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The Effect of Immuno-stimulation on the Health of Bumblebees: Assaying Behavioral and Physiological Responses

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The Effect of Immuno-stimulation on the Health of Bumblebees:

Assaying Behavioral and Physiological Responses

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Abstract

Pollinator species are declining around the world at an alarming rate, posing a major threat to both terrestrial ecosystems and the agricultural industry. The exact cause of these declines has yet to be identified, but pathogen infection claims a high position on the list of likely causes. To better understand how infection impacts the health of a native bumblebee pollinator, *Bombus impatiens*, I artificially stimulated an immune response in individual foragers and then measured their physiological (zone of inhibition assay) and behavioral (serial reversal learning assay) response. My findings will greatly improve our ability to quantify the fitness consequences of infection on bumblebees and expand the range of conservation tools available to evaluate and predict the effect of exposure to novel pathogens on different bumblebee species.

Background

Pollinators around the world are experiencing rapid and unprecedented population declines. The loss of bees in particular is distressing, since they are an invaluable part of many of the earth's ecosystems. There are more than 20,000 species of bees worldwide (Velthuis & van Doorn, 2006), and of these, the approximately 250 species of bumblebee (genus *Bombus*) have become the subject of much concern (Goulson, Lye, & Darvill, 2008). Bumblebees are quite important pollinators in their native regions of the northern hemisphere (Goulson et al., 2008). Their long tongues help them collect nectar from deep flowers in these regions, and their use of sonication or "buzz pollination" efficiently spreads pollen (Buchmann, 1985). These traits, along with ease of maintenance and handling, have made the especially important for North American and European agriculture (Kraus et al, 2010; Goulson et al., 2008). Bumblebees are now the main greenhouse pollinators of such food crops as tomatoes, bell peppers, and a variety of berries in the United States (Velthuis & van Doorn, 2006; Kraus et al., 2010). These operations currently produce about three billion dollars a year for the US economy (Danforth, 2007).

With such a large stake in our food supply and economy, the health statuses of bee species must be a priority. Currently, the cause of bee decline is unknown. However, researchers have proposed several possible environmental stressors, including habitat loss/alteration, pesticides, pathogens/disease, and climate change (Goulson et al, 2008; Whitehorn, O'Connor, Wackers, & Goulson, 2012, 2012; Henry et al., 2012; Colla, Otterstatter, Gegear, & Thomson, 2006). However, we currently have a poor understanding of how each of these stressors, and combinations thereof, affect the health of bees, particularly those native species in decline. In this study, I explore how pathogens/disease impact bee populations through sublethal effects on fitness-related behaviors.

Many animals experience changes in behavior in response to illness. Much like a human experiencing a bout with the flu, other vertebrate animals will also exhibit lethargy, decreased appetite, fever, and a number of other predictable symptoms when suffering an infection. In fact, evidence is

growing to prove that these changes are an evolutionary adaptation for the body to focus its efforts on healing (Hart, 1988). Much of these behavioral symptoms can be traced to cognitive impairment induced by the immune response to the infection (reviewed in Wilson, Finch, & Cohen, 2002). In vertebrates, it seems that sickness behavior is a concerted effort by the brain and immune system to combat disease (see Maier & Watkins, 1998 for a review). Cytokines are the main signaling molecules involved in vertebrate immune responses, and thus in vertebrate sickness behavior and cognitive impairment. For instance, Alzheimer's patients are more likely to develop delirium upon infection due to cytokine mediation (Holmes et al., 2002), and cytokines have been implicated in memory consolidation (Rachal Pugh, Fleshner, Watkins, Maier, & Rudy, 2001) and memory deficiencies (Gibertini, Newton, Friedman, & Klein, 1995) A similar type of cognitive impairment may occur in invertebrates like bumblebees, as well. It has been shown that parasitic infections can reduce learning efficiency in bumblebees (Gegear, Otterstatter, & Thomson, 2005; Gegear, Otterstatter, & Thomson, 2006). Insects, unlike humans, possess no adaptive immunity. They do, on the other hand, possess cellular and humoral innate immune functions (see Lavine & Strand, 2002 for a review). In insect innate immune responses, along with other reactions, antimicrobial peptides (AMPs) are secreted by the fat body (liver analog) in response to infection (Hoffmann, 2003). These AMPs occupy the hemolymph, which is the insect analog of mammalian blood/lymph fluid. It appears insects may even have humoral molecules that mimic the functions of cytokines in vertebrates (Wittwer, Franchini, Ottaviani, & Wiesner, 1999; reviewed in Hoffmann, 2003).

A change in bee cognition could have detrimental sublethal effects on colony performance and health. Bees have advanced capacities for learning and association, and faster learning in bumblebees has been linked to better foraging success while slower learning results in lower nectar stores (Raine & Chittka, 2008; Raine & Chittka, 2012). Other problems with foraging behavior would likely have similar effects on nectar collection. Less food would support fewer bees within a colony, which would in turn provide the colony with even less food to support the production of reproductives (males and queens), ultimately lowering the number of queens available to contribute to the population the following year. In

this way, a seemingly small, sublethal change in individual bee foraging proficiency can negatively affect the size of the bee population.

