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Does parasitic infection impair the ability of bumblebees to learn flower-handling techniques?

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Although the capacity to learn how to manipulate flowers plays an integral role in the foraging of bumblebees, little is known about the effects of parasitic infection on the motor learning and memory of host bees. In the laboratory experiment reported here, we examined whether infection by the intestinal protozoan *Crithidia bombi* affected the ability of bumblebees, *Bombus impatiens*, to learn the specialized motor pattern required to handle a novel flower type. Using videotaped records of foraging behaviour, we related the motor performance of bees to the intensity of *C. bombi* infection. Low intensities of infection had no effect on the ability of bees to learn a novel flower-handling method; however, a high intensity of infection significantly reduced both motor-learning rate and maximum handling proficiency. In addition, highly infected bees showed a 200% increase in the amount of time and the number of visits required to learn how to manipulate flowers. These results indicate that *C. bombi* can influence the foraging behaviour of host bumblebees in subtle but ecologically significant ways.

Parasites can influence a variety of host behaviours, including foraging, mate choice and predator avoidance (Sheldon & Verhulst 1996; Kavaliers et al. 1999; Adamo 2002; Moore 2002). Recently, it has been suggested that parasite-induced changes in some ecologically important behaviours may arise from impairment of the infected animal's cognitive function (Kavaliers et al. 1999; Holland & Cox 2001; Klein 2003). Several laboratory studies have found such impairment in vertebrate hosts (e.g. Olson & Rose 1966; Kavaliers & Colwell 1995; Cox & Holland 2001; Fiore et al. 2002). For example, parasitic infection with the protozoan *Eimeria vermiformis* has been found to impair the learning ability of house mice, *Mus domesticus*, in a laboratory maze task (Kavaliers et al. 1995). However, the ecological consequences of such impairments remain unclear, because past studies have relied on behavioural tasks that are not normally performed by the host under natural conditions. Moreover, relatively few data exist on how parasitic infection affects the learning and memory of invertebrate hosts. In this study, we addressed these points by determining whether parasitic infection of bumblebee foragers affected the amount of time they spent learning how to manipulate flowers.

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Crithidia bombi (Zoomastigophorea: Trypanosomatidae Lipa & Triggiani 1980) is a widespread intestinal parasite of bumblebees (*Bombus* spp.; Shykoff & Schmid-Hempel 1991). Susceptible bees contract *C. bombi* by ingesting cells that are shed in the faeces of infected bees (Schmid-Hempel & Schmid-Hempel 1993). The spread of infection within host colonies occurs when workers come in contact with infected nest materials (Schmid-Hempel 2001), and transmission between colonies is thought to occur almost exclusively at flowers via contaminated nectar (Durrer & Schmid-Hempel 1994). Although *C. bombi* is considered to be a relatively benign parasite, because infection results in death only when bees are deprived of food (Brown et al. 2000), it none the less elicits a measurable immune response (Brown et al. 2003).

Bumblebee foragers infected with *C. bombi* may have reduced abilities to learn tasks related to the exploitation of floral resources. Under natural conditions, foraging bumblebees must learn the handling technique (or motor pattern) required to efficiently extract nectar from flowers (e.g. Heinrich 1976; Laverty 1980, 1994a). Some of the flowers visited by bumblebees require extremely complicated handling techniques that involve a substantial learning investment to perfect (Heinrich 1976; Laverty 1980, 1994a). For example, Laverty & Plowright (1988) found that the generalist forager *Bombus fervidus* requires more than 50 flower visits (approximately 17 min) to reach maximum performance on the complicated flowers

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of *Aconitum napellus*. Because limitations on motor learning and memory can have profound implications for the choice behaviour of bees (Darwin 1876; Heinrich 1976; Laverty 1980, 1994b), as well as the reproductive success of flowering plants (Waser 1986), this area of bee cognition has been well studied (see Chittka et al. 1999 for review). However, the potential influence of parasitic infection on the capacity of bees to learn and remember the motor patterns required to handle flowers has yet to be explored.

In the experiment reported below, we determined motor-learning rates and maximum motor performance of inexperienced bumble bees, *Bombus impatiens* Cresson, as they accessed nectar rewards from morphologically complicated artificial flowers. We then categorized bees based on their intensity of *C. bombi* infection and tested for relationships between a bee's intensity of infection and motor performance.

