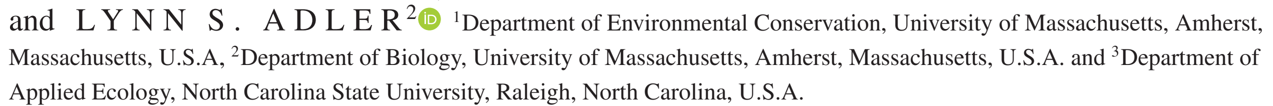
*Ecological Entomology* (2019), **44**, 702–710 DOI: 10.1111/een.12751

# Effect of timing and exposure of sunflower pollen on a common gut pathogen of bumble bees

GEORGE M. LOCASCIO,[1] RIVER PASQUALE,[2] EUGENE AMPONSAH,[2] REBECCA E. IRWIN[3] and LYNN S. ADLER[2] [1]Department of Environmental Conservation, University of Massachusetts, Amherst, Massachusetts, U.S.A, [2]Department of Biology, University of Massachusetts, Amherst, Massachusetts, U.S.A. and [3]Department of Applied Ecology, North Carolina State University, Raleigh, North Carolina, U.S.A.



**Abstract.** 1. Several bee species are declining due to multiple factors, including pathogens. Ingestion of sunflower (*Helianthus annuus*) pollen can dramatically reduce the bumble bee gut pathogen *Crithidia bombi*, but little is known about how timing and exposure to sunflower pollen consumption affects pathogen load.

2. Two experiments were carried out to investigate how exposure to sunflower pollen relative to pathogen exposure affects *Crithidia bombi* in *Bombus impatiens*. Foraging trials with pollen-producing and male-sterile (pollen absent) sunflower lines were performed to investigate whether sunflower pollen affected pathogen transmission in a single foraging bout, and 7-day laboratory trials were done to investigate whether timing and duration of exposure to sunflower pollen after infection affected *C. bombi*.

3. In foraging trials, pollen presence on inflorescences inoculated with *C. bombi* did not affect transmission (pathogen cell counts of foraging workers) 1 week later, suggesting that a brief experience with sunflower pollen concurrent with pathogen exposure is insufficient to reduce infection. In laboratory trials, consuming sunflower pollen for the first 3.5 days or all 7 days after infection reduced cell counts compared with a negative control pollen, but consuming sunflower pollen starting 3.5 days after infection did not. Consuming sunflower pollen for 7 days was significantly and substantially more effective than any other treatment. Thus, both duration and timing of exposure to sunflower pollen may affect pathogen load.

4. These results are important for understanding ecological disease dynamics in natural settings with free-flying bumble bees, and may inform decisions about using medicinal diets to manage bumble bee health commercially.

**Key words.** *Bombus impatiens*, *Crithidia bombi*, *Helianthus annuus*, medicinal pollen, pathogen, timing effects.

## Introduction

Pollinators are crucial components in agriculture and for maintaining diverse plant communities. Pollinators are important for the yield of up to 90 crops in the U.S.A., and about one-third of crops globally (Gallai *et al.*, 2009). Bumble bees in particular are used for pollination services in greenhouses and, along with other native pollinators, complement and often exceed honey bee pollination services for some crops, such as tomato (Winfree *et al.*, 2008; Russo *et al.*, 2013). However, some pollinator species are in decline (Potts *et al.*, 2010; Hallmann *et al.*, 2017). Pollinator decline is thought to be caused by several factors (Goulson *et al.*, 2015), including pathogens (Meeus *et al.*, 2011), insecticides (Pettis *et al.*, 2012), fungicides (McArt *et al.*, 2017), habitat degradation (Goulson *et al.*, 2008), and land-use change (Potts *et al.*, 2010). These declines underscore the importance of developing methods to effectively combat stressors, such as pathogens.

Managed and wild bumble bees are both affected negatively by pathogens (Murray *et al.*, 2013; Graystock *et al.*, 2014). *Crithidia bombi* [Kinetoplastea, Trypanosomatida] is a common pathogen of multiple bumble bee species (Gillespie, 2010) that

Correspondence: Lynn S. Adler, Department of Biology, University of Massachusetts, Amherst, MA 01003, USA. E-mail: lsadler@ent.umass.edu


© 2019 The Royal Entomological Society

can be contracted at flowers and transferred among nest mates via faecal-oral transmission (Durrer & Schmid-Hempel, 1994). Wild *B. terrestris* colonies infected with *C. bombi* are less likely to produce new daughter queens compared with uninfected colonies (Goulson *et al.*, 2017). *Crithidia* infection reduced queen colony founding success and colony size (Brown *et al.*, 2003), and reduced motor learning rates of flower handling in *B. impatiens* (Gegear *et al.*, 2005), which could influence how effectively bumble bees pollinate and forage (Koch *et al.*, 2017).

Both diet quantity and quality may interact with *C. bombi* pathogen infection to alter the severity of infection outcomes in bumble bees, with similar patterns for pathogen infection in honey bees. For example, mortality rates of *C. bombi*-infected *B. terrestris* under food-limited conditions were 50% higher than those of infected bees with sufficient resources (Brown *et al.*, 2000). A polyfloral pollen diet reduced *Nosema ceranae* (Microsporidia, Nosematidae, Zander) infections and improved longevity in infected honey bees (*Apis mellifera* L.; Apidae) compared with a monofloral diet (Alaux *et al.*, 2010; Di Pasquale *et al.*, 2013). Floral chemistry may also play important roles mediating bee disease; several nectar secondary compounds reduced *C. bombi* in *B. impatiens* (Manson *et al.*, 2009; Baracchi *et al.*, 2015; Richardson *et al.*, 2015), although this is not always the case (e.g. Palmer-Young *et al.* 2017). In addition, sunflower (*Helianthus annuus* L.; Asteraceae) honey reduced *N. ceranae* in honey bees compared with other types of honey (Gherman *et al.*, 2014). Furthermore, sunflower pollen reduced two pathogens in two different hosts, *C. bombi* in *B. impatiens* and *N. ceranae* in *A. mellifera* (Giacomini *et al.*, 2018). These studies suggest that the composition and identity of nectar and pollen rewards in pollinator diets can affect pathogen prevalence and influence disease dynamics.