Learning to handle different types of flowers in their natural environment also requires much of bumblebees' mental faculties (Chittka & Thomson, 2001). In order to handle flowers of varying complexity, bees must be capable of switching handling methods. This ability to switch between tasks is a product of the bee's cognitive flexibility (Chittka & Thomson, 2001; Gegear & Laverty, 2005).

Task switching proficiency and thus cognitive flexibility can be evaluated using serial reversal learning assays. In these tests, subjects are taught to associate rewards with specific tasks. The subject is then tested on completing one of the tasks, after which the tasks are reversed. There is a cost to performance inflicted by switching the tasks, and ideally, this cost is diminished with each reversal. The costs associated with switching determine the flexibility of the subject. So far, these assays have mostly been performed with humans and other vertebrates (Bond, Kamil, and Balda, 2007); however, there is evidence that this assay can be used with insects (Strang & Sherry, 2013).

The purpose of this study was to develop assays for measuring the strength of an immune response (zone of inhibition assay) and level of cognitive flexibility (serial reversal learning assay) in individual bumblebee (*Bombus impatiens*) foragers. Although *B. impatiens* is not among the bumblebee species experiencing severe declines at present (Goulson, 2008), experimental data from the current study will be used as a baseline for future assessment of bumblebee species in decline. To isolate the effects of immune-stimulation on bees, physiological and behavioral responses of healthy bees were compared to immune-stimulated bees injected with LPS (lipopolysaccharide). LPS is a component of gram-negative bacterial cell membranes, which is known to induce an immune response in many animals, and was used here to mimic pathogen invasion.

Methods

1) Flexibility Assay: Serial Reversal Learning

Bumblebees (*Bombus impatiens*) were tested in serial reversal learning trials that included a color-detection and an odor-detection task. These bees were trained using associative learning to recognize non-scented yellow flowers as rewarding (color-detection) and blue flowers with geranium scent as rewarding (odor-detection). The bee's ability to switch between these two tasks was subsequently examined in a series of trials containing distracter flowers of various colors and scents. All flowers were constructed from artificial parts, and rewarding flowers contained 30% sucrose solution compared to non-rewarding flowers, which contained 5% sucrose solution. Tests were digitally recorded for ease of data collection.

Flowers & Scents

Imitation flowers used in the experiments were constructed from 1.5ml microfuge tubes (Fig 1). Each tube was fixed with a 5.72cm diameter ring around the top edge constructed from Creatology® brand foam purchased from Michaels Stores, Inc. These rings acted as the flowers' petals. 30% sucrose was prepared by mixing ~160g sucrose and enough distilled water to make 400ml of solution, after which the concentration was confirmed with a refractometer. This solution was used as a food source for foraging bees and deposited within the microfuge tubes. Scent solutions were applied directly to the petals. Geranium, peppermint, clove, and apple scents were prepared by mixing 200µl of 98% pentane: 4µl essential oil. Geranium (*Pelargonium graveolens*), peppermint (*Mentha piperita*), and clove (*Eugenia caryophyllata*) oils were purchased from Aura Cacia®. Apple oil was purchased from The Body Shop®. Flowers used for everyday feeding were transparent with white petals, scented with 5µl apple scent solution. Test flowers were yellow or blue with yellow and blue tubes and petals, respectively. Blue geranium flowers received 5µl of geranium scent solution. Distractor flowers for the test array were constructed in the same design with purple, orange, and blue tubes and petals. Purple and orange flowers

were odorless and served as two distractor flowers for the color task. Blue flowers were odor distractors and either scented with peppermint or clove solutions.

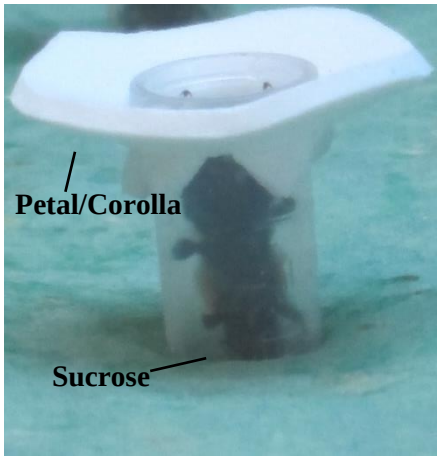

Figure 1- Forager bee collecting sucrose from an apple-scented feeder flower.

Figure 2-Forager bee restrained and injected between abdominal turga.

Colony Maintenance

Young Biobest® colonies of *Bombus impatiens* were received in a box (29.85x 0.64x22.86 cm) that contained a queen and approximately 30 workers. The colony was attached to a flight cage (133.35 x 152.4 x 99.06cm or room-sized) with a tunnel bridge made of aluminum mesh. The bridge contained two plastic gates for controlling the flow of bees into and out of the colony. Both cage frames were constructed with wood and enclosed with mesh. Cages were lit with Zoo Med Tropic Sun® 32W 5500K balanced full spectrum daylight lamps purchased from Petco®. These cages provided an open area for flight and foraging. Bees were fed daily with 30% sucrose solution delivered in white, apple-scented flowers. Flowers were arranged on a green camouflaged Styrofoam array. Bees were fed sucrose solution twice a day. Bee Feed Pollen Dust was ordered from the Epicurean Honey Company, ground, and mixed with prepared 30% sucrose solution until desired consistency was achieved. Small logs of pollen were added directly to the colony with forceps as needed for a protein source and building material.