METHODS

Bees and Parasites

Laboratory-reared bumblebee colonies, each containing a queen and approximately 20–30 workers, were obtained from a bumblebee supply company. To check colonies for *Crithidia* infection, 10 bees were randomly selected from each colony, placed in clean plastic vials, and freeze-killed at −20°C. We removed the hindgut from each worker and transferred 4 µl of faeces to a clean glass slide. Faeces samples were air dried, fixed with absolute methanol for 2 min, and stained with 10% Giemsa stain for 2 h. Each slide was examined thoroughly at 400× magnification for *Crithidia* cells (identified according to Lipa & Triggiani 1980), and classified as either infected or uninfected. We detected no parasites other than *Crithidia*.

Preliminary screening indicated that approximately 30% of colonies were infected. Among infected colonies, the intensity of *C. bombi* (number of infective cells observed per bee) varied greatly. Because colonies were raised in isolation, infection and transmission occurred naturally inside the colony. Once a colony was deemed 'infected', we connected it to a screened flight cage (2.2 × 2.2 × 2.2 m) with a tunnel constructed from wire mesh. The tunnel was gated so that the experimenter could control entry of bees into the enclosure. A door (0.4 × 0.4 m) located on one side of the enclosure allowed the experimenter access. Bees were allowed to forage freely on feeders placed on the floor of the cage. Each feeder consisted of a clear centrifuge tube (1.5-cm diameter) with the cap removed, embedded 0.5 cm into a Styrofoam block (1.4 × 1 × 0.035 m) covered in green construction paper. Feeders were filled with 30% sucrose solution (weight/weight) and were replenished immediately after being drained by bees, so that bees did not encounter unrewarding flowers. Pollen was supplied ad libitum directly into the nest. Bees that made regular foraging trips to the array were given a unique mark on the dorsal surface of the thorax and/or abdomen using various colour combinations of typewriter correction fluid.

We allowed bees to visit feeders for no more than 7 days, which is the typical duration between exposure to *C. bombi* and the occurrence of infective cells in the host's faeces (M. C. Otterstatter, unpublished data). This practice ensured that workers were infected inside the colony prior to the experiment and not while foraging on the feeders.

Previous work has shown that the motor performance (Laverty 1994a) and immune function (Doums et al. 2002) of bumblebee workers do not deteriorate up to 14 days postemergence, which is within the normal life span of bumblebees foraging under natural conditions (Rodd et al. 1980). In addition, age is unrelated to interflower flight time and handling time in *B. impatiens* workers up to 20 days postemergence (unpublished data). Therefore, we only tested bees that were less than 14 days old.

Artificial Flowers

The artificial flower type (hereafter referred to as 'flower') was modelled after the complex flower used by Gegear (1995) and Gegear & Laverty (1998); it simulated the closed-tubed flowers frequently visited by bumblebees under natural conditions (Laverty 1994a, b). We constructed each flower by bending the cap of a blue 1.5-ml centrifuge tube so that there was a 0.75-cm opening between the cap and the rim, and then fastening a 3-cm circular 'corolla' constructed from blue acetate around the entrance of the tube. To efficiently access the nectar reward of the flower, bees had to land on the corolla opposite the opening and crawl inside the tube. Naïve bumble bees typically reach maximum motor performance on this flower type in 20–30 flower visits (Gegear 1995).

Experimental Procedure

We removed feeders from the flight cage and replaced them with 15 flowers (three rows of five flowers spaced 15 cm apart) containing 4 µl of 30% sucrose (weight/weight) solution administered using a Hamilton microdispenser (Reno, Nevada, U.S.A.). We presented flowers to bees using the same method as described for feeders. We allowed a single marked bee into the cage and videotaped its foraging behaviour until the nectar from a total of 50 flowers had been taken. After the 50th flower had been visited, the bee was immediately captured, placed into a sterilized clear-plastic vial, and frozen at −20°C. We recorded a total of 22 bees in this manner. Flowers were refilled after being drained, so that test bees always encountered the same number of rewarding flowers and never experienced drained flowers during testing. Refilling occurred only as the test bee entered a subsequent flower to avoid disturbances during foraging. To eliminate any possible influence of scent marks left by previous foragers on the motor performance of subsequent bees, we replaced flowers between bees.