In epidemiology, the time period between when a subject is exposed to an infectious agent and the administration of medication can affect the outcome. For example, infections with human and simian immunodeficiency viruses were prevented in monkeys when a medicinal treatment was administered within 36 h of infection, but not after this 36-h window (Tsai *et al.*, 1995; Otten *et al.*, 2000). Furthermore, mice experienced decreased lung inflammations if they were medicated within 48 h after inhaling the irritant ragweed pollen, but not after 48 h (Sur *et al.*, 1996). In these examples, the timing of medicinal dosage mattered, and early intervention reduced or prevented irritation or infection better than late intervention. With insects, timing of exposure to potentially medicinal secondary compounds can have different benefits at different ontogenetic stages (Mason & Singer, 2015); for example, a compound may be more effective against larval than against adult parasites. However, we know little about how timing of exposure to potential medicines affects resistance to pathogens within a life stage. Previous work found that exposure to 7 days of sunflower pollen suppressed *C. bombi* in *B. impatiens* both when administered immediately after infection, and after waiting 1 week for infection to build (Giacomini *et al.*, 2018). However, these were conducted in separate experiments, limiting our ability to compare directly the effect of timing on pathogen infection. Here, we investigate how the interval between exposure relative to treatment affects *C. bombi*.

We used two experiments with individual bee manipulations to investigate whether the time between infection and receiving medicinal sunflower pollen affected *C. bombi* prevalence in *B. impatiens*. We focused on sunflower pollen given its consistent beneficial effects of reducing *C. bombi* in *B. impatiens* across multiple sunflower pollen sources and strains of *C. bombi* (Giacomini *et al.*, 2018). However, sunflower pollen is low in protein and amino acids relative to most other pollens (Nicolson & Human, 2013; Yang *et al.*, 2013). It may therefore be advantageous to supply only as much as necessary to effectively manage pathogens by determining when exposure to sunflower pollen will be most effective, as well as how much is necessary to suppress infection. First, we asked if exposure to sunflower pollen affected transmission of *C. bombi* and infection levels after single foraging bouts (hereafter referred to as the ‘exposure during foraging’ experiment). In this experiment, reduced infection levels could be due to either reduced infectivity due to pathogen contact with sunflower pollen before ingestion, or consumption of sunflower pollen and increased bee resistance to infection directly post-consumption. Second, in laboratory assays, we asked if the amount and timing of sunflower pollen consumption relative to time of infection affected pathogen prevalence (hereafter referred to as the ‘duration and timing of exposure after infection’ experiment). We hypothesised that earlier and longer exposure to medicinal sunflower pollen would prevent or reduce pathogen infection more than later or no exposure. The results of this research will have important implications for understanding how duration and timing of medicinal floral rewards affect outcomes for bee pathogen infection, which can guide future management applications.

## Materials and methods

### *Study system*

The common eastern bumble bee, *B. impatiens*, is a generalist pollinator that lives socially in colonies with an annual life cycle (Wilson, 1971). *Bombus impatiens* are commonly found in eastern North America from Maine and Ontario to the eastern Rocky Mountains and south into Florida (Williams *et al.*, 2014), and colonies are also commercially available for agricultural use. The intestinal parasite *C. bombi* is also common in northeast North America, and in western Massachusetts, U.S.A., it can be found in up to 80% of *B. impatiens* individuals at some sites (Gillespie, 2010). Sunflower, *H. annuus*, is a common early successional, self-compatible annual forb, native to central North America (Reagon & Snow, 2006). It is grown commercially in the U.S. for its oilseeds and as a cover crop (USDA, 2016), and sunflower is also an important crop globally for oilseed production (reviewed in Nicolson & Human, 2013).

### *Inoculum preparation*

For both experiments, we used *C. bombi* from wild *B. impatiens* workers originally collected at Stone Soup Farm on two dates in September 2014 in Hadley, Massachusetts (42°21′51.93″N, 72°33′55.88″W), that were used to infect

commercial *B. impatiens* colonies (Biobest Canada, Leamington, Ontario, Canada) in the laboratory. Infection was transferred to new commercial colonies when the previous colony began to decline. On days bees entered experiments, fresh *C. bombi* inoculum was prepared by dissecting five to 10 workers. We placed guts into separate 1.5-ml Eppendorf tubes mixed with 300 μl of $^1/_4$-strength Ringer's solution (Fluka 96724, Sigma-Aldrich, St Louis, Missouri), which we then homogenised with a plastic rod, vortexed for 5 s, and let settle for 3–4 h at room temperature, allowing *C. bombi* to swim up into the supernatant. From the supernatant, we collected a 10-μl sample to assess pathogen infection using a haemacytometer under a compound light microscope at 40× magnification. We counted and summed live, actively moving *C. bombi* cells in the corner and central squares of the haemocytometer grid (a total of 0.02 μl volume). We then combined multiple 150-μl samples from one to four bees and diluted with $^1/_4$-strength Ringer's solution to obtain a solution with 1200 *C. bombi* cells μl$^{-1}$. This solution was mixed with an equal amount of 50% sucrose solution to prepare an inoculum with 600 *C. bombi* cells μl$^{-1}$ in 25% sucrose, as described in Richardson *et al.* (2015). This is a common concentration of *C. bombi* that bees may come into contact with in nature (Otterstatter & Thomson, 2006). We used inoculum from seven source colonies for the 'exposure during foraging' experiment, and three source colonies for the 'duration and timing of exposure after infection' experiment.

### *Exposure during foraging*

*Plant cultivation.* We obtained *H. annuus* seeds from the United States Department of Agriculture, Agricultural Research Service through the North Central Regional Plant Introduction Station, part of the U.S. National Plant Germplasm System programme. They were sown in Sun Gro Horticulture medium (Sun Gro Horticulture Canada Ltd, Seba Beach, AB, Canada) in 50 plug trays in a greenhouse until transplanted at the Crop and Animal Research and Education Center in South Deerfield, Massachusetts (42°28′51.93″N, 72°34′55.88″W). We grew 100 cytoplasmic male-sterile (CMS), non-pollen-producing plants and 100 pollen-producing plants (non-CMS), from each of three lines (89 and CMS-89, 236 and CMS-236, and 404 and CMS-404; see Table S1 for propagation information), for a total of *c*. 600 plants. The numbers 89, 236, and 404 refer to the U.S. National Plant Germplasm System database ID plots where the seeds were grown. Thus, we used CMS or non-CMS lines as the treatment within each of three lines to assess generality of our results.

*Transmission trials.* To determine how sunflower pollen exposure (CMS versus pollen-producing sunflowers) affected *C. bombi* transmission, we allowed bumble bees to forage on an *H. annuus* inflorescence (i.e. a single capitulum) that we hand-inoculated with *C. bombi* and then determined bee infection status after 1 week. All inflorescences were enclosed with mesh bags for 48 h prior to trials to prevent pathogen contamination from wild bee visitors. We harvested each inflorescence from the field immediately before the trial began and placed the stem in a florist's tube with water in a Styrofoam holder. Before beginning each trial, we visually divided the inflorescence head into quarters, and a 10-μl *C. bombi* inoculum drop was added to one disc flower (i.e. floret) in each quarter (Fig. S1). The location was marked with a red paint marker (Uni-Paint fine line PX-21, Mitsubishi Pencil, Sanford Corporation, Oak Brook, Illinois) on the outer edge of the open whorl. All trials were conducted between 10.00 and 16.00 hours during June–August 2016.