Treatment

Active foraging bees were selected by observation of behavior on the feeding array. New bees were collected at least every other day allowing foraging age to be estimated to within 1-2 days of treatment. Viable bees were vialed and chilled at 4°C for sedation prior treatment. Bees were randomly assigned into two groups: non-injected controls and lipopolysaccharide-injected (LPS) bees. Control bees were dried on paper towel then marked with acrylic paint on the dorsal thorax and/or abdomen. These were returned to the flight cage or directly to the colony and tested within one week of their beginning to forage. LPS bees were also dried then restrained with pins secured in Styrofoam (Fig 2). The injection was made between the 4^{th} and 5^{th} dorsal turga with a 26-gauge Hamilton® needle that dispensed 2µl of insect Ringer's + LPS solution [Insect Ringer's= 7.4808g sodium chloride, 1.9977g calcium chloride, 0.0970g potassium chloride, 0.1938g sodium bicarbonate, dissolved in distilled water; LPS= 20ml Ringer's, 10mg lipopolysaccharide]. LPS bees were then marked on the dorsal thorax and/or abdomen with acrylic paint for identification and returned to the flight cage or directly to the colony. Injected bees were allowed 24 hours to recover before testing. They were tested within one week of injection.

Training

The feeding array was removed and treated foragers were trained by associative learning on both rewarding flower types—unscented yellow or geranium-scented blue—by exposure to a homogenous six-flower array. Bees had to complete at least three consecutive foraging runs (leave colony, select rewarding flower, fill honey sac, return to colony) on one flower type before the array was switched to the other flower type and the process repeated an equal number of times. Order of presented flowers for associative learning was randomized, and previous data analysis shows no effect on performance. To ensure recall of this information, training was repeated as needed within at least one day of testing.

Figure 3-(A) Six-flower training array [yellow shown] and (B) test array with geranium, yellow, and distractor flowers.

Testing

The serial reversal test allows quantification of a forager's performance efficiency when forced to switch between rewarding floral tasks of color-detection (non-scented yellow flowers) and odor-detection (geranium-scented blue flowers). The test used a Styrofoam array (120.65 x 74.93cm) covered in green camouflage paper containing both rewarding flowers and distractor flowers (color: non-scented orange and purple, odor: blue clove and peppermint) in rows spaced 11.75cm apart with 6.03cm between flowers in a row (Fig 3A). The array held a total of 90 flowers: 16 unscented yellow, 16 geranium-scented blue, 15 purple, 14 orange, 15 peppermint-scented blue, and 14 clove-scented blue. All distractors were given 2µl of distilled water. Non-scented yellow flowers and geranium-scented blue flowers received either 30% or 5% sucrose solution (5% was prepared similar to 30%, but ~64g sucrose) in alternation with trials so that one task was always dominantly rewarding during a foraging run.

Testing began by releasing the previously trained forager into the flight cage with a 33.02 x 27.31 x 3.18cm six-flower switching array. The main testing array was kept beneath this smaller array and covered with green camouflaged Styrofoam panels so it was completely hidden from sight of the bee. The six flowers were arranged in two rows of three with the positions of geranium-scented blue and unscented

yellow flowers alternating. All flowers contained 2µl of 30% sucrose solution. All but one flower were also concealed with paper cubes to direct the bee to the uncovered flower (the task of the uncovered flower was opposite that of the last flower the bee had encountered during training). Once the bee began foraging from the uncovered flower, the next flower in line was uncovered. When the bee moved to the next flower to forage, the first flower was concealed again, always giving the bee only one floral choice. This allowed the bee to begin switching rapidly between the two tasks prior to official start of the trial. The procedure continued until the bee was foraging in the last flower on the six-flower array, at which time the panels covering the main testing array were removed. When the bee moved onto the full test array, the smaller six-flower array was also removed from the flight cage.

The bee's foraging behavior was then observed on the full testing array (Fig 3B). For later analysis, the test was digitally recorded and the type of flower visit and color were verbally called out for the camera. Flower visits were defined as any time the bee crossed the threshold at the top of the flower and were classified as either "full" or "mistake." Full visits entailed the bee traveling to the bottom of a rewarding flower and collecting the sucrose reward within. Visits to any other flower type that was non-rewarding (containing 2µl distilled water or 5% sucrose) were considered mistakes. Once the bee foraged from a rewarding flower, the sucrose solution was replaced with a Rainin AutoRepE pipettor while the bee was in its next flower choice so as to not interfere with its attention. Foragers visited between 27 and 51 flowers on one foraging run before returning to the colony. When the bee left the flight cage, the rewarding flower types were removed from the array and replaced by clean set-up flowers which reversed the rewarding sucrose concentrations from the previous run. For example, if unscented yellow was rewarding first, geranium-scented blue would be rewarding second, and vice-versa. When the bee returned from the colony to forage again, it was again released into the flight cage to continue testing. This pattern continued until three trials were completed for each task (a total of six foraging runs). The bee was then captured and stored in a labeled vial in the 4°C refrigerator for later size measurements and zone of inhibition analyses.

