THE ROYAL SOCIETY PUBLISHING

PROCEEDINGS B

Bee pathogen transmission dynamics: deposition, persistence and acquisition on flowers

Laura L. Figueroa, Malcolm Blinder, Cali Grincavitch, Angus Jelinek, Emilia K. Mann, Liam A. Merva, Lucy E. Metz, Amy Y. Zhao, Rebecca E. Irwin, Scott H. McArt and Lynn S. Adler

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Note: Reports are unedited and appear as submitted by the referee. The review history appears in chronological order.

Review History

RSPB-2019-0603.R0 (Original submission)

Review form: Reviewer 1 (Matías Maggi)

Recommendation
Accept with minor revision (please list in comments)

Scientific importance: Is the manuscript an original and important contribution to its field?
Good

General interest: Is the paper of sufficient general interest?
Good

Quality of the paper: Is the overall quality of the paper suitable?
Excellent

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
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Is it clear?
N/A

Is it adequate?
N/A

Do you have any ethical concerns with this paper?
No

Comments to the Author
The manuscript provide interesting information related to adquisition of trypanosomatids parasites on bumblebees. I find the work well performed, with a clear introduction and objectives. I have some doubts that I marked on the third experiment. I have made some minor comments in the file attached (See Appendix A).

Review form: Reviewer 2 (Margarita Lopez-Uribe)

Recommendation
Accept with minor revision (please list in comments)

Scientific importance: Is the manuscript an original and important contribution to its field?
Excellent

General interest: Is the paper of sufficient general interest?
Good

Quality of the paper: Is the overall quality of the paper suitable?
Excellent

Is the length of the paper justified?
Yes

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Is it accessible?
No

Is it clear?
Yes

Is it adequate?
No

Do you have any ethical concerns with this paper?
No

Comments to the Author
This study presents results from a series of experiments to understand the mechanisms of pathogen transmission between pollinators via flowers. The main goals of the study are to: (1) determine if infected bees tend to defecate more on flowers, (2) frequency of deposition changes with flower shape, and (3) pathogen survival and transmission varies with flower morphology. it was a pleasure to read this manuscript. It is clear and well-written. The experiments were well-done and the statistical analyses are clearly presented and seem appropriate.

I only have minor comments on the current version of this manuscript.

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* L74 - This sentence is unclear. The question of whether bee infection increases deposition in flowers is easy to understand after the previous sentence. But why deposition may vary with floral morphology is not discussed until the next paragraph.

*L99 - Perhaps the authors could provide more specific predictions about how they expected pathogen survival and infectiousness to vary in flowers with different morphologies and under different environmental conditions. These predictions would help the reader understand earlier on what the different experiments are testing.

* L102-109 - This paragraph would be clearer if predictions were followed after the questions outlined here. In addition, it would probably be better to briefly describe the 3 experiments before the methods section. For example, what environmental conditions did you investigate?

Also, the phrasing of objective 4 is unclear. I suggest you change if for: “pathogen acquisition and subsequent infection of bees vary among different parts of the flower in different plant species”.

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* Figure 4 - I suggest you add the results for Monarda even if they were not significant. Or at least, mentioned in the legend in what way the data were not significant. Did all the Crithidia die or did they all survive in both treatments?

Decision letter (RSPB-2019-0603.R0)

15-Apr-2019

Dear Ms Figueroa:

Your manuscript has now been peer reviewed and the reviews have been assessed by an Associate Editor. The reviewers’ comments (not including confidential comments to the Editor) and the comments from the Associate Editor are included at the end of this email for your reference. As you will see, the reviewers and the Editors have raised some concerns with your manuscript and we would like to invite you to revise your manuscript to address them.

We do not allow multiple rounds of revision so we urge you to make every effort to fully address all of the comments at this stage. If deemed necessary by the Associate Editor, your manuscript will be sent back to one or more of the original reviewers for assessment. If the original reviewers are not available we may invite new reviewers. Please note that we cannot guarantee eventual acceptance of your manuscript at this stage.

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Best wishes,
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Associate Editor
Comments to Author:
The two reviewers were generally positive about this manuscript. They thought the manuscript is well written, the analyses correct and the results interesting and potentially appealing for the broad readership of Proceedings B. At the same time, both reviewers had several suggestions for improvement of the manuscript, particularly Reviewer 2, especially regarding the manuscript's title, the unclear relevance of some of the results, and the need for clarification or revision of parts of the methods, results and discussion sections and one figure (Fig. 4). Minor revisions should allow the authors to incorporate these suggestions.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)
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Author's Response to Decision Letter for (RSPB-2019-0603.R0)

See Appendix B.

Decision letter (RSPB-2019-0603.R1)

03-May-2019

Dear Ms Figueroa

I am pleased to inform you that your manuscript entitled "Bee pathogen transmission dynamics: deposition, persistence and acquisition on flowers" has been accepted for publication in Proceedings B.

You can expect to receive a proof of your article from our Production office in due course, please check your spam filter if you do not receive it. PLEASE NOTE: you will be given the exact page length of your paper which may be different from the estimation from Editorial and you may be asked to reduce your paper if it goes over the 10 page limit.

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Sincerely,

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Associate Editor:
Board Member
Comments to Author:
The authors have incorporated the comments made by the two reviewers. I think this is a great study of transmission dynamics of bee pathogens. I congratulate the authors for their excellent work.

Appendix A

PROCEEDINGS OF
THE ROYAL SOCIETY B
BIOLOGICAL SCIENCES

Mechanisms mediating bee pathogen transmission: deposition, persistence and acquisition on flowers

Journal:	*Proceedings B*
Manuscript ID	RSPB-2019-0603
Article Type:	Research
Date Submitted by the Author:	13-Mar-2019
Complete List of Authors:	Figueroa, Laura; Cornell University, Entomology Blinder, Malcolm; University of Massachusetts Amherst, Biology Grincavitch, Cali; University of Massachusetts Amherst Jelinek, Angus; University of Massachusetts Amherst, Biology Mann, Emilia; University of Massachusetts Amherst, Biology Merva, Liam; University of Massachusetts Amherst, Biology Metz, Lucy; University of Massachusetts Amherst, Biology Zhao, Amy; University of Massachusetts Amherst, Biology Irwin, Rebecca; North Carolina State University, Department of Applied Ecology McArt, Scott; Cornell University, Entomology Adler, Lynn; University of Massachusetts Amherst, Biology
Subject:	Ecology < BIOLOGY, Health and Disease and Epidemiology < BIOLOGY
Keywords:	Bombus impatiens, Crithidia bombi, pollinator health, disease spread, floral morphology
Proceedings B category:	Ecology

Mechanisms mediating bee pathogen transmission: deposition, persistence and acquisition on flowers

Laura L. Figueroa[1*], Malcolm Blinder[2], Cali Grincavitch[2], Angus Jelinek[2], Emilia K. Mann[2], Liam A Merva[2], Lucy E. Metz[2], Amy Y. Zhao[2], Rebecca E. Irwin[3], Scott H. McArt[1] and Lynn S. Adler[2]

[1]Department of Entomology, Cornell University, Ithaca, NY 14853, USA

[2]Department of Biology, University of Massachusetts, Amherst, MA, 01003, USA

[3]Department of Applied Ecology, North Carolina State University, 127 David Clark Labs, Raleigh, NC 27695 USA

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Abstract

Infectious diseases are a primary driver of bee decline worldwide, but limited understanding of how pathogens are transmitted hampers effective management. Flowers have been implicated as hubs of bee disease transmission, but we know little about how interspecific floral variation affects transmission dynamics. Using bumble bees (*Bombus impatiens*), a trypanosomatid pathogen (*Crithidia bombi*), and three plant species varying in floral morphology, we assessed how host infection and plant species affect pathogen deposition on flowers, and plant species and flower parts impacted pathogen survival and acquisition at flowers. We found that host infection with *Crithidia* increased defecation rates on flowers, and that bees deposited feces onto bracts of *Lobelia siphilitica* and *Lythrum salicaria* more frequently than onto *Monarda didyma* bracts. However, *Crithidia* mortality after deposition was higher on *Lobelia* and *Lythrum* than *Monarda* flowers. Among flower parts, bracts were associated with lowest pathogen survival but highest resulting infection intensity in bee hosts. These results suggest the efficiency of pathogen transmission depends on where deposition occurs and the timing and place of acquisition, which varies among plant species. This information could be utilized for development of wildflower mixes that maximize forage while minimizing disease spread.

