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Impacts and Underlying Mechanisms of Interference Competition Between Bumble Bees and Invasive Argentine Ants

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Impacts and Underlying Mechanisms of Interference Competition Between Bumble Bees and Invasive Argentine Ants

A Thesis submitted in partial satisfaction
of the requirements for the degree of

Master of Science

in

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by

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March 2018

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Dedication

I dedicate this creation to my loving parents, whose unwavering support lifts me high.

I dedicate this work to nature’s creative intelligence, that which connects us all.

ABSTRACT OF THESIS

Impacts and Underlying Mechanisms of Interference Competition Between Bumble Bees and Invasive Argentine Ants

by

Michelle Constanza Miner

Master of Science, Graduate Program in Entomology
University of California, Riverside, March 2018
Dr. Erin Wilson Rankin, Chairperson

Bees are important in both agricultural and natural ecosystems for the pollination services that they provide. However, invasive ants that exploit floral nectar in these landscapes can act as resource competitors with bees. Competitive interactions between bees and ants can have repercussions for pollinator resource acquisition, plant or bee fitness, and, ultimately, ecosystem function. There are gaps in our knowledge about the behaviors and sensory mechanisms involved in interference competition between bees and nectivorous, non-pollinator antagonists. Here, we studied how invasive ants influence bees in the context of nectar foraging. First, we performed laboratory assays to assess the foraging behavior of bumble bees (*Bombus impatiens*) in response to live Argentine ants (*Linepithema humile*) or to a subset of ant chemical cues. We found that bees were deterred by the presence of live ants as well as the combination of olfactory and gustatory ant chemical cues. Next, we characterized specific ant-bee interactions and behavioral transitions at shared nectar resources. We found that two-thirds of bee behaviors did not involve feeding, and, of those, the majority of them were aggressive interactions with ants. Similarly, 70% of behavioral transitions did not involve feeding, of which 82.3% were transitions among aggressive behaviors. Consistent with predictions of interference competition, the number of ants at a shared resource decreased the probability of a bee feeding and increased the likelihood of a bee being bitten. Similarly, getting bitten by an ant decreased the probability that a bee would subsequently feed. Finally, we examined participation and foraging success of bees during experiments with live Argentine ants, as well as the influence of ant-bee interactions on bee foraging activity and movement. We found that the majority of bees never foraged during experimental trials, consistent with high levels of non-activity in other social insects. There was further evidence of foraging specialization among bees: foragers that came into contact with ants and were nonaggressive were less successful at resource acquisition, while the foragers exhibiting aggression toward ants were more successful.

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Introduction

Insect foraging strategies must balance optimizing rewards and minimizing costs (Heinrich 1975). Shifts in these strategies can be guided by the costs associated with risk of harm (Tan et al. 2013) or competitive encounters (Ishii 2013). While modifying foraging strategy can help streamline foraging efficiency or evade predation, such responses to competition can also incur a cost to the individual or colony (Corbet *et al.* 1995). By extension, changes in foraging strategy or behavior of plant pollinators in particular can have cascading, ecosystem-wide effects (Wardle et al. 2011).

Pollinators are recognized for their crucial role in maintaining ecosystem function in natural habitats, providing ecosystem services in agriculture, and representing a key component of global biodiversity (Potts et al. 2010). Over 80 percent of terrestrial flowering plants—including both agricultural and wild species—rely on pollinators (Ollerton et al. 2011). Hence, the many additional organisms that rely indirectly on those many plants for food or shelter rely on the services of pollinators. Bees are arguably the most important subset of pollinators, and their populations face numerous, synergistic threats—including habitat loss, pollutants, pesticides, climate change, pathogens, and, increasingly, invasive species (Vanbergen 2013).

Invasive species can negatively impact ecosystems by degrading habitat, spreading disease, and competing for resources. Pollinator health is influenced by resource availability of pollen and nectar, a lack of which depresses the immune system and leaves bees more susceptible to pathogens that non-native competitors themselves may be spreading (Alaux *et al.*, 2010; Sébastien *et al.*, 2015; Levitt *et al.*, 2013; Graystock et al. 2016). Invasion by non-native species can disrupt native ecosystems by altering species interactions and community dynamics, including through interspecific competition between invaders and residents (Gibb and Johansson 2011) and by exploiting biotic resources that have not co-evolved defense strategies (Willmer et al. 2009). This has implications for species and communities both in the short term and the long term via selective pressures on surviving species at various trophic levels (Dayan and Simberloff 2005). It follows that invasion by an aggressive non-native species with whom floral resources are shared has the potential to severely impact its pollinating competitors, pollination-dependent plants, and, ultimately, ecosystem function. This is especially so when said aggressive invasive species can colonize habitat quickly and with high abundance. The Argentine ant, *Linepithema humile* (Mayr), is one such invader.

Linepithema humile is a serious pest that is globally distributed and locally abundant (Knight and Rust, 1990; Wetterer *et al.*, 2009). Habitat conversion to urban and agricultural landscape facilitates invasion by this exotic species into natural environments and the losses in native biodiversity associated with their predominance (Holway, 2005; Holway and Suarez, 2006; Buczkowski and Richmond, 2012). Argentine ants are fierce competitors that cause significant shifts in ecologically important native ant communities (Holway et al. 2002), decrease diversity across many arthropod taxa (Lach 2007), are frequent predators of both invertebrate and vertebrate nests and offspring (Sockman, 1997; Vega and Rust, 2000) and have even caused population declines in vertebrate predators of native ants (Suarez et al. 2000). They are also injurious pests of wild and crop plants because of their aphid-guarding behavior, in addition to being a household pest (Vega and Rust, 2000; Silverman and Brightwell, 2008).

Importantly, both pollinator and floral diversity may suffer as a result of ant-induced shifts in pollinator foraging (LeVan et al. 2014). Mounting evidence links Argentine ants to reductions in pollinator resource acquisition (Cembrowski et al. 2014) and plant fitness (Hanna et al. 2015) due to decreased pollinator visitation to ant-occupied or -visited flowers. Some pollinator species have been documented to avoid or spend less time at a nectar resource due to the presence of aggressive ants (Cembrowski et al. 2014), ant scent cues (Sidhu and Wilson Rankin 2016), gustatory cues from ant bodies (David Rankin, unpublished data), or inoculation of nectar with ant-associated microbes (Herrera *et al.*, 2013; Good *et al.*, 2014). However, the various mechanisms underlying this avoidance behavior remain largely unexplored.

There is evidence suggesting that chemical cues are likely involved. Ants communicate with each other through a variety of chemical means (Hölldobler and Wilson 1990), some of which other hymenopteran taxa, including bees, can learn to recognize and utilize as signals (Ballantyne and Willmer 2012) or cues (Sidhu and Wilson Rankin 2016).