Clean-up

After unscented yellow or geranium-scented flowers were used in training or testing, the petals were removed from the tubes and placed in separate containers. The tubes were also placed in separate containers and left to soak in warm water to dissolve the sugar. The tubes were then rinsed several times with water, after which the water left in the tubes was shaken out, and they were left to dry on a clean paper towel. All distracter flowers were left in the testing array for several hours to air-dry the distilled water inside them. These flowers were then stored in separate containers.

Data Analysis

A digital spreadsheet was created for each reversal learning test, which recorded each correct (rewarding) and mistake visit the bee made in every run, as well as the number of visits it took the bee to learn which flower task was rewarding. These also included total elapsed time for each run, identifying information about the bees, observations of unusual behavior, and numbers of total visits and rewarding visits during each run. Learning was defined as the visit after which at least 70% of its visits were rewarding. The learning times were compared graphically, as seen for the control bees in figure 1.

2) Physiological Assay: Zone of Inhibition

In this set of experiments, the strength of the bee immune response was quantitatively determined with a zone of inhibition study adapted from Haine et al. (2008) and Korner & Schmid-Hempel (2004). The original procedure, found in Appendix (A & B), was altered for better visualization of bacteria and clearance zones. The antimicrobial peptides within bee hemolymph should destroy the soil bacterium *Arthrobacter globiformis* if added to a culture containing the bacterium. Following this premise, plates were spread with a lawn of *A. globiformis*, and hemolymph from control and injected bees were placed on top as droplets. The sizes of the zones of no growth around droplet sites indicated the relative

concentrations of antimicrobial agents in the hemolymph, and thus the strength of the immune response. Here, two groups of bees (24hr or 48hr incubation) were tested to analyze the immune response over time.

Growing Cultures

Liquid cultures of *Arthrobacter globiformis* (ATCC 801D) were derived as needed from a stock plate culture (LB, 1% agar) streaked for isolation (incubation at 26°C). Regularly (~every 3 days) new stock plates were streaked for isolation using single colonies from the previous plates. 24 hours before anesthetizing bees for hemolymph extraction, 10ml of nutrient broth (BD® 23400) were inoculated with a single colony from plate culture and incubated in a 26°C shaker (40 rpm).

Bee Treatments

30 non-foraging female bees were collected from the colony, sedated in a 4C refrigerator, then split into three groups of 10 bees. 10 bees were injected with 2µl LPS between the 4th and 5th dorsal turga of the abdomen with a 26-gauge Hamilton® needle. 10 bees were injected with 2µl ringer's solution (vehicle). 10 bees remained as uninjected controls. The needle and syringe were rinsed with distilled water between treatments. All bees were placed in cup feeders (11.8cm diameter x 7.8cm high with mesh top; 4-5 bees in each) for 24 hours post-injection with 30% sucrose ad libitum and pollen available (Fig 4). The same procedure was performed with 30 females for 48hr incubation.

Figure 4- Females in cup feeder. Syringe pictured is of the type used for hemolymph extraction.

Assay Setup

After 23 hours, 300µl of the overnight (24hr) liquid culture were aseptically spread onto 8.5cm plates containing 20ml LB 1% agar with a plate spreader and left to dry in the hood. About 30 minutes later the female bees in feeders were then placed into a -17°C freezer for anesthetization. At 24hr post-inoculation/injection (or 48hr post-injection for the other group), the plates were labeled, and 30, 0.5ml microfuge tubes were labeled accordingly. The bees were also removed from the freezer and decapitated with a razor blade. Bees were held in place with pins lying over their backs and pinned into Styrofoam. Then, as much hemolymph as possible was extracted from the abdomens of the bees with 30 separate sterile BD® 30-gauge needle, disposable insulin syringes between the 4^{th} and 5^{th} turga. Hemolymph samples were transferred to the 0.5ml microfuge tubes, after which the bees and syringes were disposed of. Samples were spun down in a tabletop microcentrifuge for 10 seconds. 1µl aliquots were then placed on top of the bacterial lawns. Each treatment group occupied two of six positions on every plate, with each individual bee tested in triplicate (i.e. 30 bees, 2 treatments per plate, 3 plates per bee =15 plates total). This plating method helped control for plate errors. The plates were incubated at 26°C. Any remaining hemolymph was stored at 4°C.

Data Analysis

At 24hr post-plating (for both 24 and 48hr bees), the plates were photographed (Fig 5). Zones of clearance were measured digitally with ImageJ software. Of the hemolymph extracts that formed a zone, the diameter was measured twice (in cm) and the average taken. These numbers for all three replicates were averaged. Any replicates without zones were not included. The averages represented the final measurement for each bee. The measurements for each bee within a treatment group that showed zones of inhibition were then averaged. These were plotted with standard error in Figure.