Worker bees for transmission trials were isolated from laboratory colonies that were confirmed to be *C. bombi*-free via weekly subsamples of five bees. Bees were placed in individual vials and starved for 3–4 h prior to trials. Trials were conducted in 60 × 35 × 45-cm$^3$ wood-and-mesh cages with a canvas cloth at one end to allow insertion of bees and flowers. A single bee was added to the cage with a single inflorescence for each trial. We recorded the number of uninoculated flowers probed, inoculum drops probed, total time spent foraging, and researcher conducting the trial. A flower or inoculum drop was recorded as 'probed' when the bee inserted its tongue. A trial was considered complete when a bee had foraged for a minimum of 1 min, visited at least one inoculum drop, and visited at least five uninoculated flowers. Most bees foraged for much longer than 1 min; we allowed bees to complete foraging before terminating each trial, which was no longer than 20 min. Cages were kept in the sun to be sterilised for 30 min after each trial had ended to prevent potential contamination; *C. bombi* is not viable after desiccation (Figueroa *et al.*, 2019) and in previous work, ethanol used for sterilisation dissolved the wood varnish on the cages (L.S. Adler, personal observation). On days when field trials were conducted, the inoculum and bees were placed in a cooler with ice packs for transport and storage.

After each trial, the bee was recaptured into a clean individual vial, placed in a cooler, and transported at the end of the day to the laboratory. In the laboratory, we placed bees in individual vials with nectar feeder lids and reared them in an incubator at 27 °C in darkness for 7 days. Each day, bees were fed *c*. 40–60 mg of wildflower pollen (Koppert Biological Systems, Howell, Michigan) mixed with 30% sucrose to create a pollen paste, and 500 μl of 30% sucrose solution made available in a modified Eppendorf tube through a cotton wick (nectar feeder). Vials, nectar feeders, and pollen were replaced daily. After 7 days, we dissected bees and counted *C. bombi* cells as described earlier in 'Inoculum preparation'. At the time of dissection, we collected the right forewing of each bee and measured radial cell length with a dissecting microscope to estimate bee size (Harder, 1982) as a potential covariate in our statistical analysis. Final sample sizes of bees used on each *H. annuus* line from six different colonies were as follows: non-CMS 236 = 38, CMS 236 = 39; non-CMS 404 = 14, CMS 404 = 13; non-CMS 89 = 30, and CMS 89 = 23.

*Statistical analysis.* Statistical analyses were carried out in R v.3.3.1 (R Core Team, 2017) using generalised mixed linear models. Due to the nature of our zero-bounded count data, we fitted our models with a Poisson distribution and checked for overdispersion. Finding that the data were overdispersed, we

analysed data using a negative binomial error distribution with a log link function using the package LME4 (Bates *et al.*, 2015), and calculated least-squares means and standard errors with the package LSMEANS (Lenth, 2016).

To test whether sunflower pollen presence affected *C. bombi* transmission in a single foraging bout, we used raw *C. bombi* cell count as the dependent variable with a model including pollen presence (yes/no), *H. annuus* line (404, 89, 236), their interaction, and bee size (estimated by radial cell length) as independent fixed predictors. Colony of origin (to account for the genetic similarities between bees) and trial date (to account for variation in *C. bombi* inoculum preparation) were included as random effects. We initially included several fixed covariates, including the number of inoculum drops probed (to assess the amount of parasite each bee was exposed to), the number of uninoculated flowers probed, and researcher overseeing the foraging trial. However, inoculum drops probed and researcher did not affect *Crithidia* counts ($\chi_1^2 < 1.3$, $P > 0.3$ for both) and were dropped from the model. To analyse the effect of pollen presence on foraging behaviour, we used separate responses of inoculum drops probed, un-inoculated flowers probed flowers probed, and total time spent foraging, with fixed predictors of pollen presence, *H. annuus* line, their interaction, and bee size, as well as random effects of colony of origin and trial date.

### *Duration and timing of exposure after infection*

*Experimental design.* To evaluate how the duration and timing of sunflower pollen consumption after infection affected *C. bombi* counts, we inoculated individual worker bees from commercially available hives that were confirmed to be *C. bombi*-free via weekly subsamples of five bees. Inoculated bees were exposed to one of four pollen treatments: 7 days of sunflower pollen (sunflower only); 7 days of buckwheat (*Fagopyrum esculentum*) pollen (buckwheat only); 3.5 days of sunflower and then 3.5 days of buckwheat pollen (sunflower first); or 3.5 days of buckwheat pollen and then 3.5 days of sunflower pollen (buckwheat first). We used single pollen species comparisons because sunflower pollen and buckwheat pollen (both from Changge Hauding Wax Industry, Henan, China) have similar (low) protein and amino acid concentration (Yang *et al.*, 2013), but consuming buckwheat pollen results in much higher *C. bombi* infection than consuming sunflower pollen (Giacomini *et al.*, 2018; LoCascio *et al.*, 2019). Worker bees were isolated from commercial colonies in the laboratory, placed in individual vials, and starved for 2–3 h prior to inoculation. We provided bees with a 10-μl drop of inoculum from the same original source used in transmission trials, and only bees that consumed the entire drop were included in the experiment. After inoculation, bees were reared in an incubator at 27 °C in darkness for 7 days. Each day, bees were fed *c.* 40–60 mg of a fresh mixture of buckwheat or sunflower pollen paste, and 500 μl of 30% sucrose solution made available in a modified Eppendorf tube through a cotton wick. Vials, nectar feeders, and pollen were replaced daily. After 7 days, bees were dissected and *C. bombi* cells were counted, as described in the 'Exposure during foraging' section (earlier). At the time of dissection, we again collected the right forewing to measure radial cell length to estimate body size and use as a covariate in analysis. Our sample sizes were 41–45 bees per treatment, for a total of 172 bees, from four colonies that were relatively evenly distributed across pollen treatments.

*Statistical analysis.* To ask how duration and timing of sunflower consumption after infection affected raw *C. bombi* cell counts, we used a generalised linear mixed model with a negative binomial error distribution and a log link function, including pollen diet treatment and bee size as fixed effects, and random effects of colony of origin and inoculation date. We compared our model with and without pollen treatment using the ANOVA function. Finding a significant treatment effect, we used a Tukey's *post hoc* honestly significant difference (HSD) test to compare the four treatments. Graphical displays were composed with GGPLOT2 (Wickham, 2009) and COWPLOT (Wilke, 2016).

