1998). Thus, serial passage experiments often find an increase in virulence on the alternative hosts and attenuation of virulence on other host types (Ebert 1998, Yañez et al. 2020). Here, we found that serial passage

of *C. bombi* through the primary bumble bee host reduced the probability of infection and had little effect on the intensity of infection in bumble bees. Although this is opposite of what would be expected from serial passage experiments, Yourth and Schmid-Hempel (2006) also found that serial passes of *C. bombi* on bumble bees of the same colony did not increase the intensity of the infection, and Acute Bee Paralysis Virus decreased virulence in honey bees after multiple serial passes (Bailey and Gibbs 1964). Beyond bees and their parasites, Huang et al. (2019) found that the fungus *Fusarium oxysporum* decreases virulence after serial passages on susceptible cucumber cultivars. These studies, combined with our results suggest that an increase in virulence is not always the rule in serial passage experiments and that evolution does not always follow simple theoretical expectations (Yourth and Schmid-Hempel 2006). The mechanism driving the decrease in the probability of infection with serial passage of *C. bombi* through bumble bees is unknown. However, one relevant hypothesis is that for each replicate, we used workers from the same colony, and constitutive defenses of bumble bee workers, which are part of the immune response to *C. bombi* (Brown, Moret, et al. 2003, Otterstatter and Thomson 2006, Whitehorn et al. 2011), increase with colony age (Moret and Schmid-Hempel 2009), possibly making workers on later passes more resistant to *C. bombi*. This hypothesis warrants further investigation.

Serial passes through the alternative host, *Megachile rotundata*, had divergent effects compared to serial passes through the primary bumble bee host. Serial passage through the alternative host had little effect on the probability and intensity of the infection in the alternative host. It is possible that *C. bombi* needs longer exposure to the alternative host before there is a detectable increase in virulence, as we would expect a lower baseline adaptation to this host. This could be due to the rate at which virulence increases in serial passage experiments is slower

for eukaryotes than for viruses and bacteria (Ebert 1998). Interestingly, the probability and intensity of infection on the primary bumble bee host increased after serial passes through the alternative host. *Crithidia bombi* can generate new strains during infection events (Schmid-Hempel et al. 2011), and it is common to observe a genotype-by-genotype interaction between *C. bombi* and bumble bees, with individual strains producing infection that vary in intensity on different bumble bee genotypes (Shykoff and Schmid-Hempel 1991, Mallon et al. 2003, Ruiz-González et al. 2012). It is possible that the strains that are being selected in *M. rotundata* could cause maladaptation to the primary host (Gandon 2004), as higher virulence in bumble bees could reduce survival of the host to the point of decreasing between-host transmission (Leggett et al. 2013). Genetic comparison of *C. bombi* strains after serial passes through the primary and alternative host could help elucidate any potential genetic changes occurring, and also help to identify genes that are involved in the evolution of virulence (Gisder et al. 2018).

The evolution of multi-host parasite virulence is influenced by many factors, including quality of the hosts, abundance of each host, and transmission patterns among hosts (Regoes et al. 2000, Gandon 2004, Rigaud et al. 2010). The likelihood that the presence of ALCB as an alternative host for *C. bombi* would have an effect on the evolution of virulence and transmission to the primary host could depend on several factors. First, the probability of transmission of *C. bombi* on flowers between host species will depend upon the frequency with which species forage on the same floral resources (Ruiz-González et al. 2012). The flying period of the ALCB overlaps with bumble bees but does not do so across the entire bumble bee flight season (Mader et al. 2010) and not when new fall queens are produced. Thus, despite shared floral resources, the differences in phenology likely reduces the encounter rate of *C. bombi* with this alternative host and with the *Bombus* spp. reproductive caste. A reduction in the relative amount of between-host

transmission decreases the cost of specialization for the parasite, because the less abundant hosts may be rarely encountered (Gandon 2004). In addition, *M. rotundata* are significantly smaller than bumble bees and should therefore produce fewer new parasite cells, which again may reduce the likelihood that the primary host comes into contact with enough *C. bombi* cells from the alternative host to become infected. This natural history information suggests that ALCB's may have little effect on the evolution of virulence and transmission to the primary host, but more research in the natural system is needed to assess this further.

Testing the theoretical predictions of the evolution of virulence is a promising research area that is likely to provide us with new insights about the evolution of parasites (Rigaud et al. 2010). Here we found that serial passages through an alternative host did not increase the virulence of *C. bombi* on the alternative host, and it could be causing maladaptation to the primary host, the implications of which need to be assessed more thoroughly in natural systems. A better understanding of the factors that drive evolution of multi-host parasite systems would allow us to make more accurate predictions of how emerging infectious diseases, pests and parasites adapt and spread in natural and human systems (Ebert 1998, Betts et al. 2016). In the context of bee population declines, parasites are considered one of the main factors contributing to this decline (Goulson et al. 2008, Potts et al. 2010), so understanding the factors that affect parasite virulence could lead to the development of strategies to mitigate these declines. Our study is subject to the limitations of a laboratory serial passage experiment, where we used bees from commercial producers and kept them with unlimited food resources. Future studies should explore more realistic scenarios, including incorporating the effects of shared floral resources, primary and alternative host phenology overlap, and parasite dose-host response relationships.

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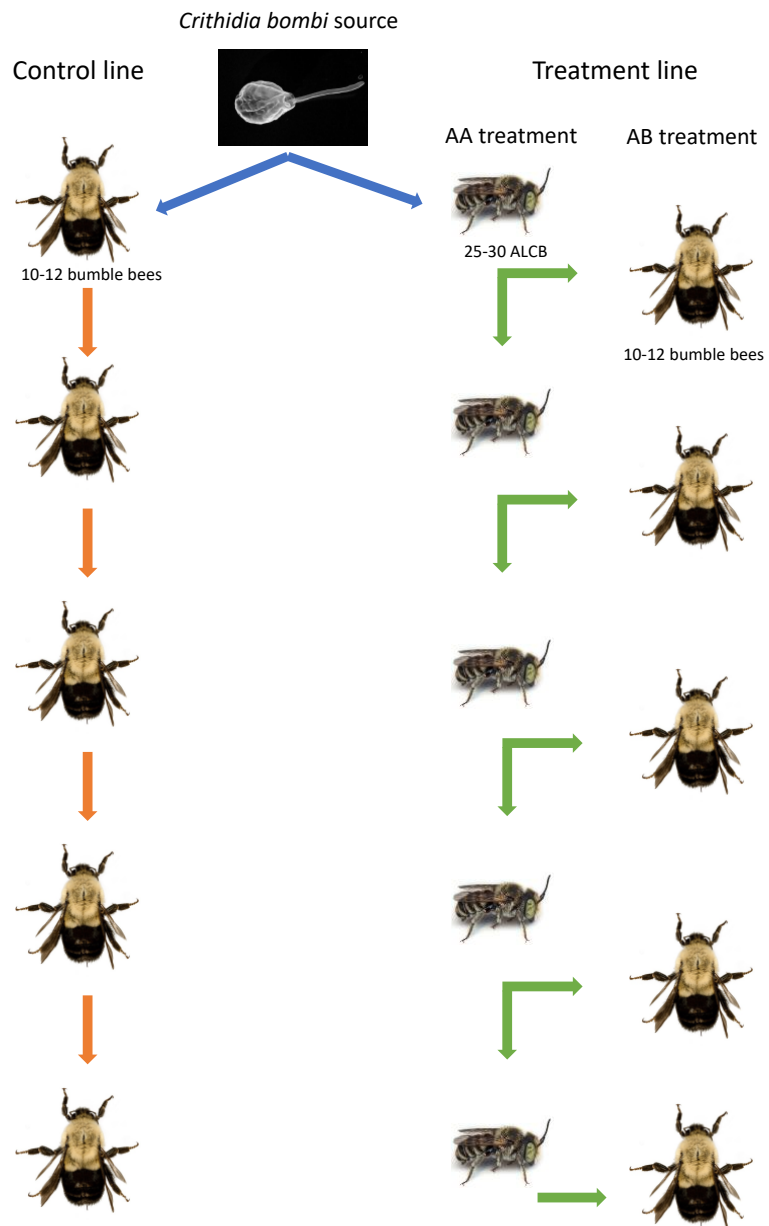


Figure 3.1. Experimental design. In this serial passage experiment, *Crithidia bombi* from a source colony was used to infect a group of bumble bees (control line) and Alfalfa leaf cutter bees (ALCB) (AA treatment). One week after infection, guts were dissected and this *C. bombi* was used to inoculate the next group of bees. In the case of ALCB, part of the inoculum was also used to infect a group of bumble bees (AB treatment).

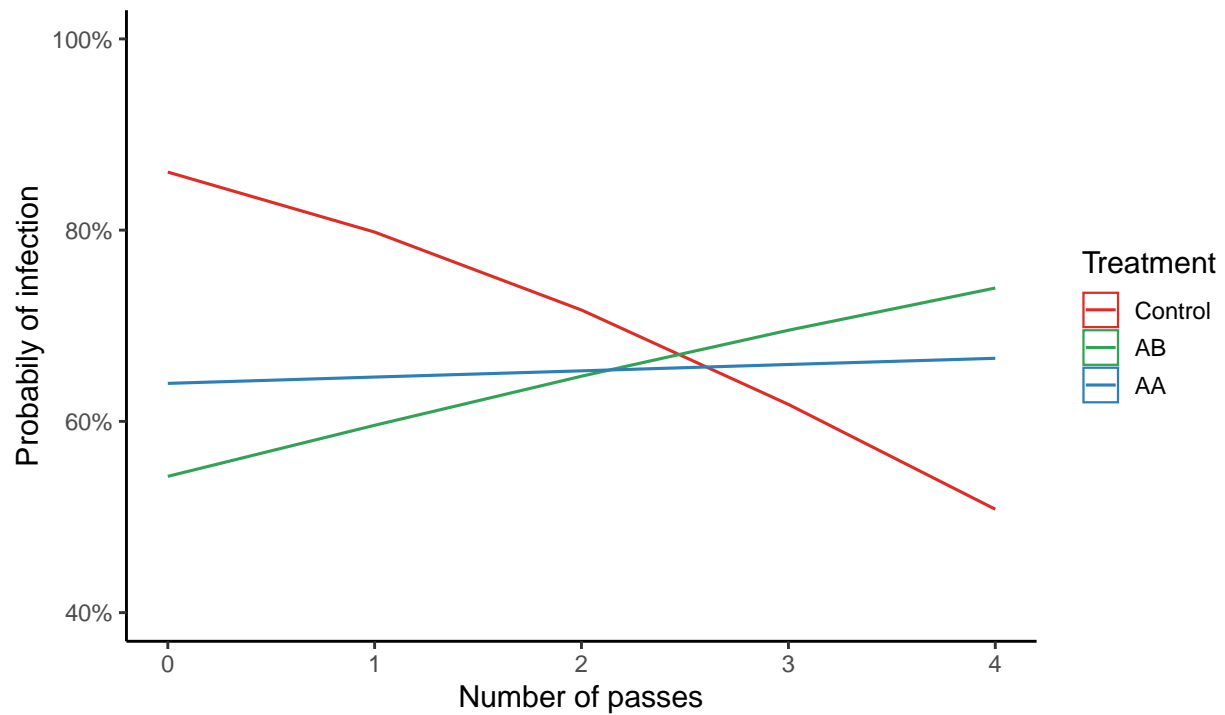


Figure 3.2. Predicted incidence of infection for: Control = *B. impatiens* to *B. impatiens*, AA = *M. rotundata* to *M. rotundata*, and AB = *M. rotundata* to *B. impatiens*. There was a significant interaction between treatment and number of passes, where the probability of infection in the control group decreases with the number of passes, while it increases for the AB and AA treatments.