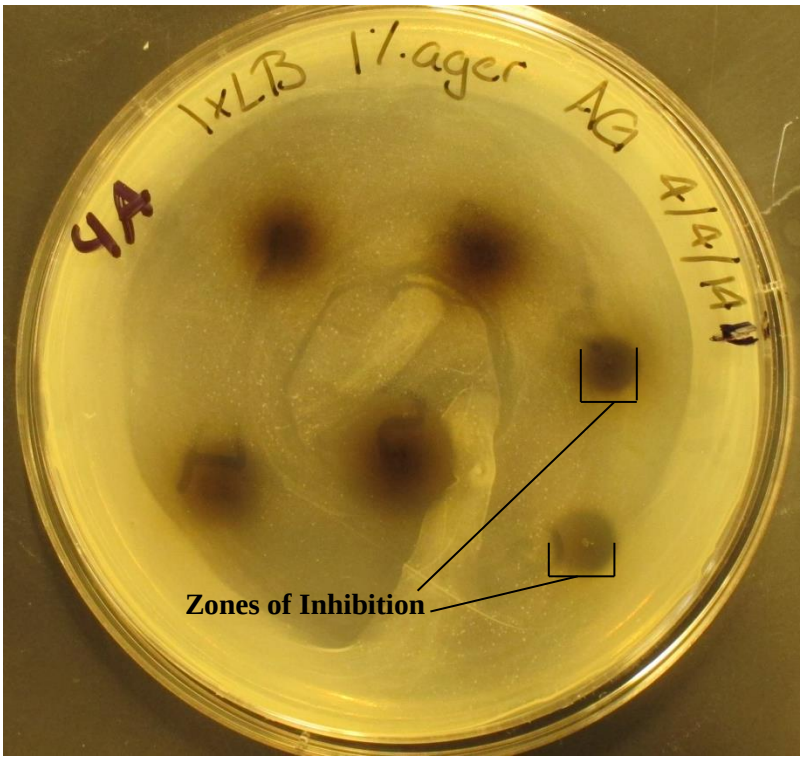

Figure 5- Example bacterial plate with zones of inhibition surrounding hemolymph extracts. Brown coloring is natural result of hemolymph exposure to air.

Results

1) Serial Reversal Learning Assay

LPS-injected and uninjected controls were run through an assay that forced them to switch between an odor-detection and color-detection task. There were six reversals, and the pattern of flower visits for each was recorded. The purpose of the reversal learning behavioral assay was to determine the numbers of visits it took bees to learn which task was rewarding after a task reversal and compare those numbers between LPS-injected and non-injected control bees. However, seven of the nine total LPS bees tested did not complete the assay. Several did not even complete the six-flower switching array meant to prime the bees for the main test array. Observations about each LPS bee test are summarized in Appendix (C).

Therefore, LPS bees could not be compared to controls. Even the two LPS bees that did complete the test presented learning efficiencies that were too highly varied to compare. However, learning was compared in control bees between those that were provided the color task first and those that were provided the odor task first. Surprisingly, there were great differences in the learning abilities

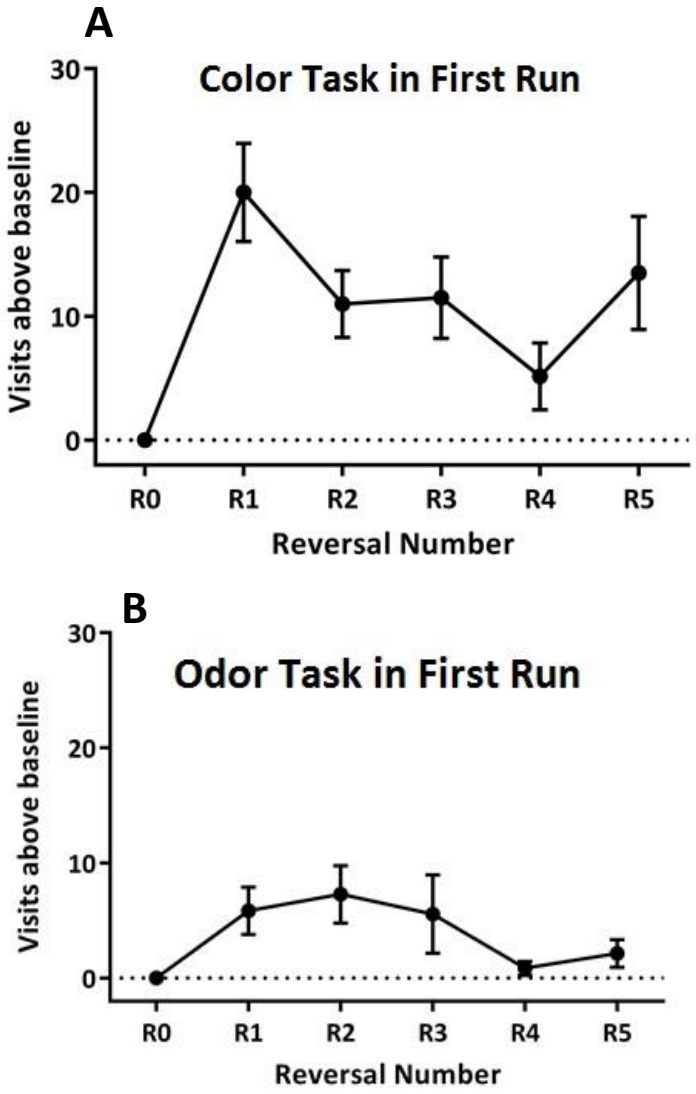

Figure 1- Learning efficiency of control bees in reversal learning assay. Numbers of visits to learn which task is rewarding compared to the first run (baseline) for uninjected bees presented with the (A) color task first (B) odor task first.