Keywords: *Bombus impatiens*; *Crithidia bombi*; pollinator health; disease spread; floral morphology

Introduction

Infectious diseases are a global concern for both humans and wildlife, with examples ranging from the shifting ecology of Ebola virus [1] to the rapid and devastating expansion of the chytrid fungus in amphibian populations [2]. Pathogens are one of the primary threats to pollinator health [3]. However, how infectious diseases spread across pollinator communities is poorly understood, limiting effective conservation. Specifically, the mechanisms mediating bee pathogen transmission through shared use of flowers are largely unknown [4, 5], despite flowers being linked to pathogen spillover and spread [6]. Increasing dependence on bees for crop pollination heightens the urgency to understand disease transmission dynamics [7].

Effective disease transmission requires that pathogens be deposited onto a plant species and flower part where they can survive long enough to be encountered by, acquired, and infect new susceptible hosts. Recent findings that transmission rates vary across flower species and floral traits [5, 8, 9] show that infected foraging bees can transmit disease to susceptible bees that subsequently visit the same flowers [8, 9]. Yet the mechanisms governing how pathogen transmission occurs on flowers, including deposition, survival, and acquisition of bee pathogens, are largely unknown. Such information could help us predict which plants are more likely than others to function as disease hubs, which is important given the increasing role that wildflower plantings play in pollinator protection efforts [10].

Infection can alter behavior and physiology in ways that facilitate or impede disease spread. For example, infection can induce changes in the social network of ant colonies in ways that suppress pathogen transmission [11]. Conversely, honey bees infected with the fecal-orally transmitted

67 microsporidian *Nosema apis* often present symptoms of dysentery, which facilitates spread
68 within the colony [ ]. Whether infection-induced changes could influence defecation rates on
69 flowers is unknown. Bumble bees infected with *Crithidia bombi*, a fecal-orally transmitted
70 trypanostomatid pathogen, are cognitively impaired [13] and less efficient foragers [14, 15],
71 spending more time learning floral information and consequently visiting each flower for more
72 time. Either of these mechanisms, physiologically induced defecation or altered foraging
73 patterns, could result in more feces deposited on flowers by infected bees. Whether infection
74 affects bee defecation patterns on flowers and how this varies across plant species represents a
75 serious knowledge gap in bee disease transmission dynamics.
76
77 The ways bees interact with flowers vary greatly across floral morphologies and architectures,
78 and depend on traits of the bees themselves, such as body size. Depending on the interaction
79 between a bee and a flower, defecation patterns and pathogen deposition may be altered [4].
80 Moreover, bee size is highly variable across and within bee species, and may play an important
81 role in pathogen deposition on flowers [5]. For example, small bodied bees may fit entirely
82 within flowers with long tubular corollas, resulting in higher likelihood of pathogen deposition
83 inside the corolla tube than for larger bees that can only access the nectar at the end of the
84 tubular corolla via their proboscis. Conversely, for flowers with short corollas, bee feces may be
85 unlikely to be deposited inside the corolla regardless of bee size, but instead may fall onto the
86 bract subtending the flower, or onto other flowers in the inflorescence. These deposition
87 dynamics could have consequences for pathogen survival and transmission, but the role of floral
88 morphology and architecture in mediating host-pathogen dynamics is largely unknown.
89

Once deposited, horizontally transmitted pathogens depend on environmental conditions to remain infectious before being encountered by a new host. For example, the bee microsporidian *Nosema apis* can remain infectious up to six years under optimal conditions, but loses infectivity within hours when exposed to ultra–violet (UV) radiation [16]. Similarly, bumble bees develop a stronger infection when inoculated with freshly prepared *Crithidia bombi* compared to inoculum that has been stored for 45 minutes [17]. Depending on where pathogens are deposited on a plant, their exposure to UV radiation and phytochemicals may vary (e. g., inside a corolla tube compared to an exposed petal). Moreover, pollen and nectar phytochemicals can have growth-inhibitory effects on *C. bombi* [18], and floral volatiles can kill certain plant pathogens [19]. Therefore, we predicted that pathogen survival and infectiousness would vary across parts within the same plant and across species.

We evaluated multiple mechanisms hypothesized to contribute to bee disease transmission through shared use of flowers. Specifically, we investigated whether: (1) infection influences fecal deposition on flowers; (2) the frequency of feces deposited varies with plant species and flower part (inside the corolla, outside the corolla, flower bract and leaves); (3) pathogen survival depends on pathogen deposition and environmental conditions across flower parts; and (4) differences in flower part among plant species affect pathogen acquisition and subsequent infection intensity in bees. This study lies at the intersection of bee foraging ecology and epidemiology, and aims to expand the current understanding of bee disease transmission.

Materials and methods

113 (a) Study system

114 All experiments were conducted using common eastern bumble bee (*Bombus impatiens*) workers
115 and the trypanosome *Crithidia bombi*. Native to eastern North America, *Bombus impatiens*
116 (Hymenoptera, Apidae) is an abundant generalist bee, frequently used for commercial pollination
117 [20]. The pathogen *Crithidia bombi* (Kinetoplastea; Trypanosomatida; hereafter *Crithidia*) is a
118 horizontally transmitted gut pathogen known to reduce bumble bee foraging efficiency and
119 increase mortality under stressful conditions, and is associated with reduced reproduction in wild
120 bumble bee colonies [14, 21, 22]. All experiments were conducted using *Crithidia* from wild *B.*
121 *impatiens* workers collected in Massachusetts, USA (GPS: 42°22'17.53"N 72°35'13.52"W) and
122 maintained in laboratory bumble bee colonies (Biobest, Leamington, Ontario); infected colonies
123 were only used as source of inoculum and not as source of bees in experimental trials. For the
124 duration of the experiments, we conducted weekly pathogen screenings of 5 bees from each
125 experimental colony to ensure colonies were *Crithidia*–free. *Crithidia bombi* species identity was
126 verified by sequencing the 18S rRNA [23].
127
128 This study compared three plant species that are visited by bumble bees in northeastern North
129 America and vary in their floral morphology and architectures: *Monarda didyma* (Lamiaceae),
130 *Lobelia siphilitica* (Campanulaceae), and *Lythrum salicaria* (Lythraceae), hereafter *Monarda,*
131 *Lobelia*, and *Lythrum* (Figure 1). *Monarda* and *Lobelia* are native to eastern North America,
132 whereas *Lythrum* is a non-native species introduced from Europe that is highly abundant and
133 attractive to pollinators [24].
134
135 (b) Experimental protocol

(i) Experiment 1: Effect of plant species and infection status on bee defecation patterns across flower parts

To evaluate the role of infection on bee defecation across plant species, we infected bees with *Crithidia*. The *Crithidia* inoculum used in the trials was prepared fresh daily by dissecting the gut of infected bees maintained in the laboratory and combining with Ringer's solution (Sigma–Aldrich, St. Louis, MO) to create a solution with 1200 cells/µl, which was then mixed with equal amount of 50% sucrose solution to create an inoculum with 25% sucrose and 600 cells per µl [25]. We used 25% sucrose in Ringer's solution without *Crithidia* for a control (sham) inoculum. We selected 18 bees from each of three experimental colonies. Half were infected, while the other half were sham-infected, for a total of 54 bees inoculated each date (13 days: July 10, 12, 16, 19, 21, 26, and 28, and August 1, 3, 9, 10, 17 and 21, 2017), by feeding 10 µl of inoculum or sucrose solution using a micropipette. Three similarly-sized bees of the same treatment and colony were maintained in microcolony containers with 30% sucrose and pollen provided *ad libitum* for 7–12 days prior to trial to allow infection to develop [26].