## Results

### *Exposure during foraging*

*Crithidia bombi* cell count after 1 week was unaffected by pollen presence at the time of foraging ($\chi_1^2 = 0.002$, $P = 0.987$), by *H. annuus* line ($\chi_2^2 = 3.512$, $P = 0.173$), or their interaction ($\chi_2^2 = 0.593$, $P = 0.741$; Fig. 1). There was no relationship between *C. bombi* cell counts and the number of inoculated flowers probed by bees ($P = 0.821$); surprisingly, however, bees that probed more uninoculated flowers had marginally higher *C. bombi* counts ($\chi_1^2 = 3.34$, $P = 0.067$).

Pollen presence, *H. annuus* line, and their interaction affected several aspects of foraging behaviour (Table 1; Fig. 2). Pollen presence significantly increased the number of uninoculated flowers probed by 25%, and this effect did not differ with line (Fig. 2a). The presence of pollen significantly decreased the average number of inoculum drops probed (by 66%), and drops probed also varied with *H. annuus* line, but the effect of pollen did not differ with line (Fig. 2b). Bees foraged on inflorescences with pollen (non-CMS) for 20% more time than on inflorescences without pollen overall, but the effect of pollen did not differ with *H. annuus* line (Fig. 2c).

### *Duration and timing of exposure after infection*

Pollen diet treatment had a significant effect on raw *C. bombi* cell counts ($\chi_3^2 = 38.239$, $P < 0.001$; Fig. 3). As expected, a pure sunflower pollen diet for 7 days strongly reduced *C. bombi* counts relative to all other treatments ($P < 0.001$ for all comparisons of 'sunflower only' with other treatments). Bees exposed to sunflower pollen for 3.5 days and then buckwheat pollen had approximately three times higher infection than those exposed to sunflower pollen only, indicating that duration of exposure is important. Although bees exposed to buckwheat pollen first had approximately twice as much *C. bombi* as bees exposed to sunflower pollen first, this comparison was not statistically

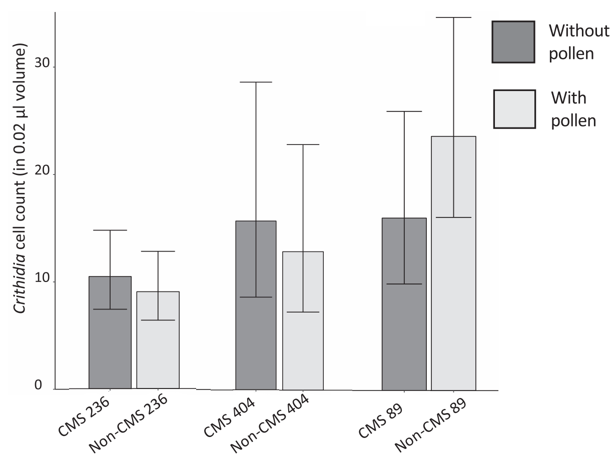



**Fig. 1.** Mean *Crithidia bombi* cell count (± SE) in bees 1 week after a single foraging bout to each cultivar. CMS, plants with cytoplasmic male sterility (i.e. that do not make pollen); non-CMS, plants that make pollen. Means and SEs were calculated by back-transforming least-square means ± least-square mean SEs.

**Table 1.** Effect of sunflower (*Helianthus annuus*) pollen presence (CMS or non-CMS), *H. annuus* line, their interaction, and bee size (estimated as length of the wing radial cell) on foraging behaviour of individual worker *Bombus impatiens* in single-bout foraging trials.

| | d.f. | $\chi^2$ | *P* |
|---|---|---|---|
| *Uninoculated flowers probed* | | | |
| Pollen presence | 1 | 4.095 | **0.043** |
| Line | 2 | 1.043 | 0.593 |
| Pollen × line | 2 | 2.817 | 0.232 |
| Bee size | 1 | 15.250 | **< 0.001** |
| *Inoculum drops probed* | | | |
| Pollen presence | 1 | 13.136 | **< 0.001** |
| Line | 2 | 15.607 | **< 0.001** |
| Pollen × line | 2 | 2.389 | 0.302 |
| Bee size | 1 | 5.575 | **0.018** |
| *Total time foraging (min)* | | | |
| Pollen presence | 1 | 4.095 | **0.043** |
| Line | 2 | 1.003 | 0.605 |
| Pollen × line | 2 | 4.426 | 0.109 |
| Bee size | 1 | 0.031 | 0.992 |

All models also included colony of origin and trial date as random effects. CMS, plants with cytoplasmic male sterility (i.e. that do not make pollen); non-CMS, plants that make pollen.
Bold font indicates significant values at $P < 0.05$.

significant in a Tukey's *post hoc* HSD test ($P = 0.441$). However, bees exposed to buckwheat pollen first did not have statistically different *C. bombi* counts compared with those exposed to buckwheat pollen only ($P = 0.525$). By contrast, bees exposed to sunflower pollen first had significantly lower infection than those exposed to buckwheat pollen only ($P = 0.023$). Bees exposed to sunflower pollen first or buckwheat pollen first were provided with the same amount of sunflower pollen but at different times following infection (i.e. immediately or 3.5 days after infection). As bees exposed to sunflower pollen first had significantly lower infection than those exposed solely to buckwheat pollen, but bees exposed to buckwheat pollen first did not, this suggests that timing of exposure to sunflower pollen is also important for determining pathogen infection.

## Discussion

The severity of *C. bombi* infection depended on the duration, and to some extent the timing, of exposure to sunflower pollen relative to infection. The first experiment demonstrated that foraging on sunflower inflorescences with pollen in a single bout was insufficient to reduce *C. bombi* transmission and infection. In the second experiment, we demonstrated that 7 days of sunflower pollen, post-infection, suppressed *C. bombi* infections to nearly undetectable levels while 7 days of buckwheat pollen resulted in a relatively high *C. bombi* infection, consistent with previous work (Giacomini *et al.*, 2018). Although exposure to 3.5 days of sunflower pollen immediately following infection was not as effective as exposure for 7 days, it was more effective than consuming only buckwheat pollen. This indicates that duration is important; consuming 3.5 days of sunflower pollen is not as effective as 7 days, but better than none. By contrast, exposure to 3.5 days of buckwheat pollen and then 3.5 days of sunflower pollen was not more effective than only consuming buckwheat pollen, indicating that timing also plays a role. In other work, however, exposure to 7 days of sunflower provided 1 week after infection still strongly reduced *C. bombi* infection, indicating that sunflower can be effective even after infection is established, as long as a sufficient amount is provided (Giacomini *et al.*, 2018). It is important to note that our experimental design does not allow us to directly compare the effects of duration and timing because both factors were not manipulated in a factorial design (i.e. the 7-day sunflower pollen duration was only imposed immediately after infection and not 3.5 days after infection). Our work suggests that both duration of exposure and timing play a role in reducing *C. bombi* infection, but their relative importance has yet to be systematically assessed.