Data Collection and Analysis

For each of the 50 visits by each bee, we measured flower access time, defined as the time from initial contact with a flower until the bee's forward motion into the tube ceased (indicating that the bee had reached the sucrose reward). Using GraphPad Prism 4.0 (San Diego, California, U.S.A.) we assessed motor performance for each bee by fitting the access time data to the one-phase exponential decay function: $Y = b + Ae^{-kX}$, where Y is access time (in seconds); b is the asymptotic access time (i.e. the access time that a bee approaches with experience), A is the amplitude, indicating the difference between access time on the first flower visit and the asymptotic access time (b); X is the flower number in the visit sequence; and k is the decay constant. Lower k values indicate the exponential curve approaches b more slowly; thus, k is our measure of motor-learning rate. Similar nonlinear models have been used in other studies to describe learning in bumblebees (Dukas & Real 1991; Chittka & Thomson 1997). To provide an indication of the costs associated with learning the motor pattern, we determined the number of visits and total amount of time in contact with flowers until access times reached 80% efficiency, which we defined as the point on the fitted curve at which access time had fallen to 80% of the initial ($b + A$) access time. We also counted the number of access errors made by each bee during the 50 flower visits. A bee was considered to have made an access error if it walked around the top of the flower and flew away without entering the tube to gain reward.

After exponential decay functions were fitted to the access time data, we assessed *Crithidia* infection for each bee using the technique previously described. In all cases, the observer was blind with respect to a bee's motor performance. We grouped bees into the following three classes of infection based on the number of *C. bombi* cells observed in faecal samples: uninfected (no cells), low (10–1000 cells), or high (>1000 cells). We estimated that workers classified as having a high infection had approximately 10 times the number of *Crithidia* cells in their faeces as bees classified as having a low infection. Of the 22 bees tested, 12 were placed in the uninfected group, four were placed in the low infection group, and six were placed in the high infection group. Bee size was uniform across the three infection classes (mean $\pm$ SE radial cell length (in mm): uninfected = 2.85 $\pm$ 0.05, low = 2.81 $\pm$ 0.04, high = 2.91 $\pm$ 0.11; ANOVA: $F_{2,19} = 0.39$, $P = 0.68$).

We tested the effect of C. *bombi* infection on motor-learning ability by using a one-way ANOVA to compare the estimated initial access time ($b + A$), the estimated learning rate (k), the number of visits required to reach 80% efficiency, the total time to reach 80% efficiency, and the number of access errors among uninfected, low and high infection groups. For each group, data were pooled irrespective of colony because several additional experiments that were conducted prior to the current study yielded no significant colony-level differences in either the motor performance or foraging behaviour of *B. impatiens* workers. We log-transformed learning rate (k) and total time to 80% efficiency to satisfy the assumption of normality. We also tested whether the maximum motor performance of bees from each group differed by comparing the average access times of the last 10 flower visits for uninfected, low and high infection groups using a one-way ANOVA.

RESULTS

The proficiency of inexperienced bees in the uninfected, low and high infection groups at accessing sucrose rewards improved with the number of flower visits (Fig. 1). We found no difference in the initial access times ($b + A$) of bees from uninfected, low and high infection groups ($F_{2,19} = 0.77$, $P = 0.48$; Fig. 2a). However, the groups differed significantly in maximum motor performance, b ($F_{2,27} = 14.5$, $P < 0.001$; Fig. 2a), and learning rate, k ($F_{2,19} = 7.2$, $P = 0.005$; Fig. 2b). Pairwise comparisons showed that bees with a high intensity of infection by *C. bombi* had 23% higher asymptotic access times and 71% lower learning rates than uninfected bees and bees with low levels of infection. In addition, bees in the high infection group made twice as many access errors as bees in the uninfected and low groups (Tukey's: $F_{2,\ 19} = 24.6$, $P < 0.001$; Fig. 2c).

The three groups also differed in their rates of reaching 80% efficiency, whether measured in flower visits (mean $\pm$ SE: uninfected = 4.71 $\pm$ 0.95, low = 6.5 $\pm$ 1.7, high = 15.1 $\pm$ 3.71; $F_{2,\ 19} = 7.4$, $P = 0.004$) or time ($F_{2,19} = 8.41$, $P = 0.002$; Fig. 2d). Pairwise comparisons showed that bees in the high infection group were approximately three times slower to reach 80% efficiency than bees in the uninfected and low infection groups, indicating that only a high intensity of *C. bombi* infection is associated with time and energy costs.