However, our knowledge of chemical communication between pollinators and their antagonists is limited.

In order to safeguard essential pollination services that bees provide, we must identify and understand the relative importance of the mechanisms underlying interference competition between invasive ants and native pollinators. In this body of work, we studied how invasive ants influence bees in the context of nectar foraging. For Chapter 1, we performed laboratory assays to assess the foraging behavior of bumble bees (*Bombus impatiens*) in response to live Argentine ants (*L. humile*) as well as ant chemical cues. In Chapter 2, we characterized specific ant-bee interactions and behavioral transitions at a shared nectar resource. In Chapter 3, we examined participation and foraging success of bees during experiments with live Argentine ants, as well as the influence of ant-bee interactions on predicting bee foraging activity and movement.

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Chapter 1: Bee foraging behavior in response to live ants and ant cues

Introduction

Pollinators are both ecologically and economically critical organisms, responsible for aiding the reproduction of the majority of wild plant species and providing vital pollination services in agriculture (Ashman et al. 2004; Potts et al. 2010). Unfortunately, important pollinators, like bees, face many threats, including invasive species (Vanbergen 2013). The Argentine ant (*Linepithema humile*) is a globally distributed pest with an aptitude for outcompeting the organisms encountered in their exotic range (Human and Gordon 1996; Wetterer et al. 2009). In order to effectively steward pollinator habitat and safeguard pollination services in natural and agricultural landscapes, it is critical that we understand the impact of invasive Argentine ants on bees.

Niche overlap between invading ants and resident bees may manifest as exploitative or interference competition (Miller 1967). Exploitative competition entails the consumption of a limiting common resource, making it less available to competitors; while interference competition occurs when an organism hinders or prevents the success of another, often through aggression. While exploitative competition with nectar-thieving ants would primarily affect nectar-collecting pollinators, interference competition may inhibit both nectar and pollen foraging.

To avoid aggressive floral visitors, pollinators could use various sensory cues. These include direct visual or chemical cues or more persistent, indirect chemical traces that indicate the identity and previous presence of the aggressor (Kats and Dill 1998). In addition to the sensory cues associated with the physical presence of combative competitors, indirect cues may mediate interspecific interactions between bees and invasive ants. Ant interaction and associated sensory cues likely play an important role in affecting pollinator fitness and shaping plant-pollinator community dynamics—perhaps in a manner similar to predator-prey interactions (Binz *et al.*, 2014; Dukas, 2001). However, more research is needed to assess the roles of direct and indirect cues in mediating pollinator-invader interactions.

Insect recognition of cuticular hydrocarbons (CHCs) is an important means of chemical communication within and amongst social Hymenoptera (Blomquist and Bagneres, 2010), and seems to be recognized by solitary species as well. The detection of scent cues like CHCs may well have a bearing on the behaviors of both social and solitary foraging pollinators. Eusocial honey bees (*Apis mellifera*) can learn to discriminate among CHC compounds (Châline et al. 2005), and non-social, gregariously nesting bees, such as *Osmia lignaria* and *Megachile rotundata*, are able to recognize their individual nests amongst others through the use of scent cues that include CHCs (Guédot *et al.*, 2006, Guédot *et al.*, 2013). In addition, bumble bees deposit CHC footprints on flowers and may utilize them to make foraging decisions (Witjes and Eltz 2009). However, little is known about how heterospecific CHCs might be used by different bee species as cues in behavioral avoidance of aggressive, invasive, nectar-thieving ant competitors.

Ants are vectors of many microbes, and the experimental inoculation of nectar with ant-associated microbes can result in reduced attractiveness and nutritive quality of nectar (De Vega and Herrera 2013). Ant-associated yeast can exaggerate the attractiveness of floral nectar to bumble bees to the detriment of the plant's fitness (Herrera et al. 2013), and ant-associated bacteria can change nectar preference and foraging behavior (Vannette et al. 2012). To date, the behavioral response of bees to nectar fed upon by invasive ants has not been thoroughly tested.

Chemosensation in insect pollinators plays an important role in foraging (Falibene et al. 2015). In bees, gustatory sensilla are found on the antennae, mouthparts, and fore tarsi. Taste sensilla are known to respond to sweetness and saline, and it is possible that, in honey bees, they also have some sensitivity to water, amino acids, and proteins (de Brito Sanchez 2011). Bumble bees are also able to detect different sugar and protein concentrations (Konzmann and Lunau, 2014). Ruedenauer *et al.* (2015) discovered that the bumble bee *Bombus terrestris* can detect nutritive substances in pollen through olfaction, but can only detect differences in their concentration through tasting. Tiedeken et al. (2014) found that bumble bees may detect compounds considered bitter (by humans) in nectar as well. While aversive compounds do not seem to be perceived as a unique quality by antennal receptors, they can act to inhibit sucrose receptor cells and cause avoidance behavior in favor of more palatable choices in laboratory assays (de Brito Sanchez 2011). However, it is unclear the extent to which gustation is involved in heterospecific communication during foraging.

Could taste influence interactions between invasive ants and pollinators? This seems to be the case for native hummingbird pollinators, which are less likely to visit nectar with visible ants and strongly repelled by the interactive tactile and gustatory cues of Argentine ants (David Rankin, unpublished data). If bee pollinators have a similar aversion to the taste of the Argentine ants, gustatory cues may well influence competitive interactions via their impact on bee foraging behavior—be it through the taste of a physically present ant perhaps unseen in a flower or the flavor the ant has left behind. Little is known on this topic despite its potential importance. Additionally, visual cues seem to be important in the context of bee predator evasion (Gonçalves-Souza et al. 2008), but it is not well understood whether visual cues are used to evade non-predatory aggression.

In this study, we sought to improve our understanding of the impact of invasive ants on bumble bee foraging, as well as the sensory mechanisms involved in bumble bee recognition and learning in the context of nectar foraging. We hypothesized that competitive interference takes place between bumble bees and Argentine ants, and we predicted that bees would show avoidance of (a) nectar sources with live ants and (b) some ant chemical cues in the absence of live ants. We performed a series of observational choice assays in the laboratory to ascertain the foraging behaviors and preferences of bumble bees (*Bombus impatiens*) in response to live Argentine ants (*Linepithema humile*) and several ant chemical cues.