By contrast, we found no effect of exposure to sunflower pollen during foraging on *C. bombi* transmission or infection level. If there had been an effect, we had envisioned at least two mechanisms that could have been involved. First, direct exposure of *C. bombi* cells to sunflower pollen on the inflorescence could have reduced viability or infectivity. This mechanism is ecologically relevant because *C. bombi* can be horizontally transmitted during foraging when uninfected bees come into contact with faeces deposited by infected bees (Durrer & Schmid-Hempel, 1994; Graystock *et al.*, 2015). Because sunflower inflorescences are very large and flat, and bees often remain for long foraging bouts relative to other plant species (Adler *et al.*, 2018), deposition of infected faeces onto flowers may be likely. However, previous work has found little evidence that *C. bombi* cells are directly sensitive to ecologically relevant concentrations of nectar secondary compounds (Manson *et al.*, 2009; Michaud *et al.*, 2019), and sunflower pollen extracts did not inhibit *C. bombi* growth *in vitro* (Palmer-Young & Thursfield, 2017). Second, it is also possible that consuming a small amount of sunflower pollen during foraging could have reduced *C. bombi* transmission or infection. During our foraging trials,

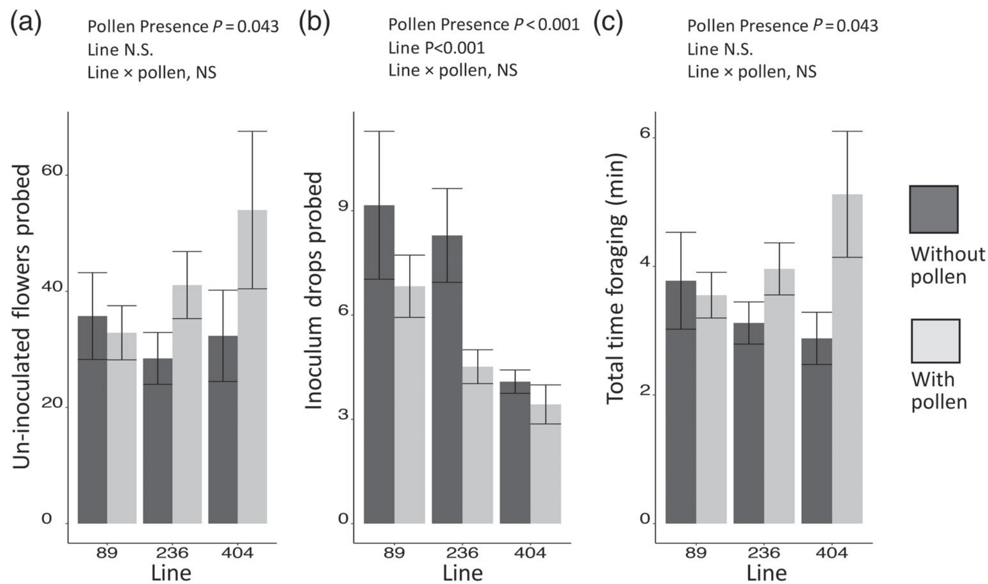



**Fig. 2.** Effect of pollen presence on bee foraging behaviour. (a) Mean (±SE) uninoculated flowers probed per inflorescence. (b) Mean (± SE) inoculum drops probed per inflorescence. (c) Mean (± SE) total time (min) foraging on each inflorescence type. Means and SEs were calculated by back-transforming least-square means ± least-square mean SEs.

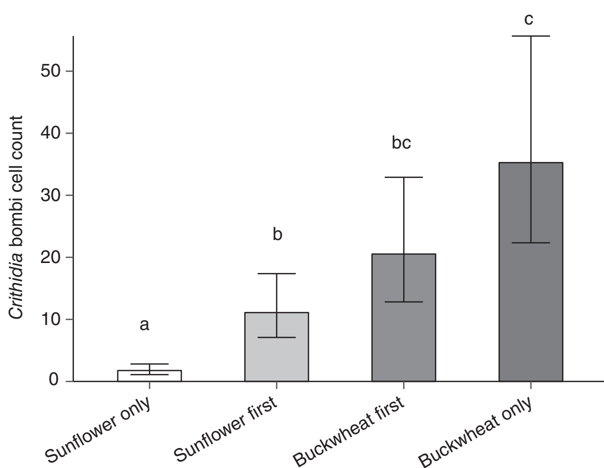



**Fig. 3.** Mean *Crithidia bombi* cell count (± SE) in response to pollen treatments (from left to right): sunflower pollen for 7 days (sunflower only); sunflower for 3.5 days and then 3.5 days of buckwheat pollen (sunflower first); buckwheat pollen for 3.5 days and then 3.5 days of sunflower pollen (buckwheat first); and buckwheat pollen for 7 days (buckwheat only). Different letters above bars denote significantly different treatments at $P < 0.05$ using a Tukey's *post hoc* honestly significant difference test. Means and SEs were calculated by back-transforming least-square means ± least-square mean SEs.

we observed bees foraging for nectar rather than pollen, but bees on pollen-producing inflorescences often became covered with pollen during the foraging process (Fig. S2). Unfortunately, we did not observe bees after the trial to see whether they groomed and consumed this pollen. However, given that 3.5 days' exposure to sunflower pollen after infection was significantly less effective than 7 days' exposure (see section on 'Duration and timing of exposure after infection' experiment), we now suspect that even if bees were confirmed to have consumed sunflower pollen acquired during a single foraging bout, this would unlikely be sufficient to significantly reduce infection.

Interestingly, although pollen presence did not affect *C. bombi* transmission or infection, it did affect foraging behavior. Bees probed more flowers and spent more time foraging when pollen was present. This is consistent with a previous experiment showing that *B. impatiens* preferred sunflower inflorescences with pollen over inflorescences without pollen (Mallinger & Prasifka, 2017). Interestingly, though, bees probed significantly more inoculum drops on inflorescences without pollen, the opposite pattern. Anecdotally, it appeared to us that when pollen was absent, it seemed easier for bees to locate inoculum drops; the presence of pollen seemed to be a minor obstacle that encouraged more foraging in place and longer foraging bouts. The sucrose reward of inoculum or the red paint could have provided visual or olfactory cues that were more detectable when pollen was absent. However, even though bees that foraged on inflorescences without pollen had greater exposure to *C. bombi*, they had similar pathogen counts to bees that foraged on inflorescences with pollen. This contrasts with prior research showing that consumption of greater volumes of inoculum led to higher infection intensity in *B. impatiens* (Otterstatter & Thomson, 2006); we would have expected that bees that consumed more inoculum would become more infected. Although we do not know why sunflowers without pollen would incur greater exposure to inoculum without affecting pathogen counts, this has interesting implications for the relationship between foraging dynamics and exposure to infectious material in agricultural settings, as male-sterile sunflowers are often grown on farms (Parker, 1981).