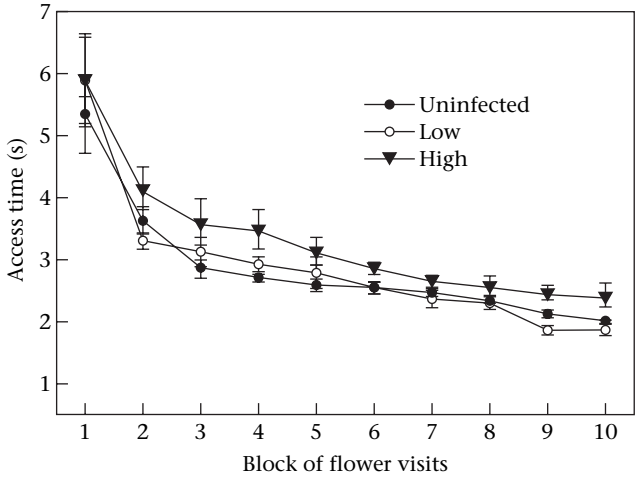

Figure 1. Access times of bees in the uninfected ($N = 12$), low ($N = 4$), and high ($N = 6$) *C. bombi* infection groups during the first 50 flower visits. Plotted points represent the mean $\pm$ SE access time of bees tested in each group over blocks of five consecutive flower visits.