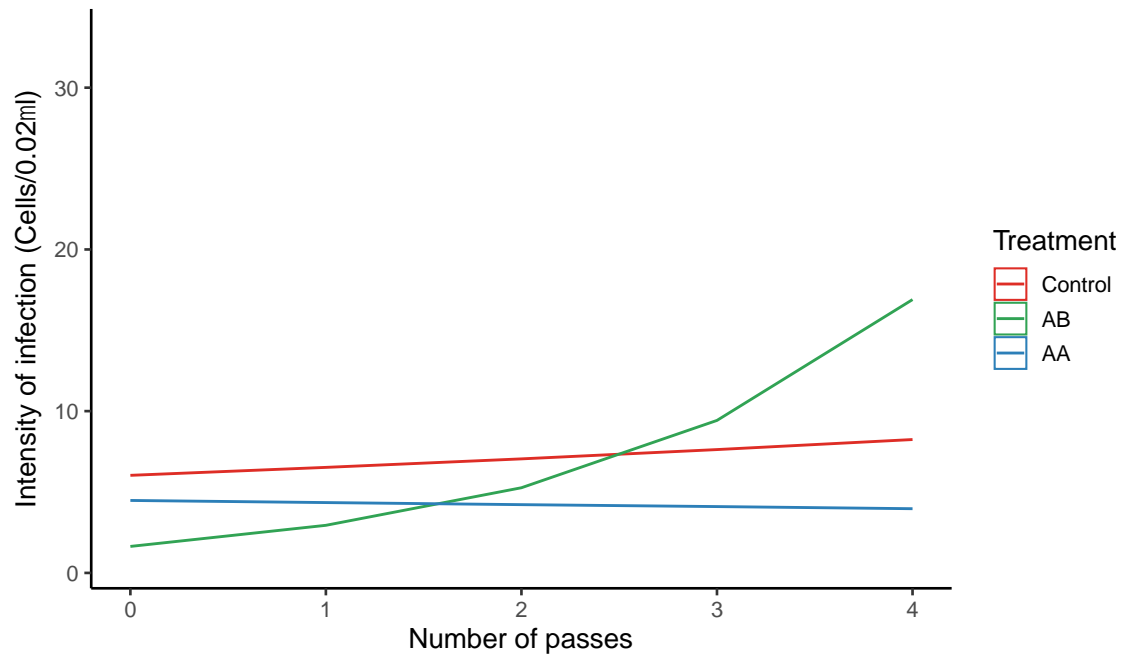


Figure 3.3. Predicted intensity of infection (cells/0.02  $\mu$ l) of *C. bombi* during serial passages from: AA = *B. impatiens* to *B. impatiens* (control), AA = *M. rotundata* to *M. rotundata*, and AB = *M. rotundata* to *B. impatiens*. There was a significant interaction between treatment and number of passes, where the intensity of infection only increases with the number of passes for the AB treatment.

## **APPENDICES**

## Appendix A: Supplementary figures and tables

Table S1.1. Pearson correlations between the first two principal components (floral size and shape) and floral morphological traits (corolla width and length). Statistically significant correlations ( $P < 0.05$ ) noted by \*. The consistent, positive correlations between PC1 and traits suggests that PC1 describes flower size. The mixture of positive and negative correlations between PC2 and traits suggests that PC2 describes flower shape.

	Floral size (PC1)	Floral shape (PC2)
Corolla width	0.731*	-0.693*
Corolla length	0.96*	0.254*

Table S1.2. Mean, minimum, maximum, standard deviation (SD) and sample size (N) for each floral trait measured for each plant species, as well as the floral size and shape.

Species	Reproductive structures per stalk				
	mean	min	max	SD	N
<i>Agastache foeniculum</i>	17	10	25	4	20
<i>Angelonia Angustifolia</i>	11	3	15	3	20
<i>Antirrhinum majus</i>	2	1	3	1	20
<i>Asclepia tuberosa</i>	11	4	35	6	20
<i>Caryopteris clandonensis</i>	186	55	1326	275	20
<i>Coreopsis verticillata</i>	24	2	64	13	20
<i>Echinacea purpurea</i>	1	1	1	0	20
<i>Kalimeris integrifolia</i>	173	40	441	109	20
<i>Lantana camara</i>	16	2	26	8	20
<i>Pentas lanceolata</i>	26	9	54	10	20
<i>Phlox paniculata</i>	19	2	39	9	20
<i>Phytolacca americana</i>	25	10	42	9	20
<i>Plectranthus</i> sp.	26	12	40	9	20
<i>Rudbeckia hirta</i>	1	1	1	0	20
<i>Solidago nemoralis</i>	26	9	49	11	20
<i>Vitex agnus-castus</i>	42	10	130	29	20
	Corolla Length (mm)				
	mean	min	max	SD	N
<i>Agastache foeniculum</i>	7	6	9	1	20
<i>Angelonia Angustifolia</i>	4	2	5	1	20
<i>Antirrhinum majus</i>	28	24	32	2	20
<i>Asclepia tuberosa</i>	6	5	6	0	20
<i>Caryopteris clandonensis</i>	7	5	8	1	20
<i>Coreopsis verticillata</i>	3	2	4	1	20
<i>Echinacea purpurea</i>	24	18	34	4	20
<i>Kalimeris integrifolia</i>	3	2	4	1	20
<i>Lantana camara</i>	12	11	14	1	20
<i>Pentas lanceolata</i>	16	12	19	2	20
<i>Phlox paniculata</i>	25	20	27	2	20
<i>Phytolacca americana</i>	2	2	2	0	20
<i>Plectranthus</i> sp.	16	1	20	4	20
<i>Rudbeckia hirta</i>	4	3	5	1	10
<i>Solidago nemoralis</i>	3	3	4	0	20

Table S1.2. (continued)

<i>Vitex agnus-castus</i>	8	6	9	1	20
	<b>Corolla Width (mm)</b>				
	mean	min	max	SD	N
<i>Agastache foeniculum</i>	1.93	1.54	2.58	0.31	20
<i>Angelonia Angustifolia</i>	5.14	4.32	6.05	0.54	20
<i>Antirrhinum majus</i>	14.89	11.41	16.82	1.49	20
<i>Asclepia tuberosa</i>	3.10	2.29	3.81	0.38	20
<i>Caryopteris clandonensis</i>	2.45	1.75	3.23	0.49	20
<i>Coreopsis verticillata</i>	6.88	5.81	7.98	0.69	20
<i>Echinacea purpurea</i>	24.38	14.57	32.01	4.39	20
<i>Kalimeris integrifolia</i>	5.55	3.04	8.42	1.52	20
<i>Lantana camara</i>	1.00	0.74	1.54	0.20	20
<i>Pentas lanceolata</i>	2.38	1.45	3.67	0.55	20
<i>Phlox paniculata</i>	3.51	2.96	4.00	0.32	20
<i>Phytolacca americana</i>	1.76	1.53	2.21	0.17	20
<i>Plectranthus</i> sp.	2.07	1.37	2.89	0.44	20
<i>Rudbeckia hirta</i>	12.11	9.79	16.81	2.08	10
<i>Solidago nemoralis</i>	1.74	1.15	2.79	0.48	20
<i>Vitex agnus-castus</i>	3.34	2.21	4.15	0.49	20
	<b>Floral size</b>				
	mean	min	max	SD	N
<i>Agastache foeniculum</i>	7.24	5.70	8.38	0.73	20
<i>Angelonia Angustifolia</i>	5.72	4.87	7.24	0.67	20
<i>Antirrhinum majus</i>	31.50	27.21	36.11	2.56	20
<i>Asclepia tuberosa</i>	6.33	5.21	7.00	0.45	20
<i>Caryopteris clandonensis</i>	6.95	5.01	8.40	0.98	20
<i>Coreopsis verticillata</i>	5.75	4.86	6.70	0.54	20
<i>Echinacea purpurea</i>	32.60	24.45	45.12	5.73	20
<i>Kalimeris integrifolia</i>	5.70	3.91	7.85	1.01	20
<i>Lantana camara</i>	11.34	10.16	12.69	0.68	20
<i>Pentas lanceolata</i>	15.15	11.38	17.60	1.58	20
<i>Phlox paniculata</i>	23.31	19.13	25.55	1.81	20
<i>Phytolacca americana</i>	2.56	2.16	2.88	0.19	20
<i>Plectranthus</i> sp.	15.33	2.43	18.47	3.49	20
<i>Rudbeckia hirta</i>	9.31	7.31	12.68	1.45	20
<i>Solidago nemoralis</i>	3.56	2.92	4.29	0.39	20
<i>Vitex agnus-castus</i>	8.19	6.81	9.46	0.69	20
	<b>Floral shape</b>				

Table S1.2. (continued)

	mean	min	max	SD	N
<i>Agastache foeniculum</i>	1.74	0.95	2.38	0.40	20
<i>Angelonia Angustifolia</i>	-2.74	-4.16	-2.06	0.52	20
<i>Antirrhinum majus</i>	0.15	-1.43	2.65	1.06	20
<i>Asclepia tuberosa</i>	-0.09	-0.69	0.53	0.35	20
<i>Caryopteris clandonensis</i>	0.98	0.32	1.58	0.37	20
<i>Coreopsis verticillata</i>	-4.71	-5.62	-3.10	0.67	20
<i>Echinacea purpurea</i>	-10.05	-13.25	-3.31	2.50	20
<i>Kalimeris integrifolia</i>	-3.23	-5.32	-1.14	1.24	20
<i>Lantana camara</i>	5.01	4.55	5.82	0.33	20
<i>Pentas lanceolata</i>	5.51	3.83	6.74	0.83	20
<i>Phlox paniculata</i>	8.65	6.27	9.64	0.88	20
<i>Phytolacca americana</i>	-0.61	-1.02	-0.42	0.15	20
<i>Plectranthus</i> sp.	5.96	-1.41	8.10	2.01	20
<i>Rudbeckia hirta</i>	-8.73	-12.24	-6.82	1.72	10
<i>Solidago nemoralis</i>	-0.05	-0.85	0.52	0.39	20
<i>Vitex agnus-castus</i>	0.64	-0.02	1.97	0.50	20

Table S1.3. Experiment 1, Deposition: Post-hoc analysis (Tukey's HSD) for the number of droplets on flowers for the species-based model for deposition.