Although the mechanisms by which sunflower pollen reduces *C. bombi* in bumble bees are unknown, the results from our

timing experiments led us to speculate about possibilities. For example, if the mechanism underlying resistance was immune system-priming, we would have expected the one-time exposure to be effective, assuming that bees consumed some sunflower pollen during or immediately following their single foraging bout on pollen-producing lines. However, because this assumption was not verified, we cannot rule out immune-priming as a possible mechanism. Priming with some nectar alkaloids has been suggested to help bumble bees infected with *C. bombi* (Manson *et al.*, 2009) and warrants further studies investigating the effects of exposure to sunflower pollen prior to and after bees are infected with *C. bombi*. Our results also indicate that a small dose of sunflower pollen is not directly toxic to *C. bombi*, or at least not enough to prevent bee infection. Rather, a longer-term exposure in the gut seems to be necessary to reduce infection, suggesting a more gradual immune (or other) response to sunflower. Alternatively, sunflower pollen may affect resistance by altering the gut microbial community, a mechanism that can only be effective post-consumption. Gut microbiota can provide protection against *C. bombi* in *B. terrestris* (Koch & Schmid-Hempel, 2011), and abundance of *Lactobacillus* ‘Firm-5’ bacteria specifically can enhance resistance to *Crithidia* (Palmer-Young *et al.*, 2019). Furthermore, pollen diet can alter gut microbiota (Billiet *et al.*, 2016), although very few studies have tested this in bees to date. Thus, we hypothesise that sunflower pollen could support growth of gut microbiota that confer resistance to *Crithidia*. Although our experiments were not designed to elucidate the mechanism, the contrasting results for single-foraging-bout exposure versus longer periods of consumption suggest that exploring whether immune function and the gut microbiota are affected by sunflower pollen would be important in future work.

Although sunflower pollen significantly reduced *C. bombi* with a 7-day exposure, several studies suggest that sunflower pollen is a nutritionally poor resource for bees. For example, sunflower pollen contains less than the minimum requirement of two essential amino acids for honey bees (Nicolson & Human, 2013). Sunflower pollen has been suggested to hinder honey bee development and reduce larval weight in *B. terrestris* when it is their sole pollen source (Tasei & Aupinel, 2008), and resulted in as much mortality as pollen starvation for honey bees infected with *Nosema* (Giacomini *et al.*, 2018). However, we note that the mechanism underlying the effect of sunflower pollen on *Crithidia* is unlikely to be solely poor nutrition, as buckwheat pollen is as low in protein as sunflower pollen but results in much higher *Crithidia* infection (Fig. 3). Aside from the specific disadvantages of a sunflower-only diet, consuming any monofloral pollen diet may reduce bee health. For example, nurse honey bees parasitised with *N. ceranae* had reduced survival when fed monofloral compared with polyfloral pollen diets (Di Pasquale *et al.*, 2013), and a polyfloral pollen mix increased immune system activity compared with monofloral diets (Alaux *et al.*, 2010). Furthermore, *B. impatiens* is a broad generalist, and so a monofloral diet is an ecologically unlikely scenario in natural situations. Our goal in the present study was to assess the conditions under which short-term exposure to sunflower pollen could reduce *Crithidia* infection; future studies using sunflower pollen to manage bee disease over longer time frames should also include other pollen sources for optimal bee health.

Our result that timing plays a role in disease management is consistent with studies using other trypanosomes that cause Chagas disease and African sleeping sickness in humans. With Chagas disease, early treatment suppressed acute symptoms more effectively than when treatment was administered after chronic symptoms began (Vallejo & Reyes, 2005; Jannin & Villa, 2007). Early intervention is crucial for African sleeping sickness (Legros *et al.*, 2002) and is necessary to prevent late-stage progression, which requires a combination of medicines to ensure drug resistance does not occur (Priotto *et al.*, 2006). In both diseases, early treatment is imperative for the disease to subside quickly or to be brought to manageable levels. Furthermore, treatment must be continued for a prolonged period of time after infection to be most effective. Although less effective than 7 days of sunflower pollen, we found that even 3.5 days’ exposure to sunflower pollen could reduce *C. bombi*, but only if sunflower pollen was administered immediately after infection. Because sunflower is thought to be a nutritionally poor resource for bees (Nicolson & Human, 2013), understanding the interplay between duration and timing of exposure is important to effectively treat pathogens while limiting exposure to suboptimal resources.

Our findings may have important applications for managed bumble bees. Because previous studies indicate that multiple cultivars of sunflower and some relatives suppress *C. bombi* (LoCascio *et al.*, 2019), future studies could directly supply bee colonies with sunflower pollen to see whether sunflower pollen supplements can benefit bees at the whole-colony level. Our experiments here suggest that colonies would need to be provided with sunflower pollen for at least 7 days for pathogen suppression, but research is needed to assess the degree to which individual bee studies scale up to the colony level. Sunflower supplements may also provide benefits to managed honey bee colonies. Giacomini *et al.* (2018) found that sunflower pollen also suppressed the widespread fungal parasite, *N. ceranae*, in honey bees. Apiary managers could investigate whether the use of sunflower pollen supplements or increased sunflower plantings improve honey bee health; our research suggests that both timing and duration should be considered to maximise pathogen reduction while minimising costs of a nutrient-poor diet. Our results suggest a phenological component to plant–pollinator–disease interactions that should be considered in future studies of non-chemical management of bumble bee disease.

## Acknowledgements

We thank J. Davis, C. Cahill, A. Fowler, K. M. Connolly and two anonymous reviewers for edits to this manuscript, L. Aguirre and Dr E. Palmer-Young for aid in statistical analysis, and BioBest for donating bumble bee colonies. Funding was provided by Big Y Grocers, Garden Club of America, Horticultural Club of Boston, Community Foundation of Western Massachusetts, Lotta Crabtree Foundation (all to GL), the University of Massachusetts at Amherst Center

for Agriculture, Food and the Environment Summer Scholars program (EA), Girls Inc. and the Eureka! extern program (RP), USDA/CSREES (Multi-state Hatch) MAS00497 (LSA), and USDA-NIFA-2016-07962 (LSA and REI) from the Pollinator Health programme. The contents are solely the responsibility of the authors and do not necessarily represent the official views of any funding agency. The authors declare there are no conflicts of interest.

## Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** The image shows how we visually quartered the sunflower inflorescence (the two 90° black lines), added one inoculum drop to each quarter, and marked that flower (floret) with a red mark. The black arrow is pointing to one of the flowers where an inoculum drop was added (top left).

**Fig. S2.** Contrasting photographs showing inflorescences with pollen (a), and inflorescences without pollen (b). The red mark on individual flowers indicated where we added *Crithidia* inoculum drops, as described in Fig. S1.