To determine defecation patterns, bees were given sucrose mixed with fluorescent dye (2.5 g of fluorescent powder (Dayglo Color, Cleveland, OH) dissolved in 500 mL of 30% sucrose) *ad libitum* 24 – 48h prior to field trials. Defecation trials were conducted during summer 2017 (*Monarda* July 10 – 19, *Lythrum* July 21 – August 3, *Lobelia* August 9 – 21). The day of the trial, bees were cooled at 4 °C and transported in a cooler to the field site in Massachusetts (42°28'45.5" N, 72°34'46.06"W). Each trial consisted of a single flight cage (45.7 cm x 71.0 cm x 55.6 cm) in which three clipped field-grown inflorescences were placed in tubes with water, held upright by tube racks. The number of flowers per inflorescence was held constant within

159 species. The bottom of each cage was lined with newspaper, which was replaced before each
160 trial to eliminate cross contamination across trials. Cooling bees prior to trials facilitated
161 foraging. Due to mortality during the period in which infection was allowed to grow, not all trials
162 included three bees; there was no difference in mortality between infected and uninfected bees
163 ($\chi^2_1 = 0.11$, $p = 0.742$), nor did number of bees in a trial affect defecation patterns ($\chi^2_1 = 1.32$, p
164 $= 0.250$ and $\chi^2_1 = 1.67$, $p = 0.200$ for presence/absence and number of fecal droplets,
165 respectively). The number of bees and time when each bee was placed in a cage and started
166 foraging were noted. If bees did not forage within 15 minutes, a flower was raised towards the
167 bees to induce foraging (20% of bees were induced). If presentation of the flower did not induce
168 foraging, that trial was excluded from the experiment. Cages were checked for bee feces three
169 hours after foraging began; the cage was brought into a darkened barn and a handheld black light
170 was used to count the number of fluorescent fecal droplets on each plant part (Escolite UV
171 Flashlight Black Light, 51 LED 395 nM). The plant parts were divided into four categories:
172 "inside" the flower (inside the corolla), "outside" the flower (surface of the corolla), on the bract
173 (on the modified leaf subtending the inflorescence), or on a leaf (excluding the bract; Figure 1).
174 We also recorded feces elsewhere in the cage, to determine the proportion of feces deposited on
175 plants for each plant species. Post-trial, bees were returned to the lab and maintained on 30%
176 sucrose until the following day, when they were dissected to confirm infection status. We
177 removed the right forewing and measured marginal cell length as a proxy for bee size [27].
178
179 *Statistical analyses*
180

Data analyses were conducted using R studio (R version 3.5.1) with the lme4 and lsmeans packages [28-30]. We excluded trials for bees that were inoculated but did not develop infection (n = 3) and control trials in which bees developed infection (n = 3), for a resulting sample size of n = 163 trials (*Lobelia* n = 54, *Lythrum* n = 61, and *Monarda* n = 48). To evaluate the factors that predicted defecation, we constructed a generalized linear mixed model (GLMM) that evaluated feces on plant (presence/absence) as the response, predicted by bee infection status (infected/uninfected), plant species, average bee size, and number of bees in the trial. To determine whether bees were defecating differently across parts of the plant, we developed a GLMM that included number of fecal droplets as the response variable and evaluated part (inside of flower, outside of flower, bract, or leaf), infection status (infected/uninfected), plant species, average bee size, and number of bees in trial as explanatory variables. Both models included observation level (trial), experimental colony, and date as random effects, and fit a Poisson distribution, which is suitable for count data [31]. Experimental colony did not explain variance in either model and affected convergence, so was removed from subsequent analyses. No variable in the model produced a Variance Inflation Factor (VIF) greater than two, indicating low co-linearity [32]. To determine the role of each explanatory variable, we employed a likelihood ratio test to compare the full model to identical models that excluded the variable in question. Significance of interactions was determined by comparing the original model with and without interactions (flower part by either average bee size, plant species, or infection status); we removed non-significant interactions. Significant interactions were evaluated using the *lstrends* function [29].

(ii) Experiment 2: *Crithidia* survival across plant species and flower parts

Pathogen survival was evaluated across plant species and parts on flowers. We made *Crithidia* inoculum based on realistic fecal volumes and sugar concentrations; we did not consider other nutrients or compounds that may be in feces. We used Ringer's solution, a saline solution often used to study insect physiology [33], as we expected it would be a more realistic proxy for bee feces than water. We determined realistic fecal volumes by placing 10 worker *Bombus impatiens* in individual vials for 2 – 4 hours and measuring fecal volume using microcapillary tubes (Sigma–Aldrich: 20 µl). The largest volume observed was 33 µl, so we used 35 µl of *Crithidia* inoculum in trials, representing the upper limit of realistic fecal quantity. Given *Crithidia*'s susceptibility to sugar [34], we evaluated the sugar concentration of bee feces using a refractometer. The values ranged from 0 – 1% sugar, and so, unlike Experiment 1 and 3, no sugar was added to inoculum.

Trials were conducted during summer 2017. Inoculum was made fresh each trial day, with at least 3,300 *Crithidia* cells per microliter of Ringer's solution (mean: 3,617, range 3,300 – 3,900); this high concentration was chosen for ease of visualization in the hemocytometer. We used the same three plant species from Experiment 1, each evaluated in one day: *Monarda* (July 12), *Lythrum* (July 21), and *Lobelia* (August 1). Because environmental conditions and inoculum strength varied between days, and flower species did not have co-occurring blooming periods, we are not able to compare viability across plant species. Flowers were bagged in the field two days prior to trial to avoid pathogen deposition from foraging bees. On the day of the trial, inflorescences were cut, individually marked, and placed in tubes with water. The experiment was conducted in large covered hexagonal tents (71 x 160.5 in). To evaluate the effect of the sun, one tent had a UV-protected cover while the other had a mesh cover that allowed UV exposure

but prevented wild bees from entering. *Monarda* was only evaluated in shaded (UV-protected) conditions due to rainy and overcast weather. Within each tent, we measured the temperature, relative humidity (AcuRite, 01083 Pro Accuracy Indoor Temperature and Humidity Monitor), and ultraviolet radiation (Apogee instruments, MU–100).

We placed 35 µl of inoculum on two parts of each inflorescence (inside corolla and bract; exception was *Monarda* where we also evaluated outside the corolla). We evaluated pathogen survival for three hours, taking five inflorescences every 30 minutes into the laboratory, where the inoculum on each part was pipetted into a hemocytometer to count mobile *Crithidia*. We did not evaluate infectivity of *Crithidia*, using mobility instead as a proxy for survival, in part because infectiousness of *Crithidia* is highly variable, even within a single day [35]. If the inoculum evaporated, we pipetted 10 µl of distilled water onto the part to collect any *Crithidia* cells and checked for mobile *Crithidia*; we were successful in detecting mobile *Crithidia* in some instances when the inoculum had visibly evaporated. The sample size for the shaded samples were: *Lobelia* n = 58 parts (29 inflorescences), *Lythrum* n = 60 (30 inflorescences), and *Monarda* n = 88 (31 inflorescences). The sample sizes for sun-exposed plants were: *Lobelia* n = 58 (29 inflorescences) and *Lythrum* n = 60 (30 inflorescences).

Statistical analyses

We conducted survival analyses using Cox proportional hazards mixed–models via the coxme package in RStudio [30, 36]. The survival analysis evaluated *Crithidia* survival (count of moving cells per 0.02 µl) by time elapsed when the flower was inspected for each of the three plant

species. The model included part on flower and shade treatment as explanatory variables, as well as individual plant as the random effect. To determine significance of the treatments (flower part and shade), we conducted a likelihood ratio test comparing the full model of each species with a model that included the same random effect structure but excluded either explanatory variable or included an additive relationship instead of an interaction. Differences in survival across flower parts were determined *post hoc* with Tukey's HSD using the *lsmeans* function [29].