Contrast	Estimate	SE	df	t-ratio	P-value
<i>Antirrhinum majus</i> - <i>Caryopteris clandonensis</i>	1.0834	0.3995	179	2.7116	0.101
<i>Antirrhinum majus</i> - <i>Coreopsis verticillata</i>	-0.8808	0.4087	179	-2.1553	0.325
<i>Antirrhinum majus</i> - <i>Lantana camara</i>	1.1217	0.4091	179	2.7422	0.094
<i>Antirrhinum majus</i> - <i>Pentas lanceolata</i>	-1.0471	0.3927	179	-2.6667	0.113
<i>Antirrhinum majus</i> - <i>Rudbeckia hirta</i>	-3.0919	0.3890	179	-7.9489	<0.0001
<i>Antirrhinum majus</i> - <i>Vitex agnus</i>	-0.0448	0.4059	179	-0.1104	1.000
<i>Caryopteris clandonensis</i> - <i>Coreopsis verticillata</i>	-1.9642	0.3134	179	-6.2673	<0.0001
<i>Caryopteris clandonensis</i> - <i>Lantana camara</i>	0.0383	0.3078	179	0.1246	1.000
<i>Caryopteris clandonensis</i> - <i>Pentas lanceolata</i>	-2.1305	0.2865	179	-7.4368	<0.0001
<i>Caryopteris clandonensis</i> - <i>Rudbeckia hirta</i>	-4.1753	0.2912	179	-14.3362	<0.0001
<i>Caryopteris clandonensis</i> - <i>Vitex agnus</i>	-1.1282	0.3054	179	-3.6945	0.005
<i>Coreopsis verticillata</i> - <i>Lantana camara</i>	2.0026	0.3211	179	6.2359	<0.0001
<i>Coreopsis verticillata</i> - <i>Pentas lanceolata</i>	-0.1663	0.3007	179	-0.5529	0.998
<i>Coreopsis verticillata</i> - <i>Rudbeckia hirta</i>	-2.2110	0.3041	179	-7.2716	<0.0001
<i>Coreopsis verticillata</i> - <i>Vitex agnus</i>	0.8360	0.3186	179	2.6237	0.125
<i>Lantana camara</i> - <i>Pentas lanceolata</i>	-2.1688	0.2911	179	-7.4496	<0.0001
<i>Lantana camara</i> - <i>Rudbeckia hirta</i>	-4.2136	0.3021	179	-13.9476	0
<i>Lantana camara</i> - <i>Vitex agnus</i>	-1.1665	0.3104	179	-3.7587	0.004
<i>Pentas lanceolata</i> - <i>Rudbeckia hirta</i>	-2.0448	0.2798	179	-7.3067	<0.0001

Table S1.3. (continued)

<i>Pentas lanceolata</i> – <i>Vitex agnus</i>	1.0023	0.2897	179	3.4601	0.012
<i>Rudbeckia hirta</i> – <i>Vitex agnus</i>	3.0471	0.2986	179	10.2052	<0.0001

Table S1.4. Experiment 1, Deposition: Post-hoc analysis (Tukey's HSD) for the number of droplets inside the corolla for the species-based model.

Contrast	Estimate	SE	df	t-ratio	p-value
<i>Antirrhinum majus</i> - <i>Caryopteris clandonensis</i>	-0.599	0.599	180	-1.0006	0.953
<i>Antirrhinum majus</i> - <i>Coreopsis verticillata</i>	-0.660	0.669	180	-0.9871	0.956
<i>Antirrhinum majus</i> - <i>Lantana camara</i>	-0.583	0.602	180	-0.9687	0.960
<i>Antirrhinum majus</i> – <i>Pentas lanceolata</i>	21.400	4950.545	180	0.0043	1
<i>Antirrhinum majus</i> - <i>Rudbeckia hirta</i>	-3.097	0.609	180	-5.0872	<0.0001
<i>Antirrhinum majus</i> – <i>Vitex agnus</i>	-0.585	0.609	180	-0.9598	0.962
<i>Caryopteris clandonensis</i> - <i>Coreopsis verticillata</i>	-0.061	0.473	180	-0.1287	1.000
<i>Caryopteris clandonensis</i> - <i>Lantana camara</i>	0.016	0.372	180	0.0433	1
<i>Caryopteris clandonensis</i> - <i>Pentas lanceolata</i>	21.999	4950.545	180	0.0044	1
<i>Caryopteris clandonensis</i> - <i>Rudbeckia hirta</i>	-2.497	0.383	180	-6.5152	<0.0001
<i>Caryopteris clandonensis</i> - <i>Vitex agnus</i>	0.014	0.384	180	0.0376	1
<i>Coreopsis verticillata</i> - <i>Lantana camara</i>	0.077	0.476	180	0.1615	1.000
<i>Coreopsis verticillata</i> - <i>Pentas lanceolata</i>	22.060	4950.545	180	0.0045	1
<i>Coreopsis verticillata</i> - <i>Rudbeckia hirta</i>	-2.437	0.485	180	-5.0215	<0.0001
<i>Coreopsis verticillata</i> - <i>Vitex agnus</i>	0.075	0.486	180	0.1549	1.000
<i>Lantana camara</i> – <i>Pentas lanceolata</i>	21.983	4950.545	180	0.0044	1
<i>Lantana camara</i> - <i>Rudbeckia hirta</i>	-2.514	0.388	180	-6.4764	<0.0001
<i>Lantana camara</i> – <i>Vitex agnus</i>	-0.002	0.389	180	-0.0043	1
<i>Pentas lanceolata</i> - <i>Rudbeckia hirta</i>	-24.497	4950.545	180	-0.0049	1

Table S1.4. (continued)

<i>Pentas lanceolata</i> – <i>Vitex agnus</i>	-21.985	4950.545	180	-0.0044	1
<i>Rudbeckia hirta</i> – <i>Vitex agnus</i>	2.512	0.399	180	6.2879	<0.0001

Table S1.5. Experiment 1, Deposition: Post-hoc analysis (Tukey's HSD) for the number of droplets outside the corolla for the species-based model.

Contrast	estimate	SE	df	t-ratio	p-value
<i>Antirrhinum majus</i> - <i>Caryopteris clandonensis</i>	23.738	6468.043	180	0.004	1
<i>Antirrhinum majus</i> - <i>Coreopsis verticillata</i>	-0.955	0.419	180	-2.280	0.259
<i>Antirrhinum majus</i> - <i>Lantana camara</i>	23.443	6943.342	180	0.003	1
<i>Antirrhinum majus</i> - <i>Pentas lanceolata</i>	-1.382	0.402	180	-3.434	0.013
<i>Antirrhinum majus</i> - <i>Rudbeckia hirta</i>	-3.040	0.397	180	-7.658	<0.0001
<i>Antirrhinum majus</i> - <i>Vitex agnus</i>	-0.090	0.419	180	-0.215	1
<i>Caryopteris clandonensis</i> - <i>Coreopsis verticillata</i>	-24.692	6468.043	180	-0.004	1
<i>Caryopteris clandonensis</i> - <i>Lantana camara</i>	-0.295	9489.235	180	0.000	1
<i>Caryopteris clandonensis</i> - <i>Pentas lanceolata</i>	-25.119	6468.043	180	-0.004	1
<i>Caryopteris clandonensis</i> - <i>Rudbeckia hirta</i>	-26.778	6468.043	180	-0.004	1
<i>Caryopteris clandonensis</i> - <i>Vitex agnus</i>	-23.828	6468.043	180	-0.004	1
<i>Coreopsis verticillata</i> - <i>Lantana camara</i>	24.397	6943.342	180	0.004	1
<i>Coreopsis verticillata</i> - <i>Pentas lanceolata</i>	-0.427	0.304	180	-1.403	0.799
<i>Coreopsis verticillata</i> - <i>Rudbeckia hirta</i>	-2.086	0.309	180	-6.755	<0.0001
<i>Coreopsis verticillata</i> - <i>Vitex agnus</i>	0.864	0.325	180	2.663	0.114
<i>Lantana camara</i> - <i>Pentas lanceolata</i>	-24.824	6943.342	180	-0.004	1
<i>Lantana camara</i> - <i>Rudbeckia hirta</i>	-26.483	6943.342	180	-0.004	1
<i>Lantana camara</i> - <i>Vitex agnus</i>	-23.533	6943.342	180	-0.003	1
<i>Pentas lanceolata</i> - <i>Rudbeckia hirta</i>	-1.659	0.282	180	-5.890	<0.0001

Table S1.5. (continued)

<i>Pentas lanceolata</i> – <i>Vitex agnus</i>	1.292	0.288	180	4.486	0.0003
<i>Rudbeckia hirta</i> - <i>Vitex</i> <i>agnus</i>	2.950	0.304	180	9.708	<0.0001

Table S1.6. Experiment 1, Deposition: Post-hoc analysis (Tukey's HSD) for the number of droplets on the calix for the species-based model.