Materials and Methods

Bumble bee colonies: We conducted a series of behavioral trials with foragers from *Bombus impatiens* colonies obtained from Biobest USA (Romulus, MI). In the lab, each colony was maintained in a plywood nestbox (30 x 30 x 30 cm) and provided 40.6% (w/w) sucrose solution and pollen (Brushy Mountain Bee Farm, Inc.) *ad libitum.* Bees were deprived of sucrose solution for at least 30 minutes prior to each trial in order to encourage participation and feeding during each trial. To allow for individual identification, each bee was marked by adhering a small, unique number tag adhered to the thorax.

Argentine ant microcolonies: A colony of *Linepithema humile* with a queen (i.e. a queenright colony) was collected from UCR's Biological Control Grove (33.973387, -117.318344), housed in a plastic container (42.5L x 30.2W x 17.8H cm, Sterilite Corporation) and supplemented, as needed, with workers collected at the same source colony. From this queenright lab colony, we created queenless experimental ant microcolonies by relocating approximately 200 ants and at least five larvae into a smaller plastic container (18.5L x 13.5W x 10H cm, Target Corporation) lined with Insect-a-Slip

Insect Barrier — Fluon (PTFE-30, DISP30, BioQuip Products, Inc.). Ants were provided *ad libitum* with sucrose water, diced German cockroaches (*Blattella germanica*), and given 15 mL nesting tubes partially filled with dH_2O, plugged with cotton, and covered with brown paper towel to provide a darkened shelter.

General experimental set up

In all experiments, we provided bees with a 40.6% (w/w) sucrose solution as experimental nectar in each of two feeders, which were presented as a choice test between a control and ant-treatment. Each feeder was constructed by drilling a hole in a plastic jar lid (5.9 cm in diameter), removing the tip of a 1.5mL micro-centrifuge tube, and securing the top of tube in the hole of the lid. To prepare a feeder for a trial, we taped a shortened 1 oz polypropylene portion container (Dart Container Corporation) to the underside of the jar lid to create a removable nectar well, filled the well with 3.5 mL of nectar, placed a nectar-soaked dental cotton wick (Johnson & Johnson) into the micro-centrifuge tube, and taped a 5.8 cm diameter disk of qualitative filter paper (Grade 1, Whatman) to cover the top surface of the feeder. Each prepared feeder was placed in a 100 x 15mm plastic petri dish (Fisherbrand), and feeder pairs were placed in the center of a plastic arena (42.5L x 30.2W x 17.8H cm, Sterilite Corporation) (Figure 1.1). Each bee colony was trained to forage in this arena for a minimum of eight hours before their first experimental trial. Bees could access feeders by flying or by crawling on a Y-shaped, metal mesh bridge. The placement of treatment and control feeders was randomized by using an online digital coin flip simulator (Google.com, search “flip a coin”).

During each 30-minute trial, we recorded, per bee: bee identity, time spent on each feeder per foraging visit, feeding duration, and, if applicable, the number and nature of ant interactions. To determine nectar consumption by bees during each trial, feeders were weighed using a Mettler PC 180 balance before and after each trial.

To ensure there were no chemical residues, all feeders underwent a chemical rinse with hexane, ethanol, and dH_2O after each trial (see Appendix 1: Chemical Rinse Protocol). Feeders were handled with sterile, disposable gloves to ensure that the control feeders were free of contamination from ant treatment materials. In addition, the arena was wiped down with 70% ethanol and dH_20 between trials.

Live Ant Presence Experiments

To test the effect of live ant presence on bee foraging behavior, a queenless *L. humile* microcolony foraged freely on the treatment feeder only, while a queenless *Bombus impatiens* colony had access to the entire foraging arena, including both control and treatment feeders (Figure 1.2). Prior to each trial, an ant microcolony was given access to the arena’s treatment feeder and allowed at least 15 minutes to establish before a bee colony was given access to the arena as well. In order to prevent ant escape from the treatment feeder space, the petri dishes in which the feeders sat were coated with Fluon. In the event that an ant was flung into the arena by a bee, strategically placed Fluon also deterred the ant’s entry into the control feeder or bee colony. Each of five bee colonies underwent three 30-minute trials on three consecutive days, for a total of 1.5 hours foraging time with live ants prior to an Ant Cue Experiment. A sixth colony required additional foraging time with ants due to low participation (with less than five bees bitten by ants), so a fourth trial with that colony is included in the analyses.

Ant Cues Experiments

To measure the effects of different ant chemical cues and to assess bee learning, we tested both naïve and ant-experienced bee colonies with one of several ant chemical cues applied to the treatment feeder (Table 1). Bee colonies were classified as "Naïve" if they had never contacted Argentine ants previously, and were classified as "Experienced" once they had at least 1.5 hours of foraging time in the arena while ants had access to the treatment feeder. Following are the preparation protocols for each ant cue treatment.

Prior visitation by ants

A fully prepared treatment feeder was placed into an ant micro-colony for one hour to ensure that a minimum of 20 ants walked upon the filter paper and fed, depositing any chemical cues associated with visitation—namely, footprint hydrocarbons as well as any pheromones associated with feeding. The control feeder sat nearby but outside of the ant colony, untouched for the same duration of time. After one hour, the treatment feeder was then retrieved, the ants gently and quickly removed with a paint brush, and both treatment and control feeders were placed in the arena to begin the trial.

Ants in nectar

To make the ant treatment sucrose solution, 50 frozen ants (0.018 g ± 0.000 g) were placed into a tissue homogenizer and crushed into 5mL of 40.6% sucrose solution. This is the equivalent of only 1 ant/100 µl and is a ratio that deters hummingbirds (David Rankin, unpublished data). One cotton wick was placed in the ant-infused nectar while a control wick was placed in 5mL of unaltered sucrose solution. Wicks were left to soak for a minimum of 10 minutes in order to completely saturate the cotton. The remaining 3.5 mL of ant nectar and 3.5 mL of control (ant-free) sucrose solution were then poured, respectively, into the treatment and control feeders. Feeders were then placed in the arena to begin the trial.

Ant pheromone (Z)-9-hexadecenal

First, 100µL of 100% ethanol (solvent) was applied in four equally spaced 25 µL droplets to the filter paper of the control feeder. Immediately following application of solvent to control filter paper, 100µL of ant pheromone solution—pure (Z)-9-hexadecenal in 100% ethanol solvent at a concentration of 1ng/µL (Sidhu and Wilson Rankin 2016)—was applied in four equally spaced 25 µL droplets to the treatment feeder. Based on Choe et al. (2012), we estimate that this is 4.3 ant-equivalents. To reduce contamination by plastic residues while still administering a known amount of liquid, solvent and pheromone solution was applied to a filter paper by using a truncated pipette tip as an adapter for a 100 µl calibrated pipet (Drummond Scientific Company). The feeders sat in the fume hood for five to seven minutes to allow evaporation of ethanol while retaining the less volatile pheromone. Feeders were weighed then placed in the experimental arena to begin the trial. Using gas chromatography – mass spectrometry (GC-MS), we verified the detectability of the 1ng of pheromone on filter paper and found that 46% of the pheromone applied was still present after 30 minutes (see Appendix 1: Pheromone Detection Protocol and Figure A1). We did not include a solvent only control because a series of pilot experiments found that bees did not discriminate between an unmanipulated control and a feeder treated with 100µL of 100% ethanol ($X^2_2 = 1.010$, $p = 0.6037$; Appendix 2).