(iii) Experiment 3: Effects of plant species and flower part on pathogen acquisition and subsequent intensity of infection

We evaluated the effect of plant species and flower part on *Crithidia* transmission by placing pathogen inoculum on flowers, allowing uninfected bees to forage, and subsequently determining infection (presence/absence and intensity) in the bees. Trials were conducted in 2016 on *Monarda* (June 30 – July 15), *Lythrum* (July 18 – Aug 9), and *Lobelia* (Aug 18 – 26). Experimental bees and inoculum were transported to the field site in a cooler with insulated ice packs. We used bees from 4 experimental colonies for *Monarda*, 5 for *Lythrum*, and 6 for *Lobelia*; colonies mostly overlapped for the first two species and had approximately 50% overlap for the second and third species. For each trial, we collected an inflorescence of the target species at the field site and placed it in a tube filled with water. Each trial was randomly assigned to one of three treatments of inoculum placement: inside corolla, outside corolla, or bract. For all the treatments, we added four 10 µl drops of inoculum (see Experiment 1 for inoculum preparation) on the inflorescence in the specified treatment part using a micropipette (Figure 1); inoculated flowers were marked using a paint pen. Inflorescences were from field-grown plants that were bagged with mesh for at least two days prior to trials to prevent *Crithidia* deposition from wild

foraging bees. We placed the prepared inflorescence in a small flight cage and released a single, chilled worker bee into the cage (see Experiment 1 for cage details). We allowed the bee to forage and recorded total time spent foraging (i.e., probing flowers, not including time moving between flowers), number of flowers probed, and number of drops probed. We also recorded the time of the trial so that we could calculate elapsed time between inoculum preparation and each trial for use as a covariate. When the bee stopped foraging (usually a clear change in behavior from probing flowers to flying around the cage), we recaptured it in a vial. Bees were excluded if they did not probe any inoculum drops or foraged for less than thirty seconds.

Bees were collected and subsequently maintained individually for one week in the laboratory to allow infection to develop. We fed each bee daily 500 µl of 30% sucrose solution and a ~0.15 g pollen ball (30% sucrose and commercial mixed wildflower pollen (Koppert Biological Systems; Linden Apiaries, Walpole, NH, USA)). We maintained the bees in an incubator set at 27 °C in darkness. After seven days, we dissected each bee and placed the gut in 300 µl of Ringer's solution. The mixture was allowed to incubate for four hours before *Crithidia* was quantified using a hemocytometer [25]. We removed the right forewing and measured marginal cell length as a proxy for size [27]. Sample sizes for each species were n = 40 bees for *Monarda*, n = 67 for *Lythrum*, and n = 89 for *Lobelia*.

Statistical analyses

Data analyses were conducted using R studio with packages lme4, DHARMa, RVAidememo and lsmeans [28-30, 37, 38]. To manage zero-inflated and overdispersed count

296 data, we used manual two-step hurdle models [39]. We first evaluated an "incidence" model
297 (evaluating presence or absence of *Crithidia* infection), followed by an "intensity" model
298 (*Crithidia* counts of the infected bees). In the first step, we modeled pathogen incidence using a
299 binomial distribution (logit link), given the binary outcome of whether bees were infected or not.
300 Next, we modeled *Crithidia* intensity when present (i.e., the non-zero outcomes) with a Poisson
301 distribution (log link). We evaluated overdispersion in the Poisson model using the
302 *overdisp.glmer* function in the RVAideMemoire package [38]. To ensure our data were well-
303 modeled by the specified distributions and to check model assumptions, we used the DHARMa
304 package [37]. Our incidence model was evaluated using a GLMM, with presence or absence of
305 infection as the response variable, predicted by flower part, plant species, their interaction, bee
306 size, foraging time, and time since the inoculum was made (related to its infectiousness). The
307 model included colony and date as random effects. The intensity model had the same random
308 effect structure as the incidence model, plus an observation-level random effect to correct for
309 overdispersion [40]. To determine significance, we conducted a likelihood ratio test by
310 comparing the full GLMM model to a model that excluded the factor of interest. Significant
311 factors were determined *post hoc* with Tukey's HSD using the *lsmeans* function [29].
312
313 **Results**
314
315 (i) Experiment 1: Effect of plant species and infection status on bee defecation patterns across
316 flower parts

317 Overall, bees defecated on plants in 65% of trials. Infected bees were more likely to defecate on
318 plants than uninfected bees ($\chi^2_1 = 4.26$, $p = 0.039$; Figure 2), although there was no relationship

between infection status and the number of fecal droplets observed ($\chi^2_1 = 1.05$, $p = 0.306$) or where bees defecated ($\chi^2_3 = 3.78$, $p = 0.287$). Flower part significantly predicted the number of fecal droplets observed ($\chi^2_4 = 23.05$, $p < 0.001$). Moreover, we found a strong plant species by part interaction ($\chi^2_6 = 166.74$, $p < 0.001$; Figure 3a and Table S1), such that the most deposition occurred on leaves and bracts for *Lobelia*, on bracts and inside the flower for *Lythrum*, and outside the flower for *Monarda*. We observed a bee size by flower part interaction for number of fecal droplets observed ($\chi^2_3 = 9.08$, $p = 0.028$; Figure 3b), whereby bigger bees defecated fewer times inside flowers (Tukey HSD: $z = -2.87$, $p = 0.004$). Plant species and average bee size did not predict presence or number of fecal droplets observed on flowers ($\chi^2_2 = 1.32$, $p = 0.517$ and $\chi^2_1 = 0$, $p = 0.991$ respectively for presence of feces; $\chi^2_2 = 0.978$, $p = 0.614$ and $\chi^2_1 = 0.50$, $p = 0.478$ respectively for number of fecal droplets). Bee size had no relationship with number of fecal droplets observed on the outside of the flower, on the bract, or on leaves ($z = 1.55$, $p = 0.122$, $z = 1.11$, $p = 0.268$ and $z = 1.34$ and $p = 0.180$, respectively). The proportion of total fecal droplets that landed on the plants (compared to elsewhere in the cage) varied across plant species ($\chi^2_2 = 28.65$, $p < 0.001$), being 0.55, 0.29 and 0.25 for *Lobelia*, *Lythrum*, and *Monarda* respectively.

(iii) Experiment 2: *Crithidia* survival across plant species and flower parts

Crithidia became non-motile within three hours of placement on flowers in 71% of trials. Furthermore, mortality varied by plant species ($\chi^2_1 = 0.001$, $p < 0.001$), at 90% for *Lobelia*, 90% for *Lythrum* and 20% for *Monarda*. *Crithidia* survival was influenced by flower part on all plant species ($\chi^2_1 = 4.67$, $p = 0.031$, $\chi^2_1 = 5.49$, $p = 0.019$ and $\chi^2_2 = 6.30$, $p = 0.043$ for *Lobelia*,

342 *Lythrum*, and *Monarda* respectively*;* Figure 4a, b). For *Lobelia* and *Lythrum*, *Crithidia* survived
343 longer inside the corolla than on the bract (Tukey HSD test: $z = 2.09$, $p = 0.037$ and $z = 2.29$, $p =$
344 0.022 for *Lobelia* and *Lythrum* respectively). *Post hoc* evaluation of *Crithidia* survival across
345 parts on *Monarda* flowers did not yield significant pairwise comparisons (Table S2), likely due
346 to low overall mortality in this species. *Crithidia* survival was also greater in shaded than sunny
347 conditions ($\chi^2_1 = 6.87$, $p = 0.009$ and $\chi^2_1 = 4.53$, $p = 0.033$ for *Lobelia* and *Lythrum* respectively*;*
348 Figure 4c, d). There was no flower part by sun exposure interaction in either species ($\chi^2_1 = 0.02$,
349 $p = 0.892$ and $\chi^2_1 = 1.48$, $p = 0.223$, for *Lobelia* and *Lythrum*, respectively).
350
351 (ii) Experiment 3: Effects of plant species and flower part on *Crithidia* acquisition and
352 subsequent intensity of infection

353 The probability of becoming infected did not depend on plant species, part where inoculum was
354 placed, their interaction, or bee size ($\chi^2 < 4.68$, $p > 0.137$ for all). However, part on flower did
355 predict *Crithidia* intensity for the infected bees ($\chi^2_2 = 13.66$, $p = 0.001$; Figure 5). Specifically,
356 when bees picked up inoculum on the bract of a flower, they developed a more intense *Crithidia*
357 infection than if they encountered the pathogen on the outside of the flower (Tukey HSD: $z =$
358 3.77, $p < 0.001$). Similarly, bees developed a marginally more intense *Crithidia* infection when
359 encountered on the bract than the inside of the flower ($z = 2.29$, $p = 0.057$). There was no
360 difference in infection intensity between the inside and outside of the flower ($z = 1.35$, $p =$
361 0.370). For infected bees, bee size did not explain *Crithidia* intensity ($\chi^2_1 = 0.83$, $p = 0.363$), nor
362 did plant species ($\chi^2_2 = 1.01$, $p = 0.602$), or plant species by flower part interaction ($\chi^2_4 = 4.54$, p
363 $= 0.338$).
364