Contrast	estimate	SE	df	t-ratio	p-value
<i>Antirrhinum majus</i> - <i>Caryopteris clandonensis</i>	-0.32	0.98	179	-0.327	1.000
<i>Antirrhinum majus</i> - <i>Coreopsis verticillata</i>	-1.50	1.02	179	-1.474	0.760
<i>Antirrhinum majus</i> - <i>Lantana camara</i>	0.27	1.02	179	0.261	1.000
<i>Antirrhinum majus</i> - <i>Pentas lanceolata</i>	21.93	12354.01	179	0.002	1
<i>Antirrhinum majus</i> - <i>Rudbeckia hirta</i>	-4.26	0.96	179	-4.442	0.000
<i>Antirrhinum majus</i> - <i>Vitex agnus</i>	23.01	18799.20	179	0.001	1
<i>Caryopteris clandonensis</i> - <i>Coreopsis verticillata</i>	-1.18	0.64	179	-1.858	0.511
<i>Caryopteris clandonensis</i> - <i>Lantana camara</i>	0.59	0.60	179	0.976	0.959
<i>Caryopteris clandonensis</i> - <i>Pentas lanceolata</i>	22.25	12354.01	179	0.002	1
<i>Caryopteris clandonensis</i> - <i>Rudbeckia hirta</i>	-3.94	0.54	179	-7.353	<0.0001
<i>Caryopteris clandonensis</i> - <i>Vitex agnus</i>	23.33	18799.20	179	0.001	1
<i>Coreopsis verticillata</i> - <i>Lantana camara</i>	1.77	0.69	179	2.555	0.146
<i>Coreopsis verticillata</i> - <i>Pentas lanceolata</i>	23.43	12354.01	179	0.002	1
<i>Coreopsis verticillata</i> - <i>Rudbeckia hirta</i>	-2.76	0.61	179	-4.508	<0.0001
<i>Coreopsis verticillata</i> - <i>Vitex agnus</i>	24.52	18799.20	179	0.001	1
<i>Lantana camara</i> - <i>Pentas lanceolata</i>	21.66	12354.01	179	0.002	1
<i>Lantana camara</i> - <i>Rudbeckia hirta</i>	-4.53	0.60	179	-7.527	<0.0001
<i>Lantana camara</i> - <i>Vitex agnus</i>	22.75	18799.20	179	0.001	1
<i>Pentas lanceolata</i> - <i>Rudbeckia hirta</i>	-26.19	12354.01	179	-0.002	1

Table S1.6 (continued)

<i>Pentas lanceolata</i> – <i>Vitex agnus</i>	1.08	22495.14	179	0.000	1
<i>Rudbeckia hirta</i> – <i>Vitex agnus</i>	27.28	18799.20	179	0.001	1

Table S1.7. Experiment 1, Deposition: Post-hoc analysis (Tukey's HSD) for the number of droplets on flowers in the cage for the species-based model.

Contrast	estimate	SE	df	t-ratio	p-value
<i>Antirrhinum majus</i> - <i>Caryopteris clandonensis</i>	1.9207	0.3557	180	5.400	<0.0001
<i>Antirrhinum majus</i> - <i>Coreopsis verticillata</i>	-0.6754	0.3607	180	-1.873	0.501
<i>Antirrhinum majus</i> - <i>Lantana camara</i>	1.4751	0.3565	180	4.138	0.001
<i>Antirrhinum majus</i> - <i>Pentas lanceolata</i>	-0.8608	0.3317	180	-2.595	0.134
<i>Antirrhinum majus</i> - <i>Rudbeckia hirta</i>	-2.3936	0.3399	180	-7.042	<0.0001
<i>Antirrhinum majus</i> - <i>Vitex agnus</i>	-0.4485	0.3434	180	-1.306	0.848
<i>Caryopteris clandonensis</i> - <i>Coreopsis verticillata</i>	-2.5961	0.2937	180	-8.840	<0.0001
<i>Caryopteris clandonensis</i> - <i>Lantana camara</i>	-0.4456	0.2864	180	-1.556	0.710
<i>Caryopteris clandonensis</i> - <i>Pentas lanceolata</i>	-2.7815	0.2542	180	-10.940	<0.0001
<i>Caryopteris clandonensis</i> - <i>Rudbeckia hirta</i>	-4.3143	0.2696	180	-16.002	<0.0001
<i>Caryopteris clandonensis</i> - <i>Vitex agnus</i>	-2.3692	0.2682	180	-8.833	<0.0001
<i>Coreopsis verticillata</i> - <i>Lantana camara</i>	2.1505	0.2915	180	7.377	<0.0001
<i>Coreopsis verticillata</i> - <i>Pentas lanceolata</i>	-0.1854	0.2599	180	-0.713	0.992
<i>Coreopsis verticillata</i> - <i>Rudbeckia hirta</i>	-1.7182	0.2757	180	-6.231	<0.0001
<i>Coreopsis verticillata</i> - <i>Vitex agnus</i>	0.2269	0.2734	180	0.830	0.982
<i>Lantana camara</i> - <i>Pentas lanceolata</i>	-2.3359	0.2492	180	-9.373	<0.0001
<i>Lantana camara</i> - <i>Rudbeckia hirta</i>	-3.8687	0.2688	180	-14.394	<0.0001
<i>Lantana camara</i> - <i>Vitex agnus</i>	-1.9236	0.2625	180	-7.327	<0.0001
<i>Pentas lanceolata</i> - <i>Rudbeckia hirta</i>	-1.5328	0.2345	180	-6.536	<0.0001

Table S1.7. (continued)

<i>Pentas lanceolata</i> – <i>Vitex agnus</i>	0.4123	0.2260	180	1.824	0.534
<i>Rudbeckia hirta</i> – <i>Vitex agnus</i>	1.9451	0.2500	180	7.779	<0.0001

Table S1.8. Percentage of trials in which all *Crithidia bombi* cells died after 3 h for each plant species.

<b>Plant species</b>	<b>n trial</b>	<b>n trials where all cells die after 3 h</b>	<b>% of trials where all cells died</b>
<i>Agastache foeniculum</i>	42	33	78.6%
<i>Angelonia angustifolia</i>	32	28	87.5%
<i>Antirrhinum majus</i>	34	8	23.5%
<i>Asclepia tuberosa</i>	42	32	76.2%
<i>Coreopsis verticillata</i>	54	51	94.4%
<i>Echinacea purpurea</i>	40	33	82.5%
<i>Kalimeris integrifolia</i>	44	39	88.6%
<i>Lantana Camara</i>	42	35	83.3%
<i>Pentas lanceolata</i>	12	7	58.3%
<i>Phlox paniculata</i>	42	9	21.4%
<i>Phytolacca americana</i>	20	15	75.0%
<i>Plectranthus</i> sp.	32	7	21.9%
<i>Solidago nemoralis</i>	22	18	81.8%
<i>Vitex agnus-castus</i>	40	22	55.0%

Table S1.9. Experiment 2, Survival: Post-hoc analysis (Tukey's HSD) for the hazard ratio of different flowers species on *C. bombi* for the species-based model.

Contrast	estimate	SE	z-ratio	p-value
<i>Agastache foeniculum</i> - <i>Angelonia angustifolia</i>	0.149	0.312	0.476	1.000
<i>Agastache foeniculum</i> - <i>Antirrhinum majus</i>	1.590	0.326	4.871	<0.0001
<i>Agastache foeniculum</i> - <i>Asclepia tuberosa</i>	0.339	0.232	1.463	0.964
<i>Agastache foeniculum</i> - <i>Coreopsis verticillata</i>	0.031	0.220	0.140	1.000
<i>Agastache foeniculum</i> - <i>Echinacea purpurea</i>	-0.292	0.283	-1.029	0.998
<i>Agastache foeniculum</i> - <i>Kalimeris integrifolia</i>	0.479	0.312	1.537	0.948
<i>Agastache foeniculum</i> - <i>Lantana Camara</i>	0.206	0.216	0.951	0.999
<i>Agastache foeniculum</i> - <i>Phlox paniculata</i>	2.121	0.364	5.824	<0.0001
<i>Agastache foeniculum</i> - <i>Phytolacca americana</i>	0.360	0.280	1.287	0.987
<i>Agastache foeniculum</i> - <i>Plectranthus</i>	2.259	0.420	5.374	0.000
<i>Agastache foeniculum</i> - <i>Solidago nemoralis</i>	0.241	0.326	0.741	1.000
<i>Agastache foeniculum</i> - ( <i>Vitex agnus-castus</i> )	0.791	0.244	3.237	0.063
<i>Angelonia angustifolia</i> - <i>Antirrhinum majus</i>	1.441	0.315	4.580	0.0003
<i>Angelonia angustifolia</i> - <i>Asclepia tuberosa</i>	0.191	0.301	0.634	1.000
<i>Angelonia angustifolia</i> - <i>Coreopsis verticillata</i>	-0.118	0.279	-0.422	1.000
<i>Angelonia angustifolia</i> - <i>Echinacea purpurea</i>	-0.440	0.188	-2.337	0.490
<i>Angelonia angustifolia</i> - <i>Kalimeris integrifolia</i>	0.331	0.216	1.533	0.949
<i>Angelonia angustifolia</i> - <i>Lantana Camara</i>	0.057	0.308	0.185	1.000
<i>Angelonia angustifolia</i> - <i>Phlox paniculata</i>	1.972	0.426	4.630	0.0003
<i>Angelonia angustifolia</i> - <i>Phytolacca americana</i>	0.212	0.337	0.628	1.000

Table S1.9. (continued)

<i>Angelonia angustifolia</i> - <i>Plectranthus</i>	2.110	0.425	4.966	0.0001
<i>Angelonia angustifolia</i> - <i>Solidago nemoralis</i>	0.093	0.248	0.375	1.000
<i>Angelonia angustifolia</i> - ( <i>Vitex agnus-castus</i> )	0.642	0.328	1.957	0.761
<i>Antirrhinum majus</i> - <i>Asclepia tuberosa</i>	-1.251	0.328	-3.815	0.009
<i>Antirrhinum majus</i> - <i>Coreopsis verticillata</i>	-1.559	0.314	-4.971	0.0001
<i>Antirrhinum majus</i> - <i>Echinacea purpurea</i>	-1.882	0.293	-6.416	0.0001
<i>Antirrhinum majus</i> - <i>Kalimeris integrifolia</i>	-1.111	0.316	-3.519	0.026
<i>Antirrhinum majus</i> - <i>Lantana Camara</i>	-1.384	0.328	-4.225	0.002
<i>Antirrhinum majus</i> - <i>Phlox paniculata</i>	0.531	0.439	1.209	0.993
<i>Antirrhinum majus</i> - <i>Phytolacca americana</i>	-1.230	0.362	-3.396	0.038
<i>Antirrhinum majus</i> - <i>Plectranthus</i>	0.669	0.458	1.458	0.965
<i>Antirrhinum majus</i> - <i>Solidago nemoralis</i>	-1.348	0.334	-4.033	0.004
<i>Antirrhinum majus</i> - ( <i>Vitex agnus-castus</i> )	-0.799	0.346	-2.311	0.509
<i>Asclepia tuberosa</i> - <i>Coreopsis verticillata</i>	-0.309	0.237	-1.304	0.986
<i>Asclepia tuberosa</i> - <i>Echinacea purpurea</i>	-0.631	0.273	-2.308	0.511
<i>Asclepia tuberosa</i> - <i>Kalimeris integrifolia</i>	0.140	0.301	0.465	1.000
<i>Asclepia tuberosa</i> - <i>Lantana Camara</i>	-0.134	0.240	-0.557	1.000
<i>Asclepia tuberosa</i> - <i>Phlox paniculata</i>	1.781	0.379	4.704	0.0002
<i>Asclepia tuberosa</i> - <i>Phytolacca americana</i>	0.021	0.295	0.071	1.000
<i>Asclepia tuberosa</i> - <i>Plectranthus</i>	1.919	0.424	4.529	0.0004
<i>Asclepia tuberosa</i> - <i>Solidago nemoralis</i>	-0.098	0.317	-0.308	1.000
<i>Asclepia tuberosa</i> - ( <i>Vitex agnus-castus</i> )	0.452	0.265	1.702	0.894