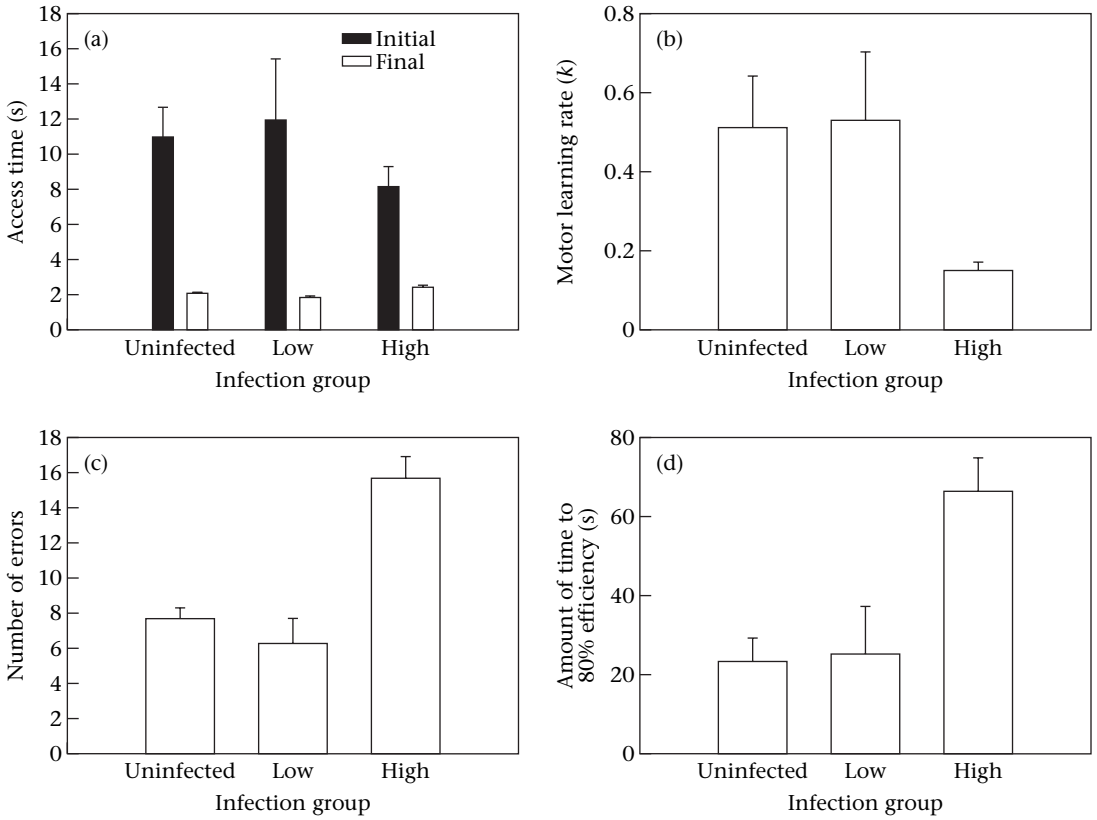

Figure 2. Mean ± SE (a) initial and final access times, (b) motor-learning rates, (c) access errors and (d) amount of time required to reach 80% efficiency for bees in the uninfected ($N = 12$), low ($N = 4$), and high ($N = 6$) infection groups. Initial access time and motor-learning rate for each bee were obtained from the exponential decay function fitted to their access time data, and represent the sum of the asymptotic access time and the amplitude ($b + A$) and the decay constant parameter (k), respectively. Final access times were based on the last 10 flower visits recorded for each bee. An access error was made when a bee walked around the top of the flower and flew away without entering the tube to gain reward.

DISCUSSION

Crithidia infection can affect ecologically important aspects of bumblebee foraging. When accessing a novel flower type requiring a specialized handling method, bumblebee foragers with a high intensity of infection by *C. bombi* had significantly slower rates of motor learning and higher asymptotic access times than did uninfected bees. By contrast, a low intensity of infection did not have any effect on the motor performance of bees, indicating that heavy infection by *C. bombi* is required before impairments to motor performance are evident. The reduced motor performance of heavily infected bees in our study was not the result of a reduced drive to visit and collect nectar from flowers. Heavily infected and uninfected bees showed similar propensities to regularly collect sucrose reward from feeders prior to the experiment and to readily extract reward from the novel flower type upon its presentation. Moreover, we did not find any evidence that a high intensity of *C. bombi* infection significantly altered the overall motor movement of bees. Heavily infected and uninfected bees required the same amount of time to obtain reward on their initial flower visit, and heavily infected bees did not appear to move sluggishly or have any visible signs of physical impairment while visiting flowers. A high intensity of infection was associated with a 23% increase in asymptotic access times, which may be explained by the fact that heavily infected bees made significantly more access errors (a 110% increase) while attempting to enter flowers than did lightly infected bees. Together, these results suggest that the reduced motor performance of bees with a high intensity of *C. bombi* infection on the novel flower type was the result of deficiencies in their learning ability.

In our experiment, we used bees that became infected with *C. bombi* naturally within their nest prior to experiments. This method allowed us to examine the motor performance of bees with the intensities of *C. bombi* infection that paralleled those of bees foraging under field conditions. However, the use of naturally infected host bees poses the problem of causation. In other words, *Crithidia* infection may have caused poor motor performance, but it is also possible that bees with poor motor performance may have been easier to infect. Although the latter explanation of our results cannot be ruled out entirely, we think that it is very unlikely because indexes of motor performance (i.e. motor-learning rate, access

errors, number of visits and total time to 80% access efficiency) in many uninfected bees were poorer than those in bees with a low intensity of *Crithidia* infection. Moreover, significant decreases in motor performance were only observed in bees with high numbers of *Crithidia*, indicating that a poor motor-learning ability was not necessary for infection to occur. Bees from each of the infection groups also did not differ in size, age or previous foraging experience (bees collected nectar from a supplemental feeder while confined to their nestbox), which all may affect both the susceptibility of bees to *Crithidia* infection and their motor performance. However, additional studies in which bees are infected experimentally are required to establish causality for certain.

The negative effect of parasitic infection on the motor performance of bumblebees parallels recent findings in both vertebrates and invertebrates. Laboratory mice infected with parasitic protozoa (*Eimeria vermiformis*) or nematodes (*Heligmosomoides polygyrus*, *Toxocara canis*) showed reduced performance in laboratory spatial tasks (Kavaliers & Colwell 1995; Cox & Holland 2001). Similarly, performance on a passive-avoidance learning paradigm was significantly reduced in mice infected with the nematode *Schistosoma mansoni* (Fiore et al. 2002). Our findings are also consistent with recent evidence that the cognitive function of honeybees (*Apis* sp.) is affected by an inducible immune response (Mallon et al. 2003). In that study, bees that had their immune system challenged by a nonpathogenic immunogenic elicitor lipopolysaccharide were less able to learn and/or remember a reward–odour association in the proboscis-extension reflex experimental paradigm.