Discussion

The intersection of bee foraging ecology and epidemiology is a novel area of research that can give rise to new understanding of pollinator disease spread and evidence-based conservation strategies. Here we show that foraging bumble bees often defecate on plants, and do so more when they are infected with *Crithidia* (Figure 2). There is not a universal part on plants where bees are more likely to defecate. That pattern depends on plant species, which may in turn be related to floral traits, such as shape or size. These deposition dynamics are also influenced by bee traits, with bigger bees defecating fewer times inside flowers (Figure 3b), possibly because they are too large to fit inside the flowers. Similarly, for pathogen survival on flowers, we found differences across flower parts for some species but not for others (Figure 4a). Moreover, the flower part where inoculum is encountered influenced the intensity of the resulting infection (Figure 5), further highlighting the complexity of bee pathogen transmission dynamics via flowers. Taken together, these data suggest variation in plant-pollinator interaction patterns, from encounter rates to trait matching, are expected to influence pathogen transmission and warrant further research.

Bees defecated on plants in 65% of trials, and did so significantly more when infected with *Crithidia* (Figure 2). Increased likelihood of defecation on plants could hasten the spread of multiple diseases, especially because bumble bees are often infected with several fecal-orally transmitted pathogens [16, 41]. Whether the increased defecation is a by-product of dysentery, as in honey bees infected with *Nosema apis* [12] or due to increased time spent on each flower by infected bees [15, 35], remains unknown.

We found a plant species by part interaction on the number of fecal droplets observed, such that each plant species had a different part where droplets were most likely to be found (Figure 3a). Differential handling of the flowers across plant species could have led to this pattern, especially given the diversity of floral morphologies and plant architectures (Figure 1). For *Monarda* (Figure 1c), the inside of the small floral tube is only accessible to the bee proboscis, likely explaining why we seldom observed feces there, compared to the outside of the corolla where the bees crawl to reach subsequent flowers. Similarly, *Lobelia* (Figure 1a) rarely had feces inside of the flower, despite an entirely different floral morphology. The floral tube of *Lobelia* is quite large, such that the entire head of the bees can fit inside, but usually the abdomen protrudes, enabling defecation onto leaves or bracts subtending the flower. However, the smallest bees in the trials fit entirely within the *Lobelia* flowers, likely contributing to the bee size by part interaction. *Lythrum* differed in that it often had feces on the inside of its flowers. This is likely because the tube of *Lythrum* is extremely short and narrow and surrounded by wide, flat petals (Figure 1b), so that bees will crawl over the entire flower after foraging to reach the next flower. These differential deposition dynamics across plant species are the first step towards horizontal transmission, which can result in transferring the pathogen to new colonies via foragers.

Horizontally transmitted pathogens must remain viable to be acquired by a new host. However, the decay rate of many pathogens outside of their host is unknown [42]. *Crithidia* survived longer on the inside of the corolla than the bract of *Lythrum* and *Lobelia* flowers (Figure 4a, b). We had predicted that the inside would provide more protection from desiccation, extending survival compared to more exposed parts. However, we did not observe that pattern for *Monarda*, which aligns with the lower overall *Crithidia* mortality on this species. Floral

411 chemistry or other unknown mechanisms could mediate the lack of differences across parts for
412 this species, as could more humid environmental conditions during the day of trial. In general,
413 we found that within three hours of being placed on flowers, most *Crithidia* had died.
414 Incorporating rate of decay between deposition by infected bees and acquisition by the incoming
415 susceptible foragers could enhance disease spread models [42].
416
417 Once pathogens have been deposited on the plant, environmental factors could influence
418 pathogen survival. *Crithidia* on sun–exposed flowers had shorter survival times than shaded
419 plants (Figure 4c, d). This may be because of UV radiation, temperature, and/or increased
420 desiccation, all of which were greater in the sun–exposed conditions. Pulsed UV radiation can
421 decrease *Crithidia* viability [43]. Otterstatter & Thompson experimentally varied the time and
422 number of *Crithidia* cells placed on *Brassica rapa* nectaries encountered by susceptible foraging
423 bumble bees. They found that most bees visiting flowers with *Crithidia* became infected when
424 the delay was less than 10 minutes, but by 85 minutes the probability was less than 15%,
425 regardless of the dose placed on the flowers [6]. They determined the half-life of *Crithidia* to be
426 77 minutes, largely mirroring our results. Floral mechanisms that maximize exposure to direct
427 sunlight, such as heliotropism, could reduce bee pathogen survival on flowers and warrant
428 further investigation.
429
430 For bees that developed an infection after foraging on inoculated plants (Experiment 3), those
431 that encountered inoculum on the bract had more intense *Crithidia* infections than when they
432 encountered it on the outside of the flower (Figure 5). This pattern may be due to fewer
433 phytochemicals from nectar and pollen encountered on the bract [44]. For *Lobelia* and *Lythrum*,

bumble bees defecated many times on the bract (Experiment 1), which was also the part associated with the most intense *Crithidia* infection (Experiment 3). However, in this part *Crithidia* survived shorter amounts of time, and so the ability to transmit *Crithidia* will depend on how quickly feces are encountered by a new host. *Lythrum* is very frequently visited by bees, especially *B. impatiens*, in its non-native North American range [24], which could then minimize the impact of short pathogen survival time and facilitate pathogen spread in the community. Conversely, foraging bumble bees seldom defecated on *Monarda* bracts, the part that resulted in the greatest infection intensity. These results suggest *Lobelia* and *Lythrum* may be more effective disease transmission hubs than *Monarda*, but transmission will also depend on frequency of visitation.

In the face of increasing dependence on bees for ecosystem services [7], there is a pressing need to understand factors that shape pollinator health. Pathogen-induced stress and spillover from commercial bees via flowers are factors consistently linked to pollinator decline [3, 6], yet the mechanisms governing how flowers serve as disease transmission venues have been largely unexplored. Flowers are multifunctional hubs, providing not only nutrition, microbial symbionts [45], and pathogen-suppressing chemical compounds [25, 46], but also many of the pathogens themselves [47]. Infection-induced changes in foraging and/or physiology are predicted to affect probability of transmission [35, 48], but had yet to be empirically evaluated until now. Understanding how flowers contribute to bee pathogen transmission is a necessary component of promoting pollinator health. Given our results, we recommend assessing floral traits associated with pathogen transmission across a diversity of plant and pollinator species, in an effort to develop wildflower mixes that not only maximize forage but also minimize disease spread.