Table S1.9. (continued)

<i>Coreopsis verticillata</i> - <i>Echinacea purpurea</i>	-0.322	0.251	-1.287	0.987
<i>Coreopsis verticillata</i> - <i>Kalimeris integrifolia</i>	0.448	0.280	1.604	0.929
<i>Coreopsis verticillata</i> - <i>Lantana Camara</i>	0.175	0.227	0.770	1.000
<i>Coreopsis verticillata</i> - <i>Phlox paniculata</i>	2.090	0.372	5.625	<0.0001
<i>Coreopsis verticillata</i> - <i>Phytolacca americana</i>	0.329	0.283	1.163	0.995
<i>Coreopsis verticillata</i> - <i>Plectranthus</i>	2.228	0.414	5.384	<0.0001
<i>Coreopsis verticillata</i> - <i>Solidago nemoralis</i>	0.211	0.298	0.708	1.000
<i>Coreopsis verticillata</i> - ( <i>Vitex agnus-castus</i> )	0.760	0.255	2.987	0.127
<i>Echinacea purpurea</i> - <i>Kalimeris integrifolia</i>	0.771	0.191	4.035	0.004
<i>Echinacea purpurea</i> - <i>Lantana Camara</i>	0.497	0.280	1.776	0.862
<i>Echinacea purpurea</i> - <i>Phlox paniculata</i>	2.412	0.406	5.945	<0.0001
<i>Echinacea purpurea</i> - <i>Phytolacca americana</i>	0.652	0.313	2.083	0.676
<i>Echinacea purpurea</i> - <i>Plectranthus</i>	2.550	0.408	6.245	<0.0001
<i>Echinacea purpurea</i> - <i>Solidago nemoralis</i>	0.533	0.226	2.361	0.472
<i>Echinacea purpurea</i> - ( <i>Vitex agnus-castus</i> )	1.082	0.302	3.587	0.020
<i>Kalimeris integrifolia</i> - <i>Lantana Camara</i>	-0.274	0.308	-0.888	1.000
<i>Kalimeris integrifolia</i> - <i>Phlox paniculata</i>	1.641	0.426	3.855	0.008
<i>Kalimeris integrifolia</i> - <i>Phytolacca americana</i>	-0.119	0.337	-0.353	1.000
<i>Kalimeris integrifolia</i> - <i>Plectranthus</i>	1.779	0.425	4.182	0.002
<i>Kalimeris integrifolia</i> - <i>Solidago nemoralis</i>	-0.238	0.250	-0.951	0.999
<i>Kalimeris integrifolia</i> - ( <i>Vitex agnus-castus</i> )	0.312	0.328	0.950	0.999
<i>Lantana Camara</i> - <i>Phlox paniculata</i>	1.915	0.371	5.168	<0.0001

Table S1.9. (continued)

<i>Lantana Camara</i> - <i>Phytolacca americana</i>	0.155	0.286	0.540	1.000
<i>Lantana Camara</i> - <i>Plectranthus</i>	2.053	0.422	4.862	<0.0001
<i>Lantana Camara</i> - <i>Solidago nemoralis</i>	0.036	0.323	0.111	1.000
<i>Lantana Camara</i> - ( <i>Vitex agnus-castus</i> )	0.585	0.254	2.308	0.511
<i>Phlox paniculata</i> - <i>Phytolacca americana</i>	-1.760	0.410	-4.297	0.001
<i>Phlox paniculata</i> - <i>Plectranthus</i>	0.138	0.513	0.269	1.000
<i>Phlox paniculata</i> - <i>Solidago nemoralis</i>	-1.879	0.436	-4.307	0.001
<i>Phlox paniculata</i> - ( <i>Vitex agnus-castus</i> )	-1.330	0.387	-3.437	0.034
<i>Phytolacca americana</i> - <i>Plectranthus</i>	1.898	0.451	4.209	0.002
<i>Phytolacca americana</i> - <i>Solidago nemoralis</i>	-0.119	0.352	-0.338	1.000
<i>Phytolacca americana</i> - ( <i>Vitex agnus-castus</i> )	0.431	0.308	1.398	0.975
<i>Plectranthus</i> – <i>Solidago nemoralis</i>	-2.017	0.439	-4.597	0.0003
<i>Plectranthus</i> – <i>Vitex agnus-castus</i>	-1.468	0.436	-3.363	0.043
<i>Solidago nemoralis</i> - ( <i>Vitex agnus-castus</i> )	0.549	0.342	1.607	0.928

Table S1.10. Experiment 2, Survival: Comparison between models that included the location of the inoculum droplet on the flower with a model that did not include the location of the droplet.

<b>Plant species</b>	<b><i>X</i><sup>2</sup></b>	<b><i>DF</i></b>	<b><i>P</i></b>
<i>Agastache foeniculum</i>	3.7386	1	0.0532
<i>Angelonia angustifolia</i>	0.1039	1	0.7472
<i>Antirrhinum majus</i>	1.8969	1	0.1684
<i>Asclepia tuberosa</i>	0.00081	1	0.9768
<i>Coreopsis verticillata</i>	0.1654	1	0.684
<i>Echinacea purpurea</i>	21.3	1	<0.0001
<i>Kalimeris integrifolia</i>	1.4302	1	0.2317
<i>Lantana Camara</i>	0.1021	1	0.7493
<i>Phlox paniculata</i>	3.5962	1	0.0601
<i>Vitex agnus-castus</i>	2.8487	1	0.0914

Table S2.1. *Nosema ceranae* spores per bee in each honey bee hive that was screened. The nine hives with the lowest spore counts were used in the experiment. The two colonies with the highest spore counts were used as inoculum source.

Hive	Estimated spores per bee	Treatment assigned
13	74400	Source of inoculum
14	57600	Source of inoculum
10	49533	
5	33333	
15	26933	
4	24467	
3	20533	Low (1%)
1	9400	Control
7	7533	High (10%)
9	600	High (10%)
6	467	Control
2	333	Low (1%)
12	67	Low (1%)
8	0	Control
11	0	High (10%)

Table S2.2. Statistical summary of parameters measured to estimate bumble bee colony performance.

Parameter	Welch t-test	DF	P-value
$\Delta$ colony weight	-0.5209	8.61	0.616
# New queens produced	1.4142	10.00	0.188
# Drones produced	0.1877	5.66	0.655
Weight drones	0.4187	5.72	0.691
Final # workers in colony	-0.1638	6.74	0.875
Weight workers	-1.0075	8.98	0.340
Total # of workers produced	-0.1390	7.49	0.893
Final # of pupa (workers)	0.7094	8.09	0.498
Weight pupa (worker)	1.0499	5.09	0.341
Final # larva	-0.9636	8.14	0.363
Weight larva	-0.3024	7.49	0.771
Final # eggs	-1.4353	9.85	0.182
Weight eggs	-1.1567	5.05	0.299

Table S3.1. Odds ratio of the incidence model.

Predictors	Odds Ratios	95% CI	<i>P</i>
(Intercept)	32.78	3.37 – 319.04	0.003
treatment [AB]	0.19	0.08 – 0.47	<0.001
treatment [AA]	0.29	0.08 – 1.00	0.051
passes	0.64	0.50 – 0.82	<0.001
wing	0.40	0.17 – 0.92	0.032
treatment [AB] * passes	1.95	1.30 – 2.91	0.001
treatment [AA] * passes	1.61	1.20 – 2.16	0.002

Table S3.2. Odds ratio of the intensity model.

<b>Predictors</b>	<b>Odds Ratio</b>	<b>95% CI</b>	<b><i>P</i></b>
(Intercept)	9.39	1.63 -54.6	0.012
passes	1.08	0.91 -1.28	0.364
treatment [AB]	0.27	0.14 -0.53	<0.001
treatment [AA]	0.74	0.29 -1.86	0.527
wing	0.77	0.40 -1.51	0.449
passes * treatment [AB]	1.65	1.23 - 2.23	0.001
passes * treatment [AA]	0.90	0.74 - 1.09	0.279

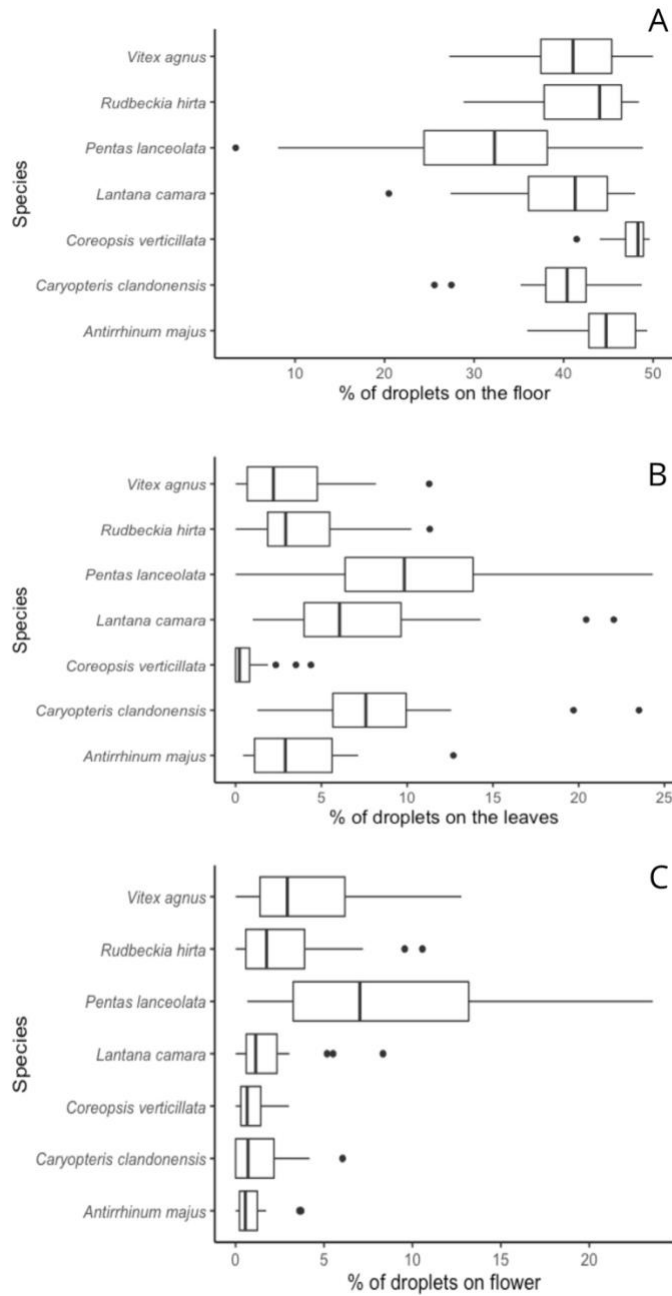


Figure S1.1. Experiment 1, Deposition: Species identity had a significant effect on the percentage of droplets per cage that ended up on (A) the floor of the cage, (B) leaves, and (C) flowers. Figures depict boxplots.