**Table S1**. Plant cultivation information. All dates were in the year 2016. The cultivar column includes whether the plant had pollen or no pollen (CMS). 'Date and total sown' describes the date each group of plants was sown at the University of Massachusetts Amherst greenhouses and the amount that was sown. 'Date trials completed' indicates the last day we used plants from that group for foraging trials. All 404 and CMS-404 plants were fertilised once on 13 May 2016 with Peters Professional 20-10-20 peat lite special (Everris NA Inc., Geldermalsen, the Netherlands); the other lines were not fertilised.

## References

Adler, L.S., Michaud, K.M., Ellner, S.P., McArt, S.H., Stevenson, P.C. & Irwin, R.E. (2018) Disease where you dine: plant species and floral traits associated with pathogen transmission in bumble bees. *Ecology*, **99**, 2535–2545.

Alaux, C., Ducloz, F., Crauser, D. & Le Conte, Y. (2010) Diet effects on honeybee immunocompetence. *Biology Letters*, **6**, 562–565.

Baracchi, D., Brown, M.J.F. & Chittka, L. (2015) Behavioural evidence for self-medication in bumblebees? [v2; ref status: indexed, http://f1000r.es/5ep]. F1000Research, **4**, 1–15.

Bates, D., Mächler, M., Bolker, B. & Walker, S. (2015) Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, **67**, 1–48.

Billiet, A., Meeus, I., van Nieuwerburgh, F., Deforce, D., Wäckers, F. & Smagghe, G. (2016) Impact of sugar syrup and pollen diet on the bacterial diversity in the gut of indoor-reared bumblebees (*Bombus terrestris*). *Apidologie*, **47**, 548–560.

Brown, M.J.F., Loosli, R. & Schmid-Hempel, P. (2000) Condition-dependent expression of virulence in a trypanosome infecting bumblebees. *Oikos*, **91**, 421–427.

Brown, M.J., Schmid-Hempel, R. & Schmid-Hempel, P. (2003) Strong context-dependent virulence in a host–parasite system: reconciling genetic evidence with theory. *The Journal of Animal Ecology*, **72**, 994–1002.

Di Pasquale, G., Salignon, M., Le Conte, Y., Belzunces, L.P., Decourtye, A., Kretzschmar, A. *et al.* (2013) Influence of pollen nutrition on honey bee health: do pollen quality and diversity matter? *PLoS One*, **8**, e72016.

Durrer, S. & Schmid-Hempel, P. (1994) Shared use of flowers leads to horizontal pathogen transmission. *Proceedings of the Royal Society of London - Series B: Biological Sciences*, **258**, 299–302.

Figueroa, L. *et al.* (2019) Mechanisms mediating bee pathogen transmission: deposition, persistence and acquisition on flowers. *Proceedings of the Royal Society of London. Series B*, (in press).

Gallai, N., Salles, J.M., Settele, J. & Vaissière, B.E. (2009) Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. *Ecological Economics*, **68**, 810–821.

Gegear, R.J., Otterstatter, M.C. & Thomson, J.D. (2005) Does parasitic infection impair the ability of bumblebees to learn flower-handling techniques? *Animal Behaviour*, **70**, 209–215.

Gherman, B.I., Denner, A., Bobiş, O., Dezmirean, D.S., Mărghitaş, L.A., Schlüns, H. *et al.* (2014) Pathogen-associated self-medication behavior in the honeybee *Apis mellifera*. *Behavioral Ecology and Sociobiology*, **68**, 1777–1784.

Giacomini, J.J., Leslie, J., Tarpy, D.R., Palmer-Young, E.C., Irwin, R.E. & Adler, L.S. (2018) Medicinal value of sunflower pollen against bee pathogens. *Scientific Reports* in press, **8**, 14394.

Gillespie, S. (2010) Factors affecting parasite prevalence among wild bumblebees. *Ecological Entomology*, **35**, 737–747.

Goulson, D., Lye, G.C. & Darvill, B. (2008) Decline and conservation of bumble bees. *Annual Review of Entomology*, **53**, 191–208.

Goulson, D., Nicholls, E., Botias, C. & Rotheray, E.L. (2015) Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science*, **347**, 1255957.

Goulson, D., O'Connor, S. & Park, K.J. (2017) The impacts of predators and parasites on wild bumblebee colonies. *Ecological Entomology*, **42**, 168–181.

Graystock, P., Goulson, D. & Hughes, W.O.H. (2014) The relationship between managed bees and the prevalence of parasites in bumblebees. *PeerJ*, **2**, e522.

Graystock, P., Goulson, D. & Hughes, W.O.H. (2015) Parasites in bloom: flowers aid dispersal and transmission of pollinator parasites within and between bee species. *Proceedings of the Royal Society B: Biological Sciences*, **282**, 20151371.

Hallmann, C.A., Sorg, M., Jongejans, E., Siepel, H., Hofland, N., Schwan, H. *et al.* (2017) More than 75 percent decline over 27 years in total flying insect biomass in protected areas. *PLoS One*, **12**, e0185809.

Harder, L.D. (1982) Measurement and estimation of functional proboscis length in bumblebees (Hymenoptera: Apidae). *Canadian Journal of Zoology*, **60**, 1073–1079.

Jannin, J. & Villa, L. (2007) An overview of Chagas disease treatment. *Memórias do Instituto Oswaldo Cruz*, **102**, 95–98.

Koch, H. & Schmid-Hempel, P. (2011) Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. *Proceedings of the National Academy of Sciences of the United States of America*, **108**, 19288–19292.

Koch, H., Brown, M.J.F. & Stevenson, P.C. (2017) The role of disease in bee foraging ecology. *Current Opinion in Insect Science*, **21**, 60–67.

Legros, D., Ollivier, G., Gastellu-Etchegorry, M., Paquet, C., Burri, C., Jannin, J. *et al.* (2002) Treatment of human African trypanosomiasis–Present situation and needs for research and development. *The Lancet Infectious Diseases*, **2**, 437–440.

Lenth, R.V. (2016) Least-square means: the R package lsmeans. *Journal of Statistical Software*, **69**(1), 1–33.

LoCascio, G.M. Aguirre, L., Irwin, R.E. & Adler, L.S. (2019) Pollen from multiple sunflower cultivars and species reduces a common bumble bee gut pathogen. *Royal Society Open Science*. **6**, 190–279.

Mallinger, R.E. & Prasifka, J.R. (2017) Bee visitation rates to cultivated sunflowers increase with the amount and accessibility of nectar sugars. *Journal of Applied Entomology*, **141**, 561–573.

Manson, J.S., Otterstatter, M.C. & Thomson, J.D. (2009) Consumption of a nectar alkaloid reduces pathogen load in bumble bees. *Oecologia*, **162**, 81–89.