457

458 *Supporting data can be accessed at*

459 *https://datadryad.org/review?doi=doi:10.5061/dryad.jc4hf80.*

460

461 *We have no competing interests*

462

463 *LLF, LSA, REI and SHM conceived and designed the study; LLF, LSA, MB, CG, AJ, EM, LM,*

464 *LM and AZ collected field data and conducted experiments; LLF carried out the statistical*

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466

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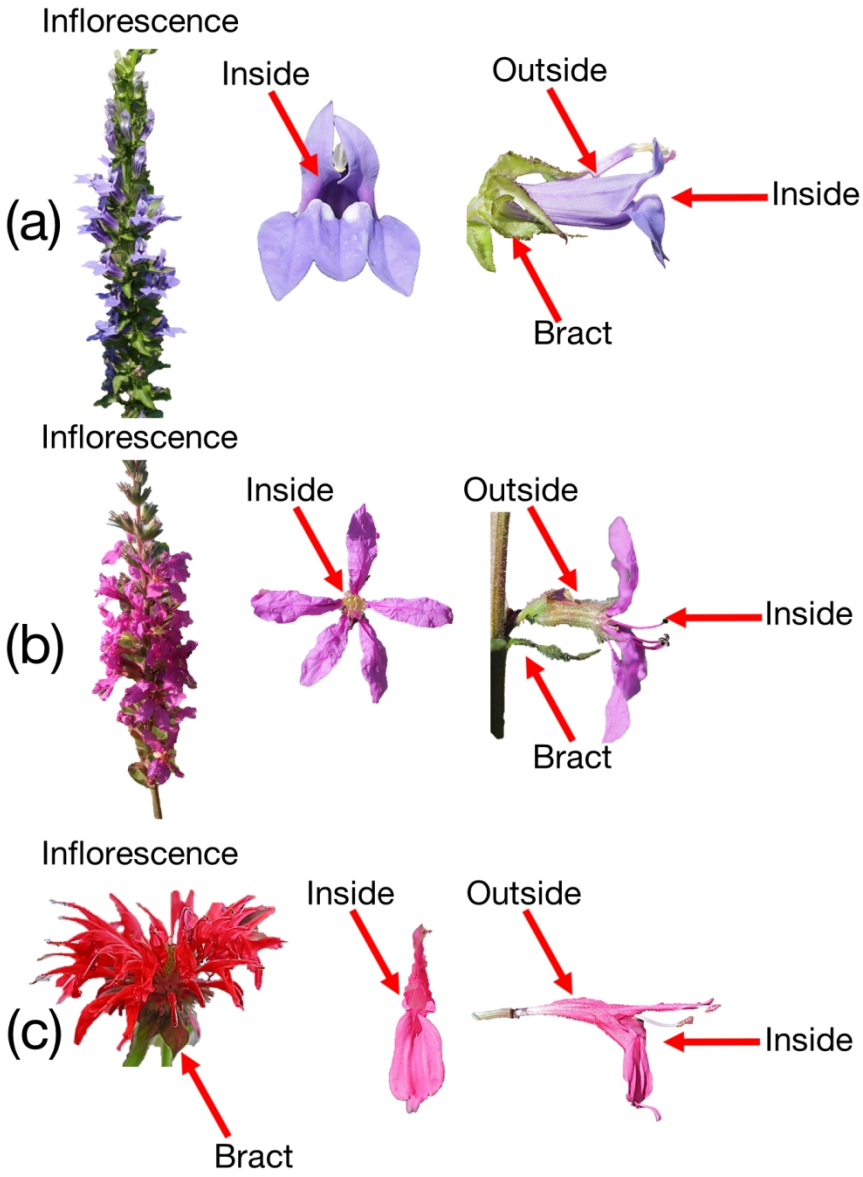

Figure 1: Flower parts where the common eastern bumble bee (*Bombus impatiens*) defecated or *Crithidia bombi* inoculum was placed on (a) *Lobelia siphilitica*, (b) *Lythrum salicaria* and (c) *Monarda didyma* (photo credit: N. Milano).

381x508mm (96 x 96 DPI)

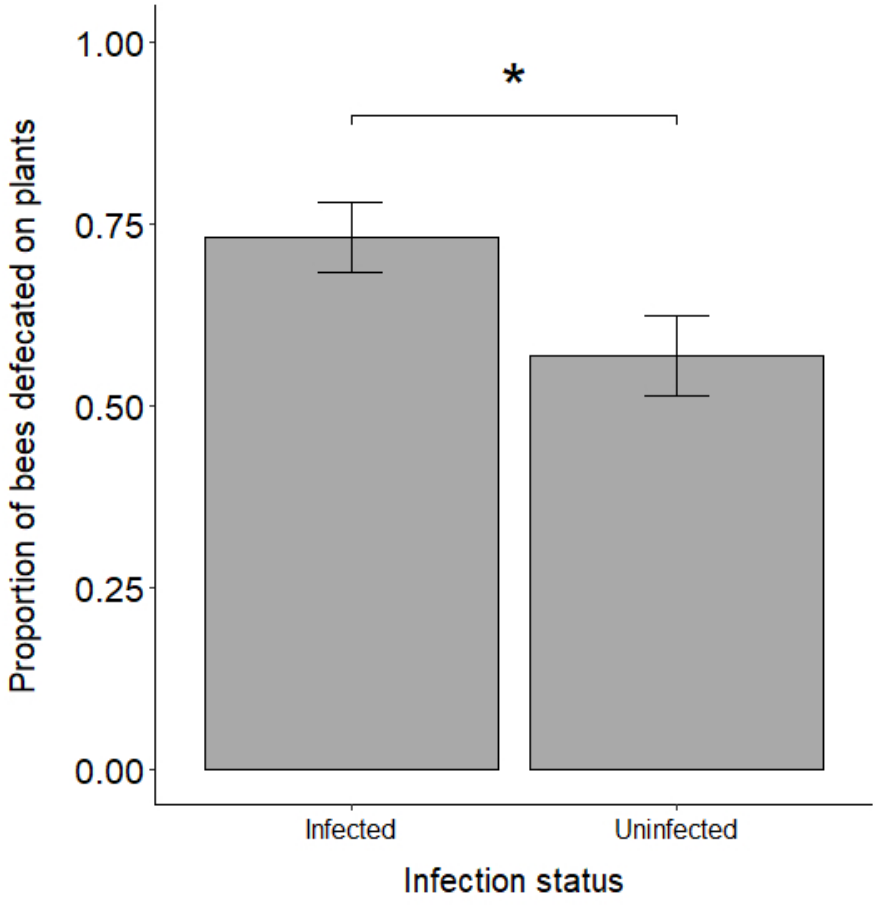

Figure 2. Experiment 1: Effect of *Crithidia* infection status on *Bombus impatiens* defecation rate on plants (mean ± s.e). Infected worker bees were more likely to defecate on plants than uninfected bees.

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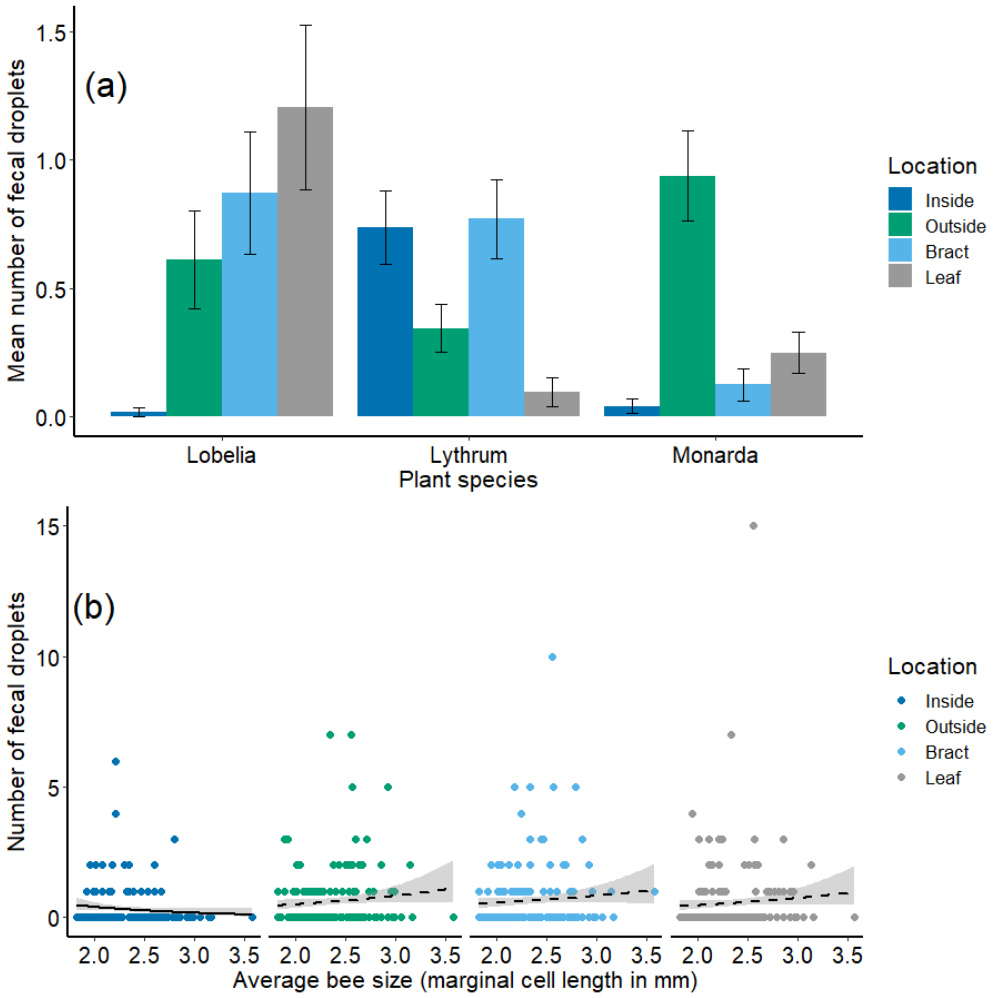

Figure 3. Experiment 1: (a) Effect of plant species and flower part on defecation by *B. impatiens* workers. Data are mean ± s.e. (for post-hoc comparisons see Table S1). (b) Effect of *B. impatiens* size on defecation among different flower parts. Solid lines indicate significance ($p < 0.05$) while dashed lines indicate no significant relationship.