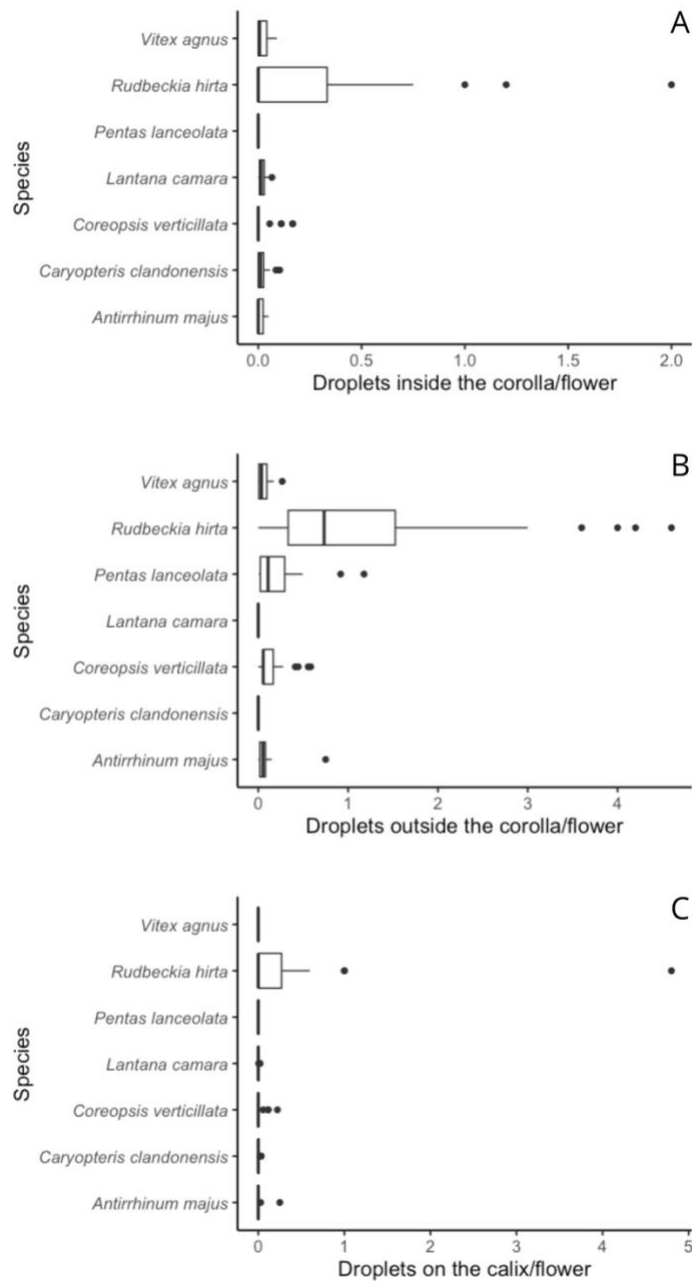
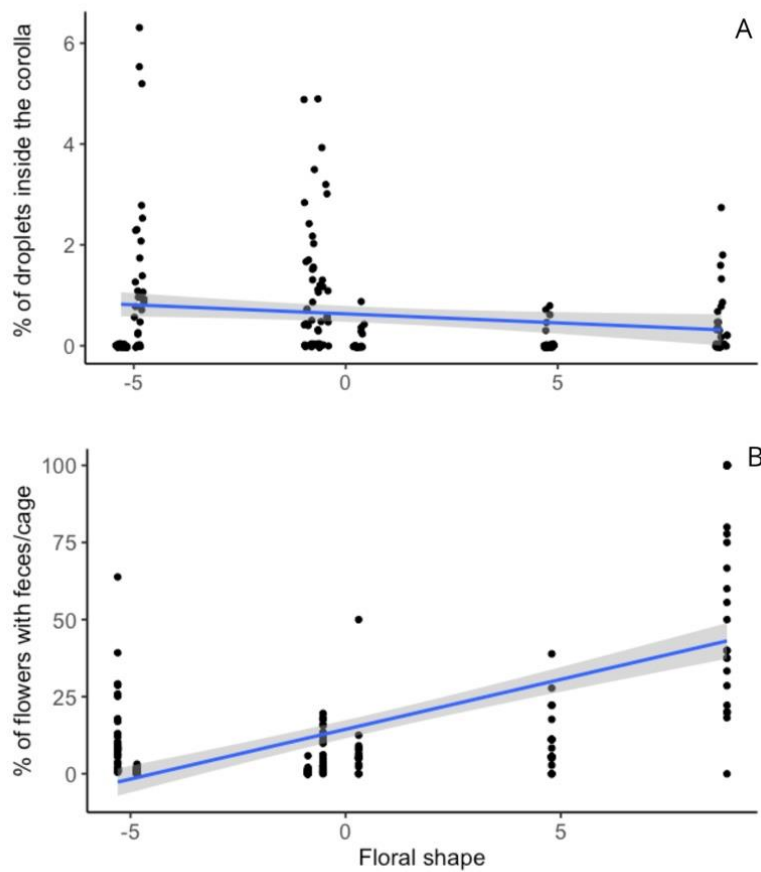


Figure S1.2. Experiment 1, Deposition: Species identity had a significant effect on the proportion of the total number of droplets per cage that ended up A) inside the corolla, B) outside the corolla and C) the calix, to the number of flowers in the cage.



Figures S1.3. Experiment 1, Deposition: Examples of a positive and negative association between floral shape and two measures of feces deposition: (A) relationship between the percentage of total number of droplets in the cage that ended up inside the corolla and floral shape, and (B) relationship between the percentage of flowers in the cage that had fecal droplets on them and floral shape.

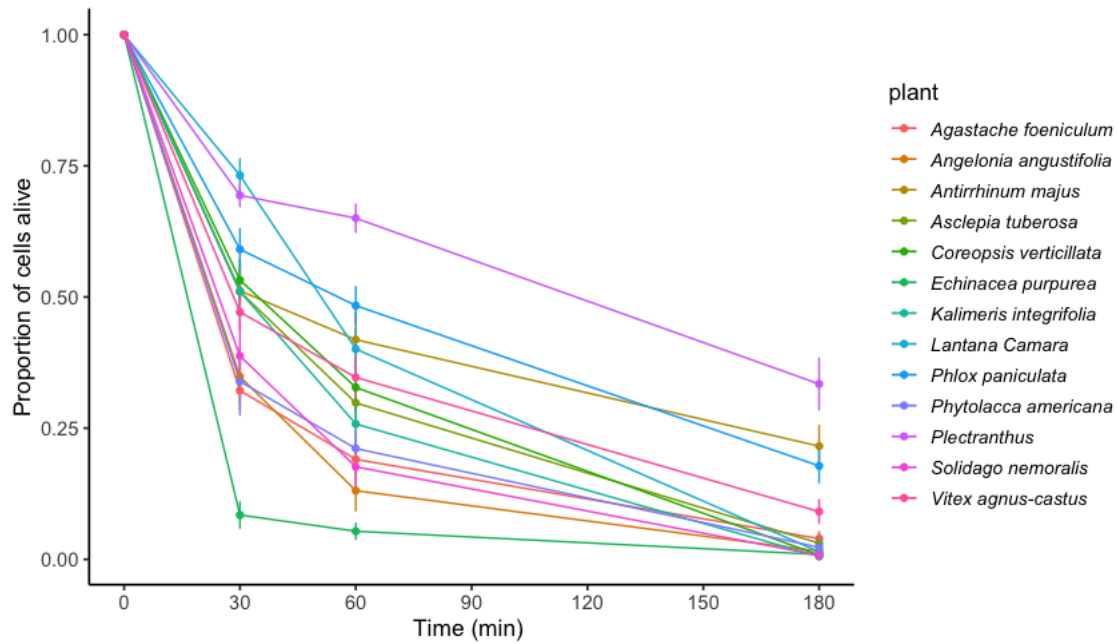


Figure S1.4. Experiment 2, Survival: Proportion of *C. bombi* cells alive per droplet on flowers after 30 min, 1 h and 3h. This figure combines survival data for both flower locations for the 11 species plant species where two locations were tested (see Table 1).

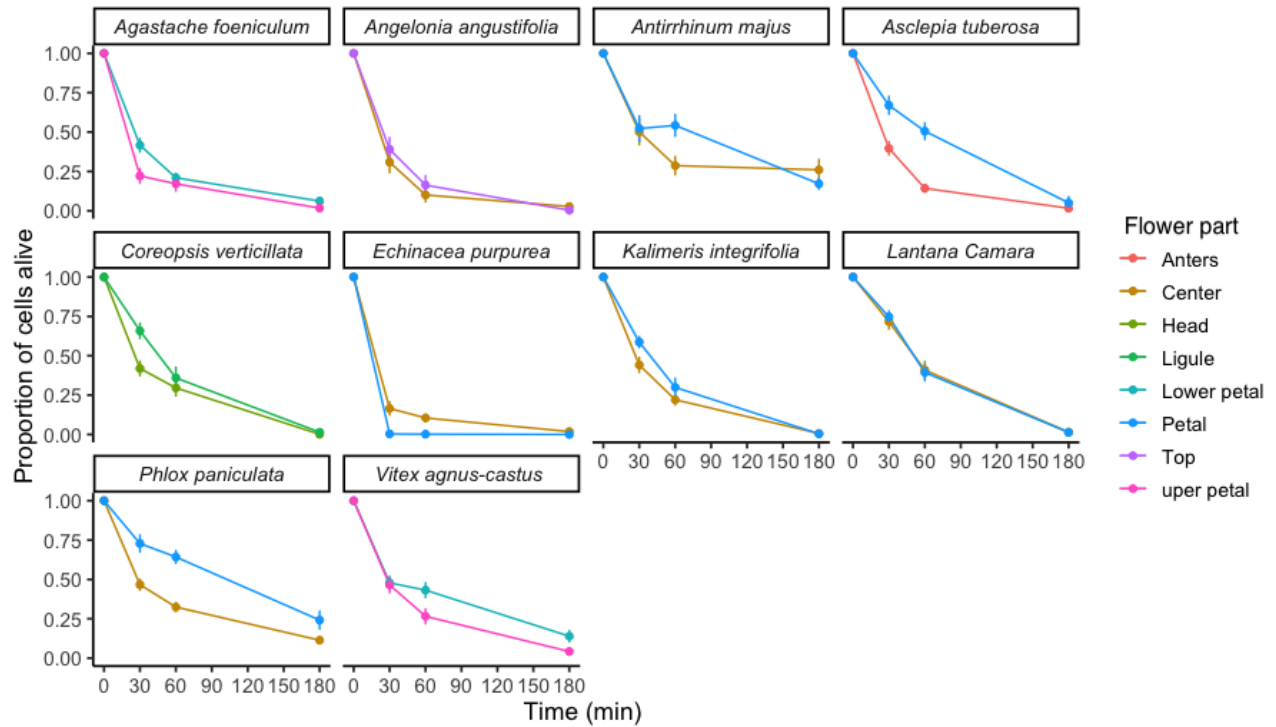


Figure S1.5. Experiment 2, Survival: Proportion of *C. bombi* cells alive per droplet on different floral parts of 10 plant species where two location per flowers were tested. *Plectranthus* sp. is not included due to high censoring for this species. We found significant differences between flower parts only for *E. purpurea*.