Mason, P.A. & Singer, M.S. (2015) Defensive mixology: combining acquired chemicals towards defence. *Functional Ecology*, **29**, 441–450.

McArt, S.H., Urbanowicz, C., McCoshum, S., Irwin, R.E. & Adler, L.S. (2017) Landscape predictors of pathogen prevalence and range contractions in US bumblebees. *Proceedings of the Royal Society B: Biological Sciences*, **284**, 20172181.

Meeus, I., Brown, M.J., De Graaf, D.C. & Smagghe, G. (2011) Effects of invasive parasites on bumble bee declines: parasites and bumble bee declines. *Conservation Biology*, **25**, 662–671.

Michaud, K.M., Irwin, R.E., Barber, N.A. & Adler, L.S. (2019) Pre-infection effects of nectar secondary compounds on a bumble bee gut pathogen. *Environmental Entomology*, https://doi.org/10.1093/ee/nvz018.

Murray, T.E., Coffey, M.F., Kehoe, E. & Horgan, F.G. (2013) Pathogen prevalence in commercially reared bumble bees and evidence of spillover in conspecific populations. *Biological Conservation*, **159**, 269–276.

Nicolson, S.W. & Human, H. (2013) Chemical composition of the 'low quality' pollen of sunflower (*Helianthus annuus*, Asteraceae). *Apidologie*, **44**, 144–152.

Otten, R.A., Smith, D.K., Adams, D.R., Pullium, J.K., Jackson, E., Kim, C.N. *et al.* (2000) Efficacy of postexposure prophylaxis after intravaginal exposure of pig-tailed macaques to a human-derived retrovirus (Human immunodeficiency virus type 2). *Journal of Virology*, **74**, 9771–9775.

Otterstatter, M.C. & Thomson, J.D. (2006) Within-host dynamics of an intestinal pathogen of bumble bees. *Parasitology*, **133**, 749.

Palmer-Young, E.C. & Thursfield, L. (2017) Pollen extracts and constituent sugars increase growth of a trypanosomatid parasite of bumble bees. *PeerJ*, **5**, e3297.

Palmer-Young, E.C., Hogeboom, A., Kaye, A.J. *et al.* (2017) Context-dependent medicinal effects of anabasine and infection-dependent toxicity in bumble bees. PLoS One, **12**, e0183729.

Palmer-Young, E.C., Raffel, T.R. & McFrederick, Q.S. (2019) pH-mediated inhibition of a bumble bee parasite by an intestinal symbiont. *Parasitology*, **146**, 380–388.

Parker, F.D. (1981) Sunflower pollination: abundance, diversity, and seasonality of bees on male-sterile and male-fertile cultivars. *Environmental Entomology*, **10**, 1012–1017.

Pettis, J.S., vanEngelsdorp, D., Johnson, J. & Dively, G. (2012) Pesticide exposure in honey bees results in increased levels of the gut pathogen *Nosema*. *Naturwissenschaften*, **99**, 153–158.

Potts, S.G., Biesmeijer, J.C., Kremen, C., Neumann, P., Schweiger, O. & Kunin, W.E. (2010) Global pollinator declines: trends, impacts and drivers. *Trends in Ecology & Evolution*, **25**, 345–353.

Priotto, G., Fogg, C., Balasegaram, M., Erphas, O., Louga, A., Checchi, F. *et al.* (2006) Three drug combinations for late-stage *Trypanosoma brucei gambiense* sleeping sickness: a randomized clinical trial in Uganda. *PLOS One: Clinical Trials*, **1**, e39.

R Core Team (2017) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.

Reagon, M. & Snow, A.A. (2006) Cultivated *Helianthus annuus* (Asteraceae) volunteers as a genetic "bridge" to weedy sunflower populations in North America. *American Journal of Botany*, **93**, 127–133.

Richardson, L.L., Adler, L.S., Leonard, A.S., Andicoechea, J., Regan, K.H., Anthony, W.E. *et al.* (2015) Secondary metabolites in floral nectar reduce parasite infections in bumble bees. *Proceedings of the Royal Society B: Biological Sciences*, **282**, 20142471.

Russo, L., DeBarros, N., Yang, S., Shea, K. & Mortensen, D. (2013) Supporting crop pollinators with floral resources: network-based phenological matching. *Ecology and Evolution*, **3**, 3125–3140.

Sur, S., Lam, J., Bouchard, P., Sigounas, A., Holbert, D. & Metzger, W.J. (1996) Immunomodulatory effects of IL-12 on allergic lung inflammation depend on timing of doses. *Journal of Immunology*, **157**, 4173–4180.

Tasei, J.-N. & Aupinel, P. (2008) Nutritive value of 15 single pollens and pollen mixes tested on larvae produced by bumblebee workers (*Bombus terrestris*, hymenoptera: Apidae). *Apidologie*, **39**, 397–409.

Tsai, C.-C., Follis, K.E., Sabo, A., Beck, T.W., Grant, R.F., Bischofberger, N. *et al.* (1995) Prevention of SIV infection in macaques by (R)-9-(2-phosphonylmethoxypropyl)adenine. *Science*, **270**, 1197–1199.

USDA. (2016) Acreage report. National Agricultural Statistics Service, Agricultural Statistics Board, US Department of Agriculture. Report 45, Washington, D.C.

Vallejo, M. & Reyes, P.P. (2005) Trypanocidal drugs for late stage, symptomatic Chagas disease (*Trypanosoma cruzi* infection). *Cochrane Database of Systematic Reviews* (ed. by The Cochrane Collaboration). John Wiley & Sons, Ltd, Chichester, U.K.

Wickham, H. (2009) *ggplot2: Elegant Graphics for Data Analysis*, Springer-Verlag, New York.

Wilke, C.O. (2016) *Cowplot: Streamlined Plot Theme and Plot Annotations for 'ggplot2'*. R Package, Austin, Texas.

Williams, P.H., Thorp, R.W., Richardson, L.L. & Colla, S.R. (2014) *Bumble Bees of North America: An Identification Guide*. Princeton University Press, Princeton, New Jersey.

Wilson, E.O. (1971) *The Insect Societies*, p. ix-592. Insect Soc., Harvard University Press, Cambridge, Massachusetts.

Winfree, R., Williams, N.M., Gaines-Day, H.R., Ascher, J.S. & Kremen, C. (2008) Wild bee pollinators provide the majority of crop visitation across land-use gradients in New Jersey and Pennsylvania, USA: crop visitation by wild pollinators. *Journal of Applied Ecology*, **45**, 793–802.

Yang, K., Wu, D., Ye, X., Liu, D., Chen, J. & Sun, P. (2013) Characterization of chemical composition of bee pollen in China. *Journal of Agricultural and Food Chemistry*, **61**, 708–718.

Accepted 29 April 2019
First published online 29 May 2019
Associate Editor: Adam Hart