238x238mm (96 x 96 DPI)

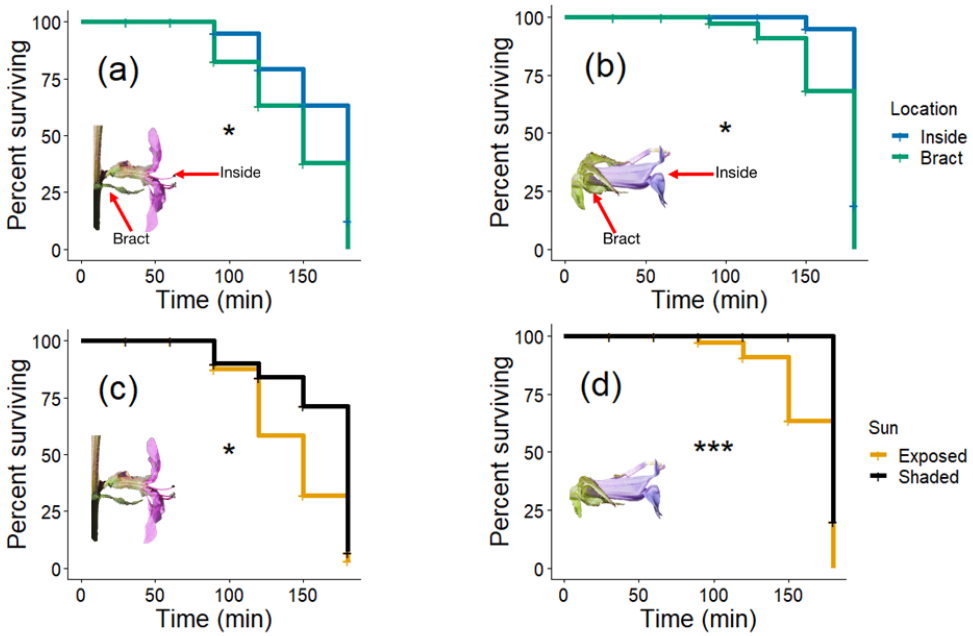

Figure 4. Experiment 2: *Crithidia* survival across plant species and flower parts. Survival differed across flower part and exposure to sun in *Lythrum* (a, c) and *Lobelia* (b, d). We did not find significant differences among flower parts on *Monarda*.

254x193mm (96 x 96 DPI)

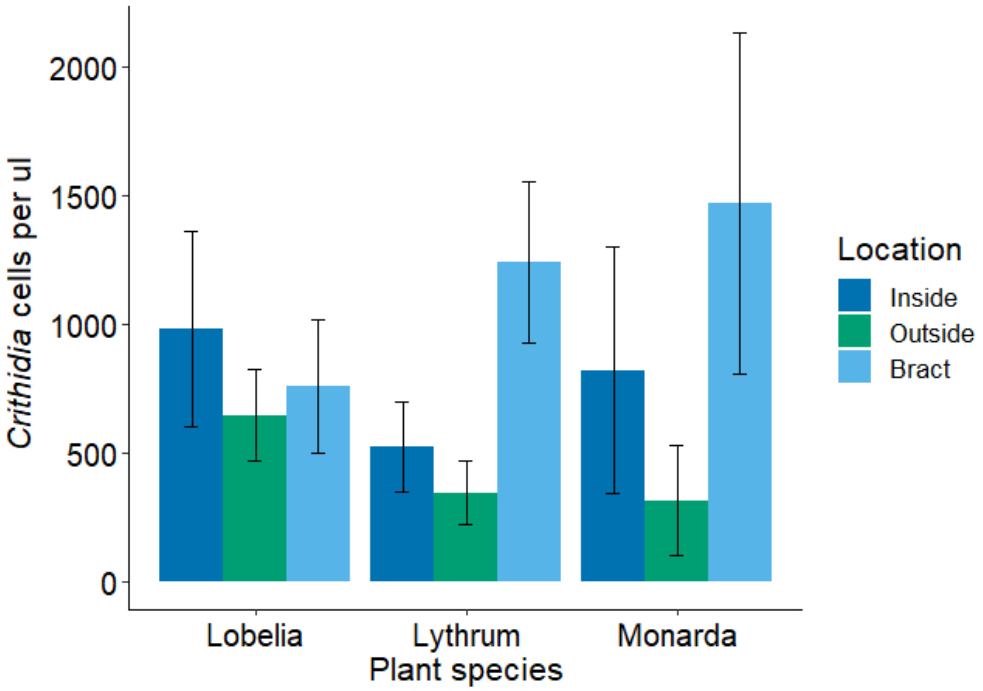

Figure 5. Experiment 3: Effects of plant species and flower part on *Crithidia* acquisition and subsequent intensity of infection (*Crithidia* cells per ul) in *B. impatiens* workers. Data are means ± s.e.

170x119mm (96 x 96 DPI)

Appendix B

Cornell University
College of Agriculture
and Life Sciences

Laura L. Figueroa
Department of Entomology

April 18, 2019

Dear Editor,

Thank you for your decision on our manuscript RSPB-2019-0603, now titled "*Bee pathogen transmission dynamics: deposition, persistence and acquisition on flowers*." We appreciate the opportunity to revise the manuscript and address reviewer comments, and feel our manuscript is much improved thanks to the reviewers' efforts.

Below we provide a point-by-point list of our responses to reviewer comments; our responses are in blue italics. Line numbers in our responses refer to the revised manuscript. All authors have approved of the final version of this resubmission.

Thank you for your time and considering our manuscript.

Sincerely and on behalf of all coauthors,

Laura Figueroa

Associate Editor
Comments to Author:

The two reviewers were generally positive about this manuscript. They thought the manuscript is well written, the analyses correct and the results interesting and potentially appealing for the broad readership of Proceedings B. At the same time, both reviewers had several suggestions for improvement of the manuscript, particularly Reviewer 2, especially regarding the manuscript's title, the unclear relevance of some of the results, and the need for clarification or revision of parts of the methods, results and discussion sections and one figure (Fig. 4). Minor revisions should allow the authors to incorporate these suggestions.

Thank you for the opportunity to resubmit our manuscript. We believe we have addressed all of the reviewers' comments.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)

The manuscript provide interesting information related to acquisition of trypanosomatids parasites on bumblebees. I find the work well performed, with a clear introduction and objectives. I have some doubts that I marked on the third experiment. I have made some minor comments in the file attached.

Thank you for these positive comments.

We addressed all edits provided by Reviewer 1:

[L45-47] Please delete this phrase, it is too general.

While we agree the first sentence of the introduction is general, we believe that placing our work in the broader ecology of infectious disease (EID) literature is important given the breadth of this field of study, the insights our study makes to the EID literature, and the broad readership of PRSB. However, if the editor feels otherwise, we can remove this sentence.

[L54] Replace "effective disease" by "effective bee disease"

We made this change.

[L68] Fries (2010) is no the correct citation. You should cite: Bailey, L., 1981. Honey Bee Pathology, second ed. Academic Press, London.

We now add the proper citation.