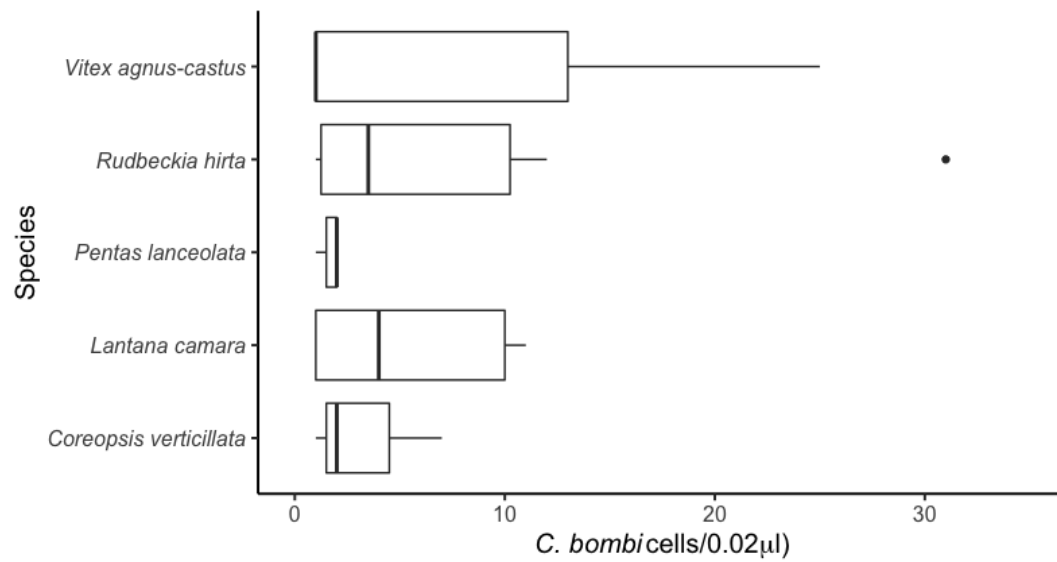


Figure S1.6. Experiment 3, Acquisition: Boxplot of the intensity of infection (cells/0.02  $\mu$ l) of bees that got infected during the acquisition trials. Species identity was not a significant predictor in the species-based model.

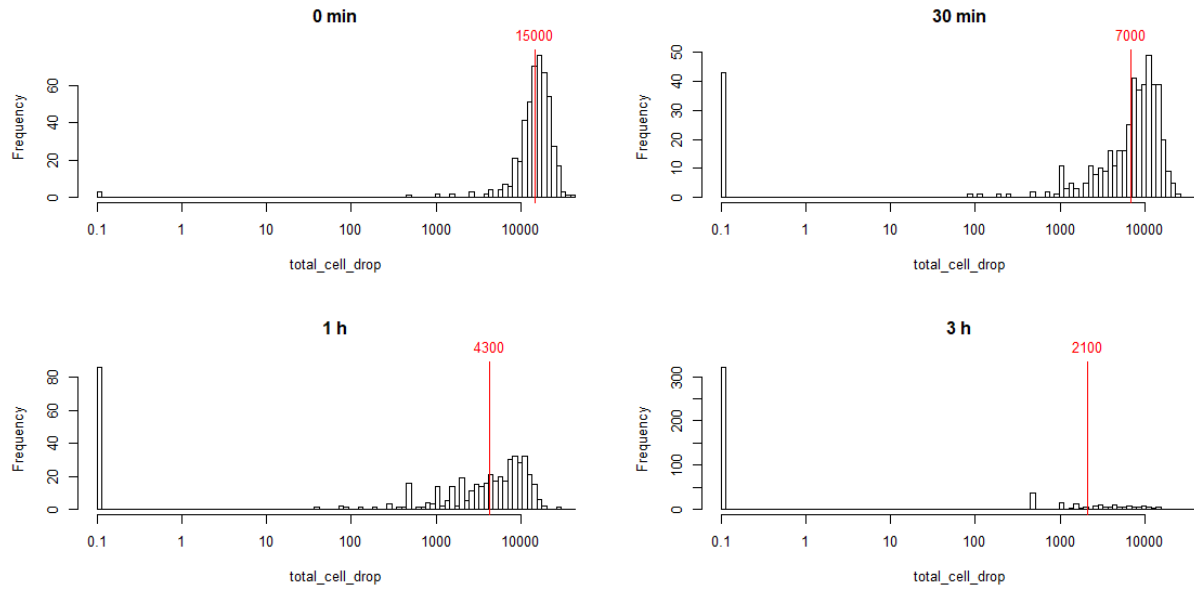


Figure S1.7. Histograms for the log transformed number of cells in a droplet, but excluding zeros. This figure combines all plant species and location of droplet. The red lines represent the geometric means, the leftwards picks in frequency indicate gradual decontamination of the droplets, while the reduction in the magnitude of the peaks over time indicates a sudden decontamination, probably due to the evaporation of the droplets.

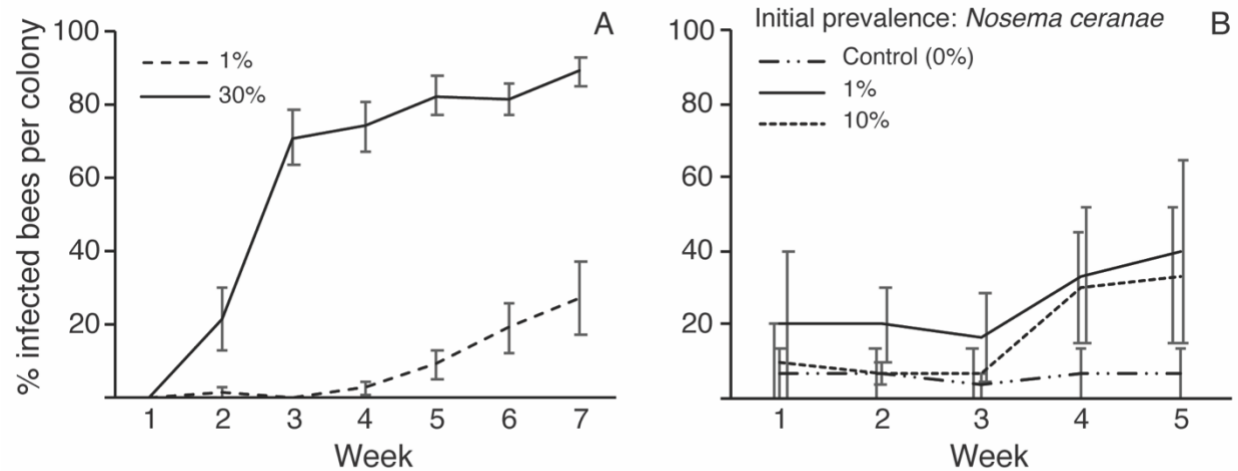


Figure S2.1. Mean ( $\pm$ SE) intensity of the prevalence of (A) *Crithidia bombi* in bumble bee (*Bombus impatiens*) colonies where 1 or 30% of initial workers were experimentally infected (six colonies per treatment) and (B) *Nosema ceranae* in honey bee (*Apis mellifera*) colonies where none (control), 1 or 10% of workers were experimentally infected (three colonies per treatment) Error bars were jittered to improve clarity.

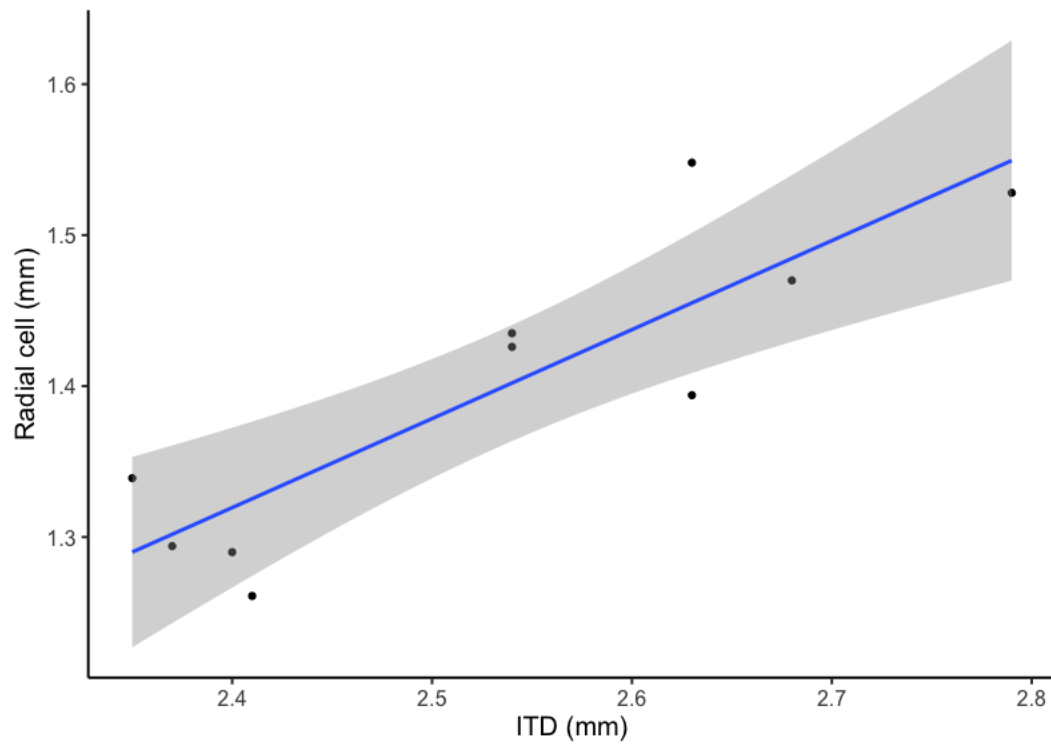


Figure S3.1. Relationship between length of the radial cell and intertegular distance (ITD) of *Megachile rotundata*. There is a significant correlation between the two variables.