Statistical Analyses

All statistical analyses were done in R v. 3.4.1 (R Core Team 2017). Outliers were identified and, when appropriate, removed using the romr.fnc function in the LMERConvenienceFunctions package (Tremblay and Ransijn 2015) prior to data analysis. All generalized linear mixed-effects models were done using the lme4 package (Bates et al. 2017). To assess colony-level visit frequency in response to live ants or ant chemical cues, we used GLMMs with a negative binomial error structure. For Live Ant Presence, visit frequency (regardless of whether or not a bee fed) was the y-response, treatment was the fixed effect, and colony ID, trial number, and date were random effects. For each of the Ant Cue experiments, visit frequency (regardless of whether or not a bee fed) was the y-response, treatment and colony's ant experience status (naïve or experienced) were the fixed effects, and colony ID, trial number, and date were random effects.

To assess total consumption by bee colonies in the presence of live ants or ant chemical cues, we used GLMMs with a gamma error structure. For Live Ant Presence, the weight of nectar consumed in grams was the y-response, feeder treatment was the fixed effect, and colony ID, trial number, and date were random effects. For each of the Ant Cue experiments, the weight of nectar consumed in grams was the y-response, feeder treatment, colony's ant experience status (naïve or experienced) and the interaction between treatment and experience status were fixed effects, and colony ID, trial number, and date were random effects. We conducted post-hoc tests on any significant interactions.

To assess per capita consumption by bee colonies in the presence of live ants or ant chemical cues, we used GLMMs with a gamma error structure and an offset of pre-trial feeder weight. For Live Ant Presence, grams of nectar consumed per capita was the y-response, feeder treatment was the fixed effect, and colony ID, trial number, and date were random effects. For each of the Ant Cue experiments, grams of nectar consumed per capita was the y-response, feeder treatment, colony's ant experience status (naïve or experienced), and the interaction between treatment and experience status were fixed effects, and colony ID, trial number, and date were random effects. We conducted post-hoc tests on any significant interactions.

To assess frequency and duration of foragers' feeding in response to live ants or ant chemical cues, we used GLMMs with a negative binomial error structure. For Live Ant Presence, the y-response was either feeding frequency or feeding duration, fixed effects were treatment and foragers' ant experience level (naïve or experienced), and random effects were bee ID, colony ID, trial number, and date. For each of the Ant Cue experiments, the y-response was either feeding frequency or duration, fixed effects were treatment, colony's experience status (naïve or experienced), the interaction between treatment and experience status and individual foragers' ant experience level (bitten or not), and random effects were bee ID, colony ID, trial number, and date. We conducted post-hoc tests on any significant interactions. All model results are reported in Tables 1.2 -1.22.

Results

Live Ant Presence

Bumble bee foragers showed a strong avoidance of live ants. In the ant presence trials, colony-level visit frequency did not differ between the two feeders (Table 1.2: $F_{1,36}$ = 0.0088, p = 0.93). However, foraging bees avoided feeding on the ant-occupied feeder. Total nectar consumption at the colony level and per capita nectar consumption were greater for the control feeder as compared to the live ant feeder (Table 1.3, Figure 1.3a: total consumption: $F_{1,34} = 53.2$, $p = 1.89\text{e-}8$; Table 1.4, Figure 1.3b: per capita consumption: $F_{1,34} = 9.07$, $p = 0.00487$). Similarly, foragers' feeding frequency and feeding duration was significantly higher on the control feeder relative to the live ant treatment feeder (Table 1.5, Figure.1.4a: $F_{1,151} = 15.68$, p = 0.000115; Table 1.6, Figure 1.4b: $F_{1,381} = 17.84$, $p = 3.006\text{e-}5$).

Prior Visitation by ants

In this experiment, we did not observe avoidance of the treatment feeders. Bees did not discriminate between the two feeders with regard to their colony-level visit frequency (Table. 1.7: $F_{1,66} = 0.9087$, $p = 0.3439$), consumption at the colony level (Table 1.8, Figure 1.5a: $F_{1,61} = 0.3885$, $p = 0.535$), consumption per capita (Table. 1.9, Figure 1.5b: $F_{1,62} = 3.2838$, $p = 0.0748$), frequency of feeding (Table 1.10, Figure 1.6a: $F_{1,494} = 0.0020$, $p = 0.9643$), or feeding duration (Table 1.11, Figure. 1.6b: $F_{1,974} = 0.9202$, $p = 0.3377$). However, feeding frequency was affected by foragers' experience with ants (Table 1.10: $F_{1,494} = 10.3372$, $p = 3.9996\text{e-}5$), such that foragers who had been bitten fed more frequently (Mean ± SE: 3.859 ± 0.338 feeding bouts) than foragers with no ant experience (3.429 ± 0.135 feeding bouts).

Ants in Nectar

We observed clear avoidance of ants in nectar at the individual forager level. Bees did not discriminate between the two feeders with regard to their colony-level visit frequency

(Table 1.12: $F_{1,68} = 6e-3$, $p = 0.9981$), consumption at the colony level (Table 1.13, Figure 1.7a: $F_{1,64} = 2.2089$, $p = 0.1420$) and nectar consumption per capita (Table 1.14, Figure 1.7b: $F_{1,61} = 1.5094$, $p = 0.2239$). However, there was an effect of treatment when we examined individual foraging behavior. Individual bees fed more frequently from the control feeder than treatment feeder (Table 1.15, Figure 1.8a: $F_{1,677} = 4.2059$, $p = 0.0407$). There was an interaction with colony experience level (Table 1.15: $F_{1,677} = 3.8424$, $p = 0.0500$), such that only foragers from naïve colonies fed more frequently on the control feeder than the treatment feeder (Table 1.16, Z = 2.68, p = 0.037). Feeding frequency was also associated with foragers' experience with ants (Table 1.15: $F_{1,677} = 16.7214$, $p = 1.726e-10$), such that foragers with prior experience of being bitten fed more frequently (Mean ± SE: 3.382 ± 0.240 feeding bouts) than foragers with no ant experience (2.397 ± 0.086 feeding bouts). In addition, foragers from both naïve and experienced colonies fed for a shorter duration on the treatment feeder than the control feeder (Table 1.8b, Figure 1.12: $F_{1,1832} = 18.792$, $p = 1.537e-5$).