of these two control groups. For each group, the number of visits required to learn the rewarding task in the first run was set as the baseline for learning. This baseline was subtracted from subsequent numbers of visits before learning. Control bees given the color task first (fig 1A) showed a sharp increase in the number of visits required to learn the rewarding task right after the first run. The numbers of visits for learning decrease over time, but never reach baseline again. Contrastingly, controls given the odor task first (fig 1B) show a more gradual increase in visits before learning after the first run. The visits also start to decrease gradually after Run 3 and return to baseline.

Zone of Inhibition Assay

This assay was used as an indication of whether LPS could induce a physiological response in bees that would correspond to behavioral changes. Hemolymph samples from LPS-injected, Ringer-injected, and uninjected bees were placed on culture plates spread with *Arthrobacter globiformis* lawns.

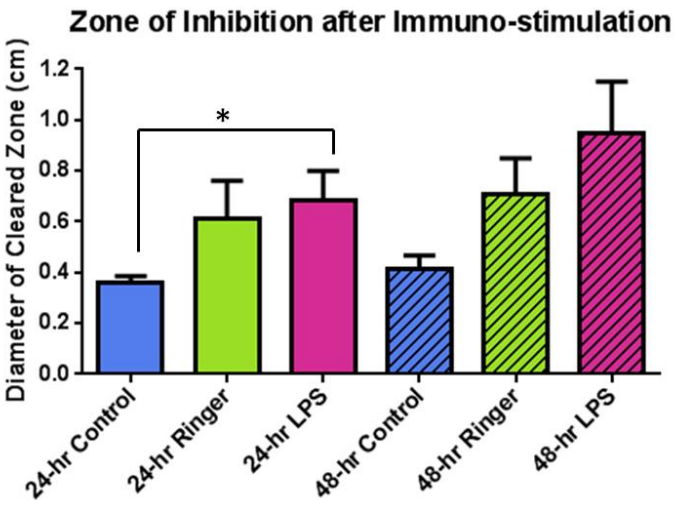

Figure 2- Antimicrobial response to immuno-stimulation. Average diameter of zones of inhibition compared for control, Ringer, and LPS bees at 24 and 48 hours after injection.

The sizes of zones of no bacteria growth around the hemolymph samples indicate the antimicrobial peptide activity within the hemolymph.

All three treatment groups presented a portion of the hemolymph samples with zones of inhibition, including the controls (fig 2). LPS bees possessed the largest diameter zones of inhibition for the bees incubated at both 24 hour and 48 hours. After 24 hours, the difference between LPS and control bees was significant. Although the significance of this relationship was not evaluated for 48 hours, LPS and control bees also had the largest difference in diameters for that incubation group. Although the LPS in the 24-hour group were not significantly

different from Ringer controls, and the same appears to be true for the 48-hour group, LPS trended toward larger diameters than Ringer controls. In addition, the data suggest that antimicrobial responses for all treatments are heightened at 48 hours compared to at 24 hours.

Discussion

In previous voluntary task switching (VTS) assays, we have found that *Bombus impatiens* display perseverance behavior if stimulated with LPS. In other words, when presented with an array possessing rewarding color and odor tasks together, bees treated with LPS appear to lose all ability to switch between tasks, exclusively performing only one of the tasks. This study helps complete the picture of this seeming loss in cognitive flexibility with immune stimulation. Here, we explored LPS's effect on behavior when bees were forced to switch between tasks in a serial reversal assay.

The serial reversal learning assay was performed on non-injected control bees and LPS-injected bees. While control bees performed in a similar manner to uninjected bees in the previous VTS assays, most of the LPS-injected bees in this study did not complete the assay. This suggests that, similar to the VTS results, LPS stimulation of the bee immune system may impair cognition enough to eliminate switching, even when task switching is forced upon the bee. Not completing or even starting the assay may be the result of an inability to make a cognitive switch between the last task type encountered and the first task type given in the test. Of those that did complete the test (n=2), the number of visits it took before the bees learned the rewarding task for each run was highly variable.

While it is possible that other factors besides immuno-stimulation could have influenced the non-completion behavior, the behavior persisted over time exclusively in LPS-injected individuals. Although previous investigation showed no correlation between the trauma of injection and long-term behavior changes, an injected control bee (injected with Ringer's solution) was run after the LPS bees to ensure that the injection process itself or colony-wide sickness were not factors in the results. The results of the Ringer control contrasted with those of uninjected bees, but also with those of the LPS bees. It is most likely, therefore, that this bee was an outlier. Running more Ringer-injected controls through the assay would be helpful for future studies to better understand the extent of injection trauma's effects on bees.