## **Appendix B: Floral trait measurements**

Figure S4. The following images depict what we measured as corolla length and width for each flower species. In all pictures below, ‘CL’ indicates corolla length and ‘CW’ indicates corolla width. The blue dots indicate the location(s) where we placed the inoculum for the survival experiment. Not all species were included in the survival experiment, and for some species we only teste one location.

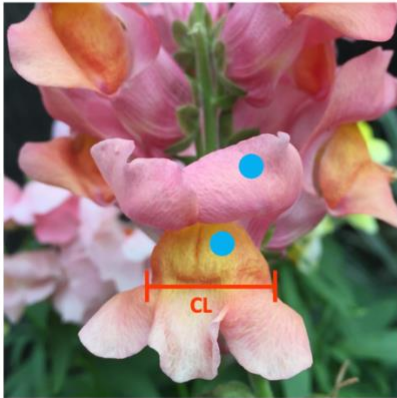
*Agastache foeniculum*



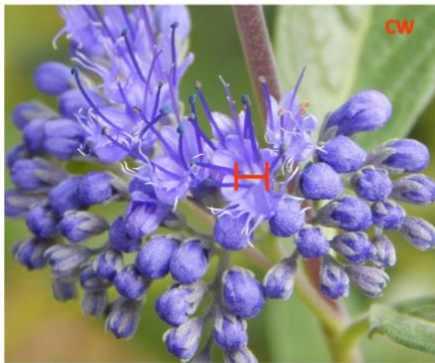
*Angelonia angustifolia*



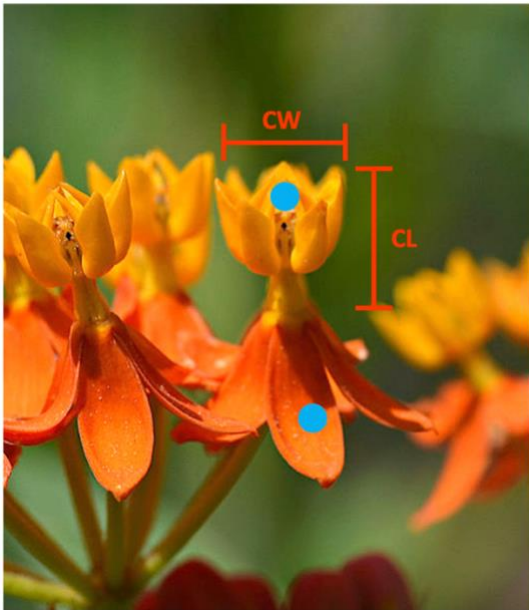
*Antirrhinum majus*



*Caryopteris clandonensis*



*Asclepia tuberosa*



*Lantana camara*



*Coreopsis verticillata*



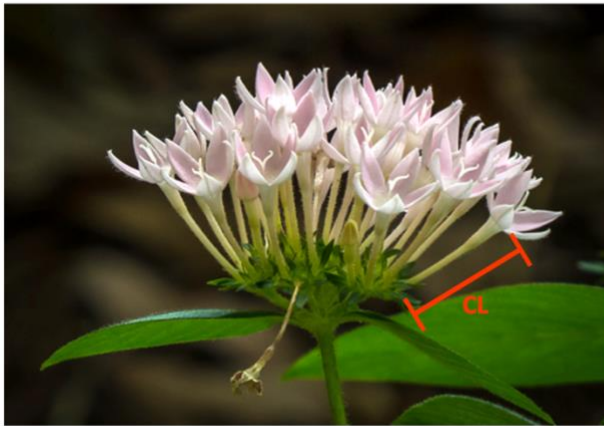
*Echinacea purpurea*



*Kalimeris integrifolia*



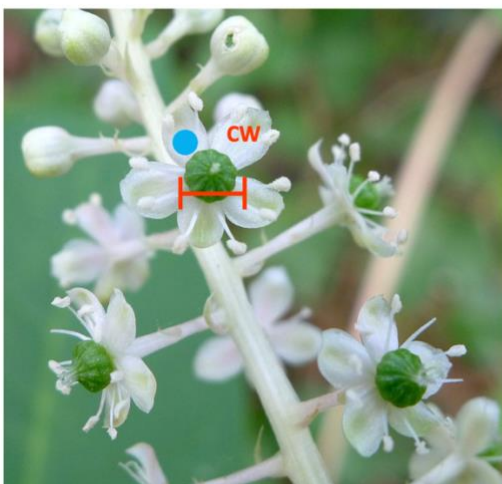
*Pentas lanceolata*



*Phlox paniculata*



*Phytolacca americana*



*Plectanthus* sp.



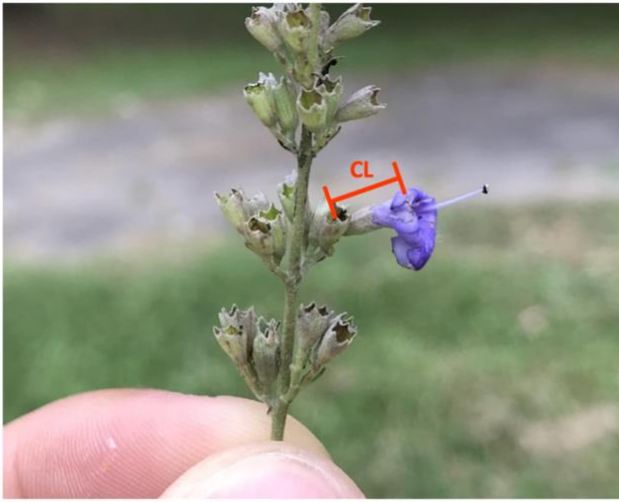
*Rudbeckia hirta*



*Solidago nemoralis*



*Vitex agnus-castus*



## Appendix C: Predicting droplet lifetimes using the Cox proportional hazards

The lifetime of a fecal droplet is given by the area under the survival curve. However, since the survival curves were censored at 3 hours, it is impossible to estimate the area without assuming a parametric form of the survival curve. At the same time, the assumption of proportional hazards in the Cox model implies that once we know the survival curve  $S_0(t)$  for droplets on a plant with risk score  $r_0$ , we will automatically know the survival curve  $S(t)$  for droplets on any plants with risk score  $r$ , since  $S(t) = S_0(t) \wedge (r/r_0)$ . This motivated the following procedure.

First, we generated empirical droplet survival curves for each plant species. Barring uncertainties in the estimated proportions, the survival curves appeared to be concave for slowly-decaying species, linear for intermediate species, and convex for rapidly-decaying species. In the proportional hazards framework, this suggested that a linear parametric form  $S_0(t) = 1 - a \cdot t$  at some intermediate (but for now unknown) risk score  $r_0$  could be appropriate, since then  $S(t) = (1 - a \cdot t) \wedge (r/r_0)$  would be convex for species with  $r > r_0$  (rapid), linear for species with  $r = r_0$  (intermediate), and concave for species with  $r < r_0$  (slow), in agreement with the empirical curves.

Next, we calculated the risk score of each species using the best traits-based Cox model. We then constructed a dataset, where each "observation" contained the time elapsed  $t$ , the risk score  $r_i$  for some species  $i$ , and the surviving proportion of droplets  $S_{it}$  for that species at time  $t$ . We then fitted the dataset using the nonlinear least squares model  $S_{it} = (1 - a \cdot t) \wedge (r_i/r_0) + \varepsilon_{it}$  to estimate the unknown parameters  $a$  and  $r_0$ . With  $a$  and  $r_0$ , we could then estimate the lifetime of

a droplet on any plant with risk score  $r$  using the area under the corresponding parametric survival curve, which turned out to have the very simple form  $1 / [a \cdot (r/r_0 + 1)]$ .

Fig. S1.8 shows for each plant species, (a) the empirical survival curve, (b) the parametric survival curve estimated using the above procedure but with risk scores from a species-based Cox model, and (c) the parametric survival curve from a trait-based Cox model. The purpose of this figure is to show that the procedure can indeed generate reasonable-looking parametric survival curves, especially for the species-based Cox model where there are enough model degrees of freedom for a much closer fit of the species risk scores to the data.

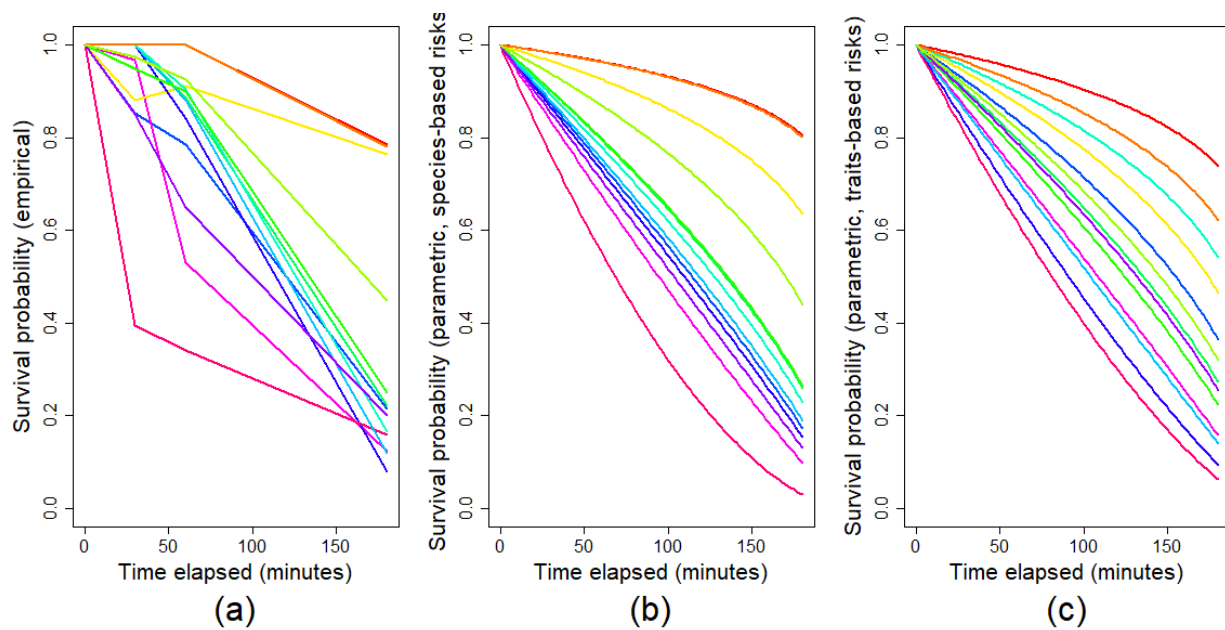


Figure S5. Survival curves of droplets on different plant species. Each color corresponds to one species. (a) Empirical. (b) Parametric with proportional hazards, based on risk scores calculated from a species-based Cox model. (c) Parametric with proportional hazards, based on risk scores calculated from a traits-based Cox model.