Ant Pheromone, (Z)-9-hexadecenal

In this experiment, we saw no avoidance of the Argentine ant aggregation pheromone. Bees did not discriminate between the two feeders with regard to their visit frequency (Table 1.18: $F_{1,68} = 0.6378$, $p = 0.4273$), consumption at the colony level (Table 1.19, Figure 1.9a: $F_{1,67} = 0.0650$, $p = 0.7995$), consumption per capita (Table 1.20, Figure 1.9b: $F_{1,67} = 0.3323$, $p = 0.8559$), forager feeding frequency (Table 1.21, Figure 1.10a: $F_{1,740} = 0.2000$, $p = 0.6549$) or feeding duration (Table 1.22, Figure 1.10b: $F_{1,2083} = 0.9563$, $p =$

0.3846). However, feeding frequency was associated with foragers' experience with ants (Table 1.21: $F_{1,740} = 8.8914$, $p = 0.0001528$), such that foragers with prior experience of being bitten fed more frequently (Mean ± SE: 3.431 ± 0.267 feeding bouts) than foragers with no ant experience (2.604 ± 0.092 feeding bouts).

Discussion

We have shown that, indeed, live Argentine ants very clearly deter bumble bees, and the flavors or scents associated with ants can elicit this effect even in the absence of a physical threat. Both exploitation and interference enable invasive Argentine ants (*Linepithema humile*) to displace native ant species (Human and Gordon 1996). Both may be at work in Argentine ants' competition with bees as well (Schaffer et al. 1983, Buys 1987, Cembrowski et al. 2014). In this study, we disentangle these two types of competition and focus on interference. The nectar wells of our experimental feeders were filled to exclude the possibility of exploitative competition from ants. We saw that bumble bees modified their foraging behavior in response to aggressive encounters and detectable cues associated with their ant antagonists, supporting the hypothesis that interference competition with the ants does occur.

In all four experiments, colony-level visit frequency demonstrated that bees approached both feeders at similar rates regardless of treatment. However, for the live ant presence and crushed ants in nectar experiments, we observed foragers preferring the control feeders upon arrival to a feeder. This indicates the importance of physical contact with ants and short-range chemical cues at a nectar resource.

The average feeding frequency and duration decreased significantly with both the presence of live ants and of dead ants in nectar. The general trend of experienced colonies' lower consumption of nectar containing crushed ants is consistent with our results for feeding frequency and duration, but a decrease in nectar consumed was only significant when live ants were present. These findings demonstrate that chemical cues associated with ant bodies clearly shape forager decision-making, while the physical presence of belligerent ants has the greatest overall effect on bumble bee foraging behavior, particularly as a strong impediment to bees' ability to collect nectar.

Experience with enemies is known to shape subsequent forager decisions (Jones and Dornhaus 2011). We observed that naïve bee colonies made more frequent feeding visits to ant-free nectar. This suggested an innate detection of and aversion to some component(s) associated with the bodies of Argentine ants. While bees in experienced colonies also visited the ant-free nectar more frequently, this difference was not statistically significant. Nonetheless, our results suggest that gustation is involved to some degree in the relationship between ants and foraging bees. Furthermore, bees from both naïve and experienced colonies fed for a shorter duration on the nectar containing ants. By feeding less frequently and for shorter durations of time, foragers may decrease the amount of resources being brought back to the nest. Additional research is needed to assess whether this level of decreased resource collection would be sufficient to decrease colony fitness or productivity.

Interestingly, some foragers on the feeder with ant-nectar displayed the same aggression (i.e. gaping mandibles and attacking) that they showed toward live ants, often before making any physical contact with the ant-infused nectar. This suggests that scent is an important aspect of ant-experienced bees' recognition of Argentine ants. At least one bee in each of the six colonies tested gaped their mandibles while on the treatment feeder, and in three of those colonies at least one bee bit the cotton wick as though it were attacking an ant. This behavior was very distinct in that it was not observed during any other ant cue trials, including for naïve bees with the ant nectar.

By crushing ants in nectar, we exposed bees to all the external and internal chemicals that they might encounter from the ants. We have shown that even in the absence of the physical threat of ants, a chemical stimulus prompted bees to behave similarly to when live ants are present. This may be due to an innate aversion to the contamination of nectar with the flavor of ants (as is likely the case for the naïve colonies tested). Any such aversion may be reinforced by bees learning to associate ant flavors and smells with their aggressive interactions with ants (as may be the case for experienced colonies tested). Foraging bumble bees may be deterred from flowers as a response to both tactile chemosensation (e.g. inadvertently licking or antennating an ant) and close-range scent (e.g. ant pheromone, CHC's). Further study is needed to disentangle and identify the specific components of taste and smell to which the bumble bees respond with avoidance and aggression.

Crushed ants in nectar was the only ant cue experiment during which bees showed a feeder preference. Foragers from naïve colonies frequented the control feeder more often than the treatment feeder, and foragers from both naïve and experienced colonies fed for a longer duration on the control feeder compared to the treatment. For the other two cue experiments—prior visitation and the ant pheromone (Z)-9-hexadecenal—feeding duration and frequency were consistent irrespective of feeder treatment and colony-level ant experience. The marked differences between these two cues and the live ant treatment demonstrate that bumble bees are not able to detect if ants have fed from a nectar source shortly beforehand and do not avoid the ant aggregation pheromone tested.

Interestingly the ant pheromone which elicits avoidance in honey bees (Sidhu and Wilson Rankin 2016) did not elicit a similar response in bumble bees. (Z)-9-hexadecenal is known to evoke trail following behavior in the Argentine ant (*L. humile*) as well as in the Southeast Asian ant *Dolichoderus thoracicus* (Van Vorhis Key and Baker 1982; Attygalle et al. 1998). This substance is also found in other insect taxa where it can function as a sex pheromone (e.g. Kainoh et al. 1991) or as a kairomone (DeLury et al. 1999). Choe et al. (2012) found that although (Z)-9-hexadecenal was inside the bodies of Argentine ants, ant trails were characterized by two other chemicals, and (Z)-9-hexadecenal was not actually detected by GC-MS in the trails. Future research could test the response of bees to these chemicals that are the primary components of ant trails and to the chemical substances used in an offensive or defensive manner by Argentine ants.