Although it is difficult to say conclusively from the behavioral assay what LPS's effect on bee behavior is, there is strong support for its effective disruption of cognitive flexibility to the point of eliminating task switching. Preliminary results from the 24-and 48-hour zone of inhibition assays also provide some physiological evidence of LPS causing a heighten immune response, giving support to the link between LPS and behavior changes. This has grim implications for bumblebees in the wild and in agriculture. If the response to LPS does indeed mimic the response in the field to pathogens, bumblebee foragers could face serious deficits in their ability to utilize multiple types of flowers for food, reducing the fitness of the colony as a whole. Another possibility for future investigation might be assessing behavior with varying concentrations of injected LPS, as 2µl might elicit too strong of an immune response in the bee.

The most surprising finding of the behavioral assay was that although previous studies indicated the opposite, this study saw the task type presented at the beginning of the test making a difference in learning patterns for normal, uninjected bees. When starting on a color task, the bees appeared to have a much harder time normalizing their number of visits before learning than when starting on an odor task. Only odor-first controls came back down to a baseline learning efficiency with time. This is an interesting anomaly for which there is not yet an explanation, but it will be important information to keep in mind for future testing. The starting task type may need to be tested for significance again on its own. Or, future researchers might consider using only the odor task first.

Although there are inconsistencies in the data from the zone of inhibition study (i.e. the amount of antimicrobial peptide activity of hemolymph among bees in the same injection group is different) LPS appears to produce slightly higher levels of antimicrobial peptide activity than injection alone (Ringer-injected).

Unexpectedly, a number of the control bees in each incubation group produced zones of inhibition, suggesting a baseline level of immune system activity occurring in these bees without artificial stimulation. This may represent a pre-existing infection in the bees, or simply a maintenance level of antimicrobial peptides in the hemolymph. Still, the higher levels in LPS bees are consistent with the hypothesis of a heightened response in the presence of a stimulator. The Ringer bees in this study also showed a heightened response, but less so than that of the LPS bees. An immune response is expected for a short time after injection itself, and although the response appears to increase from 24 to 48 hours, 48 hours may be representing a peak in a transient response, while LPS sustains a high immune response for several days. Korner & Schmid-Hempel (2004) provide evidence of the LPS antibacterial response in *Bombus terrestris* peaking at 48 hours post-injection, which would fit with our data. However, they also present a peak for Ringer-injected bees at 12 hours. The increase at 48 hours in this study could be the result of a small sample size or the difference in bumblebee species. Inconsistencies with bacterial plating and visibility issues when measuring zones of inhibition may have also contributed to these results.

Clearly, the zone of inhibition study must be further developed for future analysis of the bumblebee immune response. Future investigations will include several incubation groups over seven days to reproduce the Korner and Schmid-Hempel findings that show *Bombus terrestris* recovery from LPS after one week. Plates will be systematically labeled to ensure the identities of each zone of inhibition. Better lighting for pictures will also improve the precision and accuracy of diameter measurements.

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Appendix

A. First zone of inhibition study following Haine et al. (2008) protocol.

Growing Cultures

Freeze-dried *A. globiformis* (ATCC) in a double-glass vial was rehydrated and resuspended in 500µl nutrient broth under a sterile hood [nutrient broth: DI water, beef extract, peptone, autoclaved]. The suspension was transferred to a 5.5ml of broth (BD® 23400) in a glass tube. The tube was placed in a 40 rpm, 27°C shaker for two days. 500µl of culture was then aseptically pipetted onto an 8.5cm LB 1% agar plate to ensure bacterial viability. The plate was incubated at 30°C for two days. Since bacterial growth was confirmed from this plate, two new plates were aseptically aliquoted 750µl of the liquid culture (more liquid culture was thought to ensure better bacterial spread). Plates were incubated at 30°C for two days. 1ml of the liquid culture was also passaged into 9ml fresh nutrient broth. Both the original and new liquid stocks were kept in the 40rpm, 26°C shaker. After two days' incubation, single bacterial colonies were indistinguishable on plates. An isolation plate was streaked for each culture plate using a sterile loop. The isolation plates were incubated at 30°C for two days. Three single colonies were picked from the plates under sterile hood and used to inoculate 3 tubes of 5ml fresh nutrient broth. The tubes were placed in the shaker at 40rpm and 26°C overnight.

The three clones were streaked for isolation on new agar plates after 24 hours. Two days later, one colony from each of the three plates were picked and placed in fresh 10ml LB broth. They were incubated for 32 hours in the 26°C shaker, at which time 100µl of each culture was added to 50ml of sterile LB 1% agar. 7ml of inoculated medium was added to 6, 8.5cm plates for each culture, and the plates left in the sterile hood to solidify. The plates were then transferred to a 4°C cold room for 24 hours.

Bee Treatments

Six non-foraging female bees were collected from an extra colony (not attached to flight cage) and injected with 2µl LPS/Ringer solution the day after the LB 1% agar plates were poured. Six control female workers were collected from the same colony at this time and not injected. Injected bees and control bees were kept in separate cup feeders (11.8cm diameter x 7.8cm high with mesh top; 4-5 bees in each) with 30% sucrose ad libitum and pollen available.