The *C. bombi*-induced reductions in motor performance found in our study represent a significant additional cost to foraging bees. Highly infected bees showed a 200% increase in the amount of time and number of flower visits required to reach maximum motor performance on the novel flower type. Under natural conditions, bees can take up to 1 h to become proficient at handling flowers (Laverty 1994a). Our data indicate that bees with an intense infection of *C. bombi* require an additional time investment of 2 h to reach maximum performance on these same flowers. Consequently, these substantial time costs could have profound effects on the foraging decisions of bees. Numerous studies have suggested that foraging decisions of bumblebees are related to the amount of time required to learn and remember flower-handling methods (e.g. Heinrich 1979; Laverty 1980, 1994a, b; Gegear & Laverty 1998; Chittka et al. 1999). Bees may avoid novel flowers that require a greater learning investment because initial time costs may far exceed the energetic value of the reward that they contain. In addition, the decision of whether to specialize on the flowers of one plant species or adopt a more generalist foraging strategy has traditionally been considered in terms of the amount of time invested into handling methods (Darwin 1876; Heinrich 1976; Waser 1986). Previous studies of bumblebees have shown that there are costs associated with alternating between flower types requiring different handling methods (Woodward & Laverty 1992; Gegear & Laverty 1995; Chittka & Thomson 1997), and these costs limit flower selection (Gegear & Laverty 1998). Parasitic infection may act to amplify these effects through either an increase in the time invested per flower or a reduction in the number of motor patterns that can be held in memory. Indeed, the propensity of bumblebee foragers to visit flowers requiring less complicated handling methods has been shown to increase in bees parasitized by conopid flies (Schmid-Hempel & Schmid-Hempel 1990; Schmid-Hempel & Stauffer 1998). However, the extent to which parasite-induced cognitive impairments contribute to these changes has yet to be determined.

Our results raise the intriguing mechanistic question of how a protozoan gut parasite can affect the learning ability and memory of bees. Because *C. bombi* triggers a general (constitutive) immune response in bumblebees even though it does not cross the gut barrier into the haemolymph of infected bees (Brown et al. 2003), the answer most likely involves the action of a chemical messenger that is common to the processes of immune defence and cognitive function (Kavaliers et al. 1999; Mallon et al. 2003). In the case of the intestinal trypanosome *C. bombi*, one plausible chemical messenger is nitric oxide, although others are possible (Mallon et al. 2003). Nitric oxide is produced by epithelial cells in the gut of insects upon invasion by trypanosomes and is thought to play an integral role in signalling the fat body (equivalent to the mammalian liver) to initiate an immune response (Luckhart et al. 1998; Hao et al. 2001, 2003). Studies of vertebrate and invertebrate nervous systems have revealed that nitric oxide also acts as a diffusible signal molecule and is important in the formation of long-term memories (Muller 1996, 1997; Prast & Philippu 2001). However, high levels of nitric oxide have recently been shown to have detrimental effects on cognitive function, probably due to the role of nitric oxide as a free radical (dos Reis et al. 2002; de la Torre et al. 2003). Therefore, it is possible that an intense infection by *C. bombi* impairs the motor learning of bees through either a trade-off in the use of nitric oxide for both immune and cognitive functions or through the adverse effects of increased free-radical nitric oxide in the haemolymph.

From the perspective of *C. bombi*, the behavioural changes of highly infected bumblebees appear to be beneficial. There are many examples of host–parasite relationships in which the parasite induces behavioural changes in its host that facilitate further transmission (see Poulin 2000 for review). Because horizontal transmission of *C. bombi* between bumblebee colonies occurs almost exclusively through the shared use of flowers by infected and uninfected bees (Durrer & Schmid-Hempel 1994), it is reasonable to expect that any behavioural changes in bumblebees favouring the transmission of *C. bombi* should involve flower visits. We have shown that a high intensity of infection by *C. bombi* increases the amount of time bumblebee workers spend extracting nectar from flowers. Assuming that there is a link between the amount of time spent on a flower and the probability that a bee will deposit infective cells, our results suggest that behavioural changes in highly infected bumblebee workers may promote horizontal transmission of *C. bombi*. Moreover, because bumblebees generally spend greater amounts of time on, and are often the main visitors of, flowers

requiring more specialized handling methods, the occurrence and frequency of *C. bombi* transmission may be a function of floral complexity. Durrer & Schmid-Hempel (1994) found that bumblebees visiting the flowers of *Echium vulgare* had a higher probability of becoming infected by *C. bombi* than bees visiting the relatively less complicated flowers of *Rubus caesius*.

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