Bees' aggression toward the ant-infused nectar prior to making physical contact suggest that they do use olfactory cues in their recognition of Argentine ants, though it appears that bumble bees are responding to odors other than (Z)-9-hexadecanal. It may be that *B. impatiens* does not detect (Z)-9-hexadecenal, does not use it as a foraging cue, has a higher response threshold compared to honey bees, or responds differently based on experience level with ants. As such, further studies of bee response thresholds regarding ants and ant cues are warranted. In comparison to Sidhu & Wilson Rankin (2016), we have shown that there is a marked difference between two eusocial bee species. Both of these species inhabit areas where the Argentine ant has invaded (Ruggiero et al 2018; Williams et al, 2014,Tsutsui and Suarez 2003), while only honey bees overlap in distribution with Argentine ants in the latter's native range (Maggi et al, 2016). While we may posit that shared evolutionary history may influence bee responses, we still lack any data about the responses of many other non-*Apis* bees. There may even be heterospecific differences within the genus *Bombus* alone. These results provide a reminder that we should be cautious and not to draw definitive predictions about all bees based on the behaviors of honey bees.

To date, only honey bees and bumble bees—both social species—have been the subjects of study in the chemical ecology of competition with Argentine ants. Social insects tend to have highly evolved chemical communication through odor, including the recognition of colony and nest mates and the organization of their societies (Blomquist and Bagneres, 2010; Nunes *et al*, 2009; Nunes *et al.*, 2011). The limited research that has been done to compare the reactions of several bee species, including both social and solitary ones, to the scent signals left on flowers by previous intra- and inter-specific floral visitors shows species differences in behavioral response (Gawleta *et al.*, 2005; Yokoi and Fujisaki, 2008). Thus, different bee species may respond in a non-uniform fashion to invasive ants. Future research should investigate the foraging impacts, interactions, and differences in sensory perception regarding antagonistic, non-pollinator competitors across a variety of bee species. With this information we could better predict community-level responses or resilience in the face of invasive species.

Despite the difference in responses to feeder treatment across cue experiments (i.e. bumble bees only preferred the control when the treatment feeder had ant-infused nectar), for all three ant cue experiments, foragers with a known history of ant interaction fed more frequently overall than foragers without ant experience. Regardless of the direction of causation with feeding frequency and foragers' ant experience, there could be individual-level determinants or traits that determine the relationship between a bee's foraging behavior and her interaction with ants. It is likely that our most active or elite foragers were the most likely to encounter ants.

It is common in social insects for a few foragers to conduct the majority of the foraging activity (Hurd et al. 2003, Jandt et al. 2012, Tenczar et al. 2014, Charbonneau et al. 2017), and size may be predictive of a bumble bee's foraging activity (Goulson et al. 2002). We observed that during our experiments with live ants present, the bulk of the foraging in each colony was conducted by a select group of foragers (see Chapter 3). Further, bees of different body sizes may behave differently in the physical presence of aggressive, ant floral visitors, although size did not influence bees' foraging response to a laboratory simulation of predator attack (Jones and Dornhaus 2011). Bee body size was not recorded as part of this study. Future research should consider both intra-colony and heterospecific differences in body size in relation to foraging interactions between bees and invasive ants. Gonzálvez *et al.*, (2013) found that resource guarding by native weaver ants filtered out a smaller, less efficient bee pollinator while the larger, more effective bee pollinator tenaciously shook off confrontational ants to continue feeding. In this context of plant bodyguarding, native ant aggression had a positive effect on plant fitness, but a positive effect of pollinator deterrence rarely seems to be the case (Ness 2006), especially in the context of nectar-thieving ant invasions (Hanna et al. 2015).

In this study, we examined how bumble bees respond to encountering ants or their cues at feeders. While we focused on nectar collection, future research could extend these experiments to pollen foraging and the impacts of foraging shifts on colony-level fitness. Here, we showed the bumble bees avoid feeders with live ants and preferentially collect nectar on feeders without live ants. We examined several cues associated with the presence of ants and determined that ants did not avoid baits that had previously been visited by ants, suggesting that bees were not avoiding ant CHC footprints. However, CHC's from other parts of the ant body could have been detected in ant-infused nectar by bees. Nor did the bees avoid ant aggregation pheromone, neither in the context of any pheromone laid down during prior visitation nor in the application of the pheromone alone. The physical interaction with ants and the chemosensation of ants in the nectar were sufficient to elicit avoidance. Predator simulation has shown that after bumble bees experience physical attack, they will decrease foraging or switch to a less rewarding nectar resource (Jones and Dornhaus 2011). Experience with Argentine ants may elicit similar responses that result in resource collection of a lower quality or quantity. Decreased nectar collection and quality has serious implications for the maintenance of healthy pollinator colonies. This study highlights the fact that ants are a serious pest to consider when developing conservation and management strategies for pollinators and pollination services.

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Tables

Table 1.1 Argentine ant treatments and the sensory systems through which they may be perceived. In addition to testing bee response to the presence of live ants on the treatment feeder, we tested both naïve and ant-experienced bee colonies with four chemical cues associated with ant presence.

Treatments	Tactile	Olfactory	Gustatory	Visual
Ant presence – ants free to forage (duration of trial, "ant experience")	✓	✓	✓	✓
Prior visitation – ants forage on feeder (for 1 hr before trial)		✓	✓	
Ants in nectar (50 crushed ants/5 mL)		✓	✓	
Pheromone (Z)-9-hexadecenal on filter paper (100 uL of 1 ng/uL ethanol)		✓		

Table 1.2 Ant presence. Bee colony visit frequency in response to treatment.

Model: glmer.nb(Visit Frequency ~ Treatment + (1|ColonyID) + (1|Trial.Num) + (1|Date))

	Sum Sq	**Mean Sq**	**Num Df**	**Den DF**	**F value**	**P value**
Treatment	0.0087756	0.0087756	1	36	0.0088	0.9258

Table 1.3. Ant presence. Total consumption by bee colonies in response to treatment. Model: glmer(Consumption (g) ~ Treatment + (1|Tr.Feeder.Side) + (1|ColonyID) + (1|Trial.Num) + (1|Date), family=Gamma())

	Sum Sq	**Mean Sq**	**Num Df**	**Den DF**	**F value**	**P value**
Treatment	5.9451	5.9451	1	34	53.248	1.884e-8***

Table 1.4. Ant presence. Per capita consumption by bee colonies in response to treatment.

Model: glmer(Per capita consumption (g)~ Treatment + (1|Tr.Feeder.Side) + (1|ColonyID) + (1|Trial.Num) + (1|Date), family=Gamma())

	Sum Sq	**Mean Sq**	**Num Df**	**Den DF**	**F value**	**P value**
Treatment	1.1981	1.1981	1	34	9.0746	0.00487**

Table 1.5. Ant presence. Forager feeding frequency in response to treatment and experience with ant bites. Model: glmer.nb(Feeding Frequency ~ Treatment + Bites + (1|ColonyID) + (1|Trial.Num) + (1|BeeID) + (1|Date)

	Sum Sq	**Mean Sq**	**Num Df**	**Den DF**	**F value**	**P value**
Treatment	15.682	15.6822	1	151	15.6822	0.000115***
Bites	0.777	0.3885	2	151	0.3885	0.6787

Table 1.6. Ant presence. Forager feeding duration in response to treatment and ant experience. Model: glmer.nb(Feeding duration (sec) ~ Treatment + Bites + (1|Trial.Num) + (1|Date) + (1|BeeID) + (1|ColonyID))

	Sum Sq	**Mean Sq**	**Num Df**	**Den DF**	**F value**	**P value**
Treatment	17.8422	17.8422	1	381	17.8422	3.006e-5***
Bites	1.5097	0.7548	2	381	0.7548	0.4708

Table 1.7. Prior visitation. Bee colony visit frequency in response to treatment and colony status of ant experience (naïve or experienced). Model: glmer.nb(Visit Frequency ~ Treatment*Status + (1|ColonyID) + (1|Trial.Num) + (1|Date))

	Sum Sq	**Mean Sq**	**Num Df**	**Den DF**	**F value**	**P value**
Treatment	0.90870	0.90870	1	66	0.9087	0.3439
Status	1.26605	1.26605	1	66	1.2661	0.2646
Treatment:Status	0.85884	0.85884	1	66	0.8588	0.3575

Table 1.8. Prior visitation. Total consumption by bee colonies in response to treatment and colony status of ant experience (naïve or experienced). Model: glmer(Consumption (g) ~ Treatment*Status + (1|ColonyID) + (1|Trial.Num) + (1| Date), family=Gamma())

	Sum Sq	**Mean Sq**	**Num Df**	**Den DF**	**F value**	**P value**
Treatment	0.141079	0.141079	1	61	0.3885	0.5354
Status	0.185711	0.185711	1	61	0.5114	0.4773
Treatment:Status	0.032576	0.032576	1	61	0.0897	0.7660

Table 1.9. Prior visitation. Per capita consumption by bee colonies in response to treatment and colony status of ant experience (naïve or experienced). Model: glmer(Per capita consumption (g)~ Treatment*Status + (1|ColonyID) + (1|Trial.Num) + (1| Date), family=Gamma())

	Sum Sq	**Mean Sq**	**Num Df**	**Den DF**	**F value**	**P value**
Treatment	2.5576	2.5576	1	62	3.2838	0.0748.
Status	2.5534	2.5534	1	62	3.2784	0.0750.
Treatment:Status	1.4962	1.4962	1	62	1.9210	0.1707

Table 1.10. Prior visitation. Forager feeding frequency in response to treatment, colony status of ant experience (naïve or experienced), and forager experience with ant bites. Model: glmer.nb(Feeding Frequency ~ Treatment*Status + Bitten + (1|ColonyID) + (1|BeeID) + (1|Date) + (1|Trial.Num))

	Sum Sq	**Mean Sq**	**Num Df**	**Den DF**	**F value**	**P value**
Treatment	0.0020	0.0020	1	494	0.0020	0.9643
Status	2.0538	2.0538	1	494	2.0538	0.1525
Treatment:Status	0.0034	0.0034	1	494	0.0034	0.9535
Previously bitten (Y/N)	20.6744	10.3372	2	494	10.3372	3.9996e-5***

Table 1.11. Prior visitation. Forager feeding duration in response to treatment, colony status of ant experience (naïve or experienced), and forager experience with ant bites. Model: glmer.nb(Feeding duration (sec) ~ Treatment * Status + Bitten + (1|Trial.Num) + (1|Date) + (1|BeeID) + (1|ColonyID))

	Sum Sq	**Mean Sq**	**Num Df**	**Den DF**	**F value**	**P value**
Treatment	0.92021	0.92021	1	974	0.9202	0.3377
Status	0.01235	0.01235	1	974	0.0124	0.9114
Treatment:Status	0.72599	0.72599	1	974	0.7260	0.3944
Previously bitten (Y/N)	0.84018	0.42009	2	974	0.4201	0.6571

Table 1.12. Ants in nectar. Bee colony visit frequency in response to treatment and colony status of ant experience (naïve or experienced). Model: glmer.nb(Visit Frequency ~ Treatment*Status + (1|ColonyID) + (1|Trial.Num) + (1|Date))

	Sum Sq	**Mean Sq**	**Num Df**	**Den DF**	**F value**	**P value**
Treatment	0.0060083	0.0060083	1	68	6e-03	0.9981
Status	0.0000016	0.0000016	1	68	0e+00	1
Treatment:Status	0.0000870	0.0000870	1	68	1e-04	0.9921

Table 1.13. Ants in nectar. Total consumption by bee colonies in response to treatment and colony status of ant experience (naïve or experienced). Model: glmer(Consumption (g) ~ Treatment*Status + (1|ColonyID)+(1|Trial.Num)+(1|Date), family=Gamma())

	Sum Sq	**Mean Sq**	**Num Df**	**Den DF**	**F value**	**P value**
Treatment	0.7477	0.7477	1	64	2.2089	0.1420
Status	13.4452	13.4452	1	64	39.7193	3.061e-8***
Treatment:Status	0.5328	0.5328	1	64	1.5739	0.2142

Table 1.14. Ants in nectar. Per capita consumption by bee colonies in response to treatment and colony status of ant experience (naïve or experienced). Model: glmer(Per capita consumption (g)~ Treatment*Status + (1|ColonyID)+(1|Trial.Num)+(1|Date), family=Gamma())

	Sum Sq	**Mean Sq**	**Num Df**	**Den DF**	**F value**	**P value**
Treatment	0.34886	0.34886	1	61	1.5094	0.2239
Status	2.79169	2.79169	1	61	12.0788	0.000945***
Treatment:Status	0.00001	0.00001	1	61	0.0000	1

Table 1.15. Ants in nectar. Forager feeding frequency in response to treatment, colony status of ant experience (naïve or experienced), and forager experience with ant bites. Model: glmer.nb(Feeding Frequency ~ Treatment*Status + Bitten + (1|ColonyID) + (1|BeeID) + (1|Date) + (1|Trial.Num))

	Sum Sq	**Mean Sq**	**Num Df**	**Den DF**	**F value**	**P value**
Treatment	4.206	4.2059	1	677	4.2059	0.0407*
Status	2.384	2.3841	1	677	2.3841	0.1230
Treatment:Status	3.842	3.8424	1	677	3.8424	0.050*
Previously bitten (Y/N)	50.164	16.7214	3	677	16.7214	1.726e-10***

Table 1.16. Ants in nectar. Post-hoc testing for experience level and treatment on feeding frequency of model described in above Table 15. P-value adjustment: tukey method.

Comparison	**Estimate**	**SE**	**Z ratio**	**P value**
Control,Experienced - Treatment,Experienced	1.051282	0.067058	0.78402	0.8618
Control,Experienced - Control,Naive	0.623751	0.096457	-3.05227	0.0122*
Control,Experienced - Treatment,Naive	0.84932	0.146007	-0.95002	0.7777
Treatment,Experienced - Control,Naive	0.593324	0.09238	-3.35272	0.0044**
Treatment,Experienced - Treatment,Naive	0.80789	0.139652	-1.23411	0.6051
Control,Naive - Treatment,Naive	1.361634	0.156867	2.679451	0.037*

Table 1.17. Ants in nectar. Forager feeding duration in response to treatment, colony status of ant experience (naïve or experienced), and forager experience with ant bites. Model: glmer.nb(Feeding duration (sec) ~ Treatment * Status + Bitten + (1|Trial.Num) + (1|Date) + (1|BeeID) + (1|ColonyID)

	Sum Sq	**Mean Sq**	**Num Df**	**Den DF**	**F value**	**P value**
Treatment	18.7920	18.792	1	1832	18.792	1.537e-5***
Status	12.4223	12.422	1	1832	12.422	0.0004347***
Treatment:Status	2.6670	2.667	1	1832	2.667	0.1026
Previously bitten (Y/N)	7.0799	2.360	3	1832	2.360	0.0698.

Table 1.18. (Z)-9-hexadecenal. Bee colony visit frequency in response to treatment and colony status of ant experience (naïve or experienced). Model: glmer.nb(Visit Frequency ~ Treatment*Status + (1|ColonyID) + (1|Trial.Num) + (1| Date))

	Sum Sq	**Mean Sq**	**Num Df**	**Den DF**	**F value**	**P value**
Treatment	0.6378	0.6378	1	68	0.6378	0.4273
Status	18.2684	18.2684	1	68	18.2684	6.121e-5***
Treatment:Status	0.6136	0.6136	1	68	0.6136	0.4362

Table 1.19. (Z)-9-hexadecenal. Total consumption by bee colonies in response to treatment and colony status of ant experience (naïve or experienced). Model: glmer(Consumption (g) ~ Treatment*Status + (1|ColonyID) + (1|Trial.Num) + (1|Date), family=Gamma())

	Sum Sq	**Mean Sq**	**Num Df**	**Den DF**	**F value**	**P value**
Treatment	0.01768	0.01768	1	67	0.0650	0.7995
Status	0.33069	0.33069	1	67	1.2162	0.2740
Treatment:Status	0.00904	0.00904	1	67	0.0332	0.856

Table 1.20. (Z)-9-hexadecenal. Per capita consumption by bee colonies in response to treatment and colony status of ant experience (naïve or experienced). Model: glmer(Per capita consumption (g)~ Treatment*Status + (1|ColonyID) + (1|Trial.Num) + (1| Date), family=Gamma())

	Sum Sq	**Mean Sq**	**Num Df**	**Den DF**	**F value**	**P value**
Treatment	0.093369	0.093369	1	67	0.3323	0.8559
Status	0.025574	0.025574	1	67	0.0910	0.7638
Treatment:Status	0.046045	0.046045	1	67	0.1639	0.6869

Table 1.21. (Z)-9-hexadecenal. Forager feeding frequency in response to treatment, colony status of ant experience (naïve or experienced), and forager experience with ant bites.

Model: glmer.nb(Feeding Frequency ~ Treatment*Status + Bitten + (1|ColonyID) + (1|BeeID) + (1|Date) + (1|Trial.Num))

	Sum Sq	**Mean Sq**	**Num Df**	**Den DF**	**F value**	**P value**
Treatment	0.2000	0.2000	1	740	0.2000	0.6549
Status	0.0008	0.0008	1	740	0.0008	0.9774
Treatment:Status	1.4136	1.4136	1	740	1.413	0.2349
Previously bitten (Y/N)	17.7829	8.8914	2	740	8.8914	0.0001528***

Table 1.22. (Z)-9-hexadecenal. Forager feeding duration in response to treatment, colony status of ant experience (naïve or experienced), and forager experience with ant bites.

Model: glmer.nb(Feeding duration (sec) ~ Treatment * Status + Bitten + (1|Trial.Num) + (1|Date) + (1|BeeID) + (1|ColonyID))

	Sum Sq	**Mean Sq**	**Num Df**	**Den DF**	**F value**	**P value**
Treatment	0.0590	0.0590	1	2083	0.0590	0.8081
Status	6.6663	6.6663	1	2083	6.6663	0.00989**
Treatment:Status	0.1732	0.1732	1	2083	0.1732	0.677
Ant Experience	1.9126	0.9563	2	2083	0.9563	0.3846

Figures

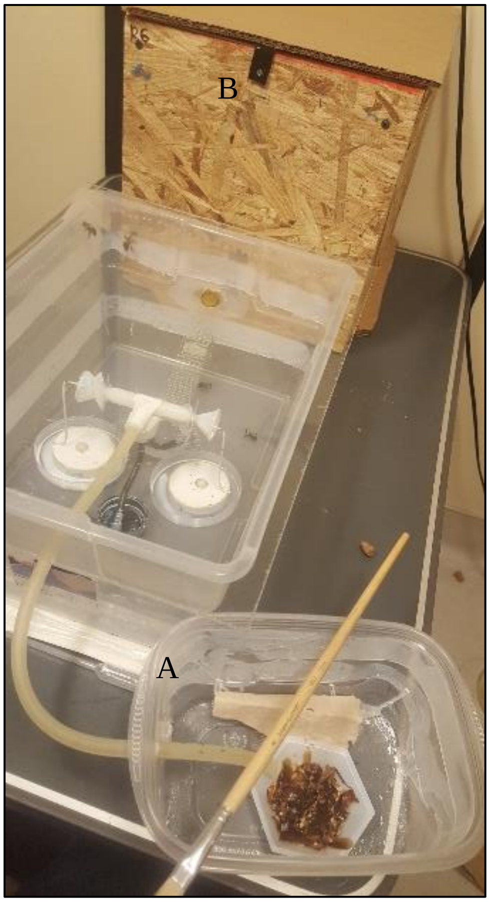

Figure 1.1. Full experimental set-up for live ant interaction: ant micro-colony (A) and bee colony nesting box (B) both attached to experimental arena.