Assay Setup

Three inoculated LB 1% agar plates were removed from the cold room and left to warm at room temperature for 30 minutes. Female bees in feeders were then placed into a -17°C freezer for anesthetization. Four wells were then formed in each of the plates by aspiration with a standard glass Pasteur pipette and nipple. Wells were labeled, and 12, 0.5ml microfuge tubes were labeled similarly. At 24 hours post-injection, the sedated bees were decapitated with a razor blade. Bees were secured to Styrofoam by placing pins across their backs. As much hemolymph as possible was then extracted from the abdomen of the bee with sterile BD® 30-gauge needle, disposable insulin syringes between the 4^{th} and 5^{th} turga. Hemolymph samples were transferred to the 0.5ml microfuge tubes, after which the bees and syringes were disposed of. Samples were spun down in a tabletop microcentrifuge for 10 seconds. A 2µl aliquot of each hemolymph sample was then micropipetted to each plate well, with two injected and two control bee's samples on each plate. The colony, which was several months old, had few females of average size remaining, so injected bees were paired in size to the control bees on their plates as much as possible to eliminate size as a variable. The plates were incubated at 30°C for optimal bacterial growth and hemolymph diffusion, as suggested by Haines. Any remaining hemolymph was stored at 4°C.

Data Analysis

At 24 and 48 hours of incubation, the first three plates were photographed. Zones of inhibition were expected to form around the wells of LPS-injected bees. However, there were no zones because bacterial growth was not clearly visible or consistent throughout the plate. A longer incubation time (48hr) did not improve bacterial visibility.

B. Further zone of inhibition studies to assess lawn growth and *E.coli* use

It was predicted that a bacterial lawn, rather than infusing the agar with bacteria, would improve bacterial growth and visibility in the zone of inhibition assay. This is similar to many standard zone of inhibition procedures for non-insect samples. It was also thought that a more standard bacterial model, *E. coli*, would produce clearer results.

Bee Treatments

Eight non-foraging females were taken from the colony and sedated in the 4°C refrigerator. Four of the bees were injected with 2µl of LPS solution, as done in the previous zone of inhibition study. The

other four bees were left as uninjected controls. All bees were placed in cup feeders for 24 hours post-injection.

Culturing and Assay Setup

New plates containing 20ml LB 1% agar without bacteria were poured and solidified in the hood overnight. At 23 hours post-injection of the bees, three new LB plates were spread with 300µl *A. globiformis* and left to dry until 24hrs. Bees were put in freezer after spreading bacterial lawns. At 24 hours post-injection, all bees were decapitated with a razor blade and pinned to Styrofoam. Hemolymph was extracted from the abdomens with sterile BD® disposable 30-gauge needle insulin syringes. Hemolymph was collected in 0.5ml microcentrifuge tubes and spun down for 10s in tabletop microcentrifuge. 2 µl hemolymph from control and LPS-injected bees were placed in wells of pre-made LB 1% agar + bacteria plates. 1µl drops of hemolymph from each treatment groups were also plated on LB 1% agar with the bacterial lawn. After incubation for 24 hours at 30°C, photographs were taken of the plates. The bacterial lawn plates showed adequate bacterial growth (was cloudy after 24 hours) and was much clearer and more defined than the well plates, which had no visible bacterial growth. The lawn technique was chosen to move forward with the assay.

Another similar experiment was done comparing lawns of *A. globiformis* and *E. coli*. *A. globiformis* zones of inhibition appeared larger and more well-defined compared to zones for *E.coli*. Therefore, *A. globiformis* was maintained as the model bacteria for this assay.

C. LPS-injected Forager Behavior Observations

Bee #	Outcome	Runs completed	Notes and Observations
135	Complete		On geranium run #2 let into colony and came out to finish; had to shake away from a flower several times because it was not moving
136	Incomplete	Did switching array fine 1 run, stopped during second	First run only correctly foraged ~4 times and most of the time it was away from the array, then returned to colony; second run it frequently flew away or tried to return to colony then not consistent once it learned; during 2nd run bee had to be shaken from a flower; only did a few during 2nd run; test was ended purposely
137	Complete		On 5th run yellow and geranium had 5ul of their sucrose solutions
138	Incomplete	Switching array force first (geranium) and second to last (geranium) 1 run stopped during second	Ended since bee was not going to rewarding (did not try forcing+ waited less than 20 minutes); first run distractor flowers had no odor
140	Incomplete	Did not finish switching array	Would not forage from 2nd to last flower (geranium)
141	Incomplete	Completed switching array	Completed 1 run; on 1st run had to be disturbed into continuing to forage several times; forced back to colony and did not return
142	Incomplete	Helped to all switching array flowers except last one (B3)	End on 1st run; had to help on 6-flower switching array all except last flower; crawling/flying away most of the time; helped back to array once; forced once unsuccessfully; tried giving reward directly from pipet and bee took it but did not forage
143	Incomplete	Ended during switching array	Trouble completing 6-flower switching array (forced for some and did not quite forage from them) allowed back in colony before completing all 6 and did not return; 30% sucrose was more like 33%
144	Incomplete	Completed switching array	Did switching array (may not have actually foraged from last flower [geranium]); ended on 1st run; tried forcing to learn twice and bee foraged

			but did not learn; returned to colony and came back and did same behavior