[L264-266] How authors have assessed this overlapping? please clarify.

We clarified the use of overlapping bumble bee colonies on L278-281, by referring to the statistical methods section: "We used bees from 4 experimental colonies for Monarda, 5 for Lythrum, and 6 for Lobelia; colonies mostly overlapped for the first two species and had approximately 50% overlap for the second and third species. We accounted for colony origin in the analyses (see Statistical analyses)".

In the statistical analyses section L322-324, we clarified that the random effect structure corrects for overlap in colonies during trials: "The model included colony and date as random effects, thus accounting for overlap in colonies during trials".

Referee: 2

Comments to the Author(s)
This study presents results from a series of experiments to understand the mechanisms of pathogen transmission between pollinators via flowers. The main goals of the study are to: (1) determine if infected bees tend to defecate more on flowers, (2) frequency of deposition changes with flower shape, and (3) pathogen survival and transmission varies with flower morphology. it was a pleasure to read this manuscript. It is clear and well-written. The experiments were well-done and the statistical analyses are clearly presented and seem appropriate.

Thank you for these positive comments.

I only have minor comments on the current version of this manuscript.

* The title emphasizes that this study reveals new mechanisms of pathogen transmission. However, these mechanisms are not clearly stated or discussed in the abstract and the discussion. Even though the results of this study do not directly allow to test how generalizable these patterns are (meaning do deeper and longer corollas generally facilitate pathogen transmission?), it would benefit the paper to mention hypotheses that emerge from this study about general patterns linking flower morphology and pathogen transmission.

We have reworded the title to be more representative of the manuscript: "Bee pathogen transmission dynamics: deposition, persistence and acquisition on flowers". We agree that evaluating floral morphology is an important future direction in the field of bee disease transmission. We now add hypotheses related to morphology in the discussion. Specifically, in L463-467 we state: "We hypothesize that floral morphologies that facilitate overlap in where pollinator feces are deposited and acquired (e.g. flat composites on which bees walk and forage for long periods of time) would result in higher rates of disease transmission compared to morphologies for which deposition and acquisition may be disjointed (e.g., Solanaceous plants that are visited for short periods of time and do not have a landing platform)".

* The results of the pathogen persistence under different environmental conditions are important, clean but are not highlighted in the paper. These results are not mentioned in the abstract and only briefly discussed.

Thank you for this feedback. We agree and have incorporated the environmental conditions in the abstract and discussion. Specifically, in the abstract on L34-37 we now add "Additionally, we found that Crithidia survival across locations was reduced with sun exposure. These results suggest that efficiency of pathogen transmission depends on where deposition occurs and the timing and place of acquisition, which varies among plant species and environmental conditions". In addition, we dedicate the fifth paragraph of the discussion to the importance of environmental conditions. In L445-448 we now add areas for future research: "Similarly, whether environmental gradients that affect exposure to UV radiation (e.g., along an altitudinal gradient or from the forest canopy to the ground layer) influence bee pathogen transmission dynamics on flowers is entirely unknown and is an important area for future research".

* L74 - This sentence is unclear. The question of whether bee infection increases deposition in flowers is easy to understand after the previous sentence. But why deposition may vary with floral morphology is not discussed until the next paragraph.

We revised the sentence to conclude the paragraph only discussing the question of whether bee infection increases deposition on flowers, L71-73: "Whether infection affects bee defecation patterns on flowers represents a serious knowledge gap in bee disease transmission dynamics". The question of floral morphology, as noted, is introduced and discussed in the next paragraph.

*L99 - Perhaps the authors could provide more specific predictions about how they expected pathogen survival and infectiousness to vary in flowers with different morphologies and under different environmental conditions. These predictions would help the reader understand earlier on what the different experiments are testing.

Thank you for this suggestion. We now specify our prediction in L97-100, stating "Therefore, we predicted that pathogen survival and infectiousness would vary across floral parts within the same plant and across species and environmental conditions, and would be lowest for floral parts more exposed to the sun's UV radiation, such as outside the corolla and on flower bracts".

* L102-109 - This paragraph would be clearer if predictions were followed after the questions outlined here. In addition, it would probably be better to briefly describe the 3 experiments before the methods section. For example, what environmental conditions did you investigate? Also, the phrasing of objective 4 is unclear. I suggest you change if for: "pathogen acquisition and subsequent infection of bees vary among different parts of the flower in different plant species".

Thank you for these suggestions. In the third aim, we now specify that the environmental condition evaluated was sun exposure (L105-107: "pathogen survival depends on pathogen deposition and environmental conditions (sun exposure) across flower parts"). We now briefly describe the three experiments and our predictions in the final paragraph of the introduction in L108-121: "We asked these questions by conducting three experiments. In the first experiment (questions 1 and 2), we allowed experimentally infected and uninfected bees fed fluorescent diet to forage on three flower species, and determined how many times and where they defecated on the plants. We predicted that infected bees would defecate more on flowers than uninfected bees, and that defecation patterns would depend on how the bees interact with the morphology of each plant species. In the second experiment (question 3), we placed pathogen inoculum on three flower parts and determined survival for three hours across three plant species, either in sun exposed or shaded conditions. We predicted that the pathogen would survive longer inside the flower corolla and under shaded conditions, due to reduced exposure to UV radiation. In the third experiment (question 4), we allowed uninfected bees to forage on flowers upon which we had placed inoculum on a discrete flower part, and quantified the resulting infection loads one week after exposure. We predicted that resulting infections would be lowest when inoculum was encountered inside the flower corolla, due to increased presence of phytochemicals in pollen and nectar". Finally, we also made the suggested change in wording for objective four (L107-108).

* Will the R scripts be shared as supplementary information? If so, can you indicate that in the text?

The R scripts will be shared as supplementary information in Dryad alongside the data. We now add that to L482.

* L325 - While the authors clarify this later in the text, I think the sentence "bigger bees defecated fewer times inside flowers" is misleading. As mentioned later on, it is likely that the bees were defecating at equal rates but they were doing that outside the flowers because they were larger. Could the authors rephrase? Maybe: "Fewer droplets were detected inside flowers visited by larger bees".

That you for this clarifying comment. We now rephrased as "We observed a bee size by flower part interaction for number of fecal droplets observed (χ^2_3 = 9.08, p = 0.028; Figure 3b), whereby fewer droplets were detected inside flowers visited by larger bees (Tukey HSD: z = – 2.87, p = 0.004)" (L341-343).

*L385 - The authors mention in L306 that "foraging time" was collected for experiment 3. Could the authors investigate the role of time spent in the flower on how many pathogens were left behind after a visit? Or does foraging time mean something else?

In experiment 3, uninfected bees foraged on inoculated flowers and we quantified the resulting Crithidia infections. Primarily to ensure foraging behavior was normal, we measured foraging time in trials. Foraging time did not predict either Crithidia incidence or intensity (χ^2_1 = 0.94, p = 0.333 and χ^2_1 = 2.08, p = 0.150, respectively). Nonetheless, time the bee spent foraging and time since the inoculum was made (related to its infectiousness) were both included as covariates in our model to increase accuracy. The question of foraging time and how many pathogens were left after a visit could only be evaluated if we had calculated foraging time for experiment 1, which, for logistical reasons, we did not do. In the discussion, we recommend evaluating foraging time as an important future direction in the field of bee pollinator disease transmission (L463-467).

*L411- Add "across floral parts"

We made this change.

* L423 - Sentence is unclear.

We clarified the sentence as "Otterstatter & Thompson experimentally varied the time and number of Crithidia cells placed on Brassica rapa nectaries encountered by susceptible foraging bumble bees. They found that most foraging bees became infected when exposed to Crithidia that had been placed on the flower for less than 10 minutes; by 85 minutes the probability of infection was under 15%" (L438-442).

* Figure 4 - I suggest you add the results for Monarda even if they were not significant. Or at least, mentioned in the legend in what way the data were not significant. Did all the Crithidia die or did they all survive in both treatments?

Thank you for this comment. We expand the legend and clarified the conditions for Monarda: "Monarda was only evaluated in shade conditions (see methods); we did not find significant differences among flower parts in Monarda, likely due to a high overall Crithidia survival (80%)" (L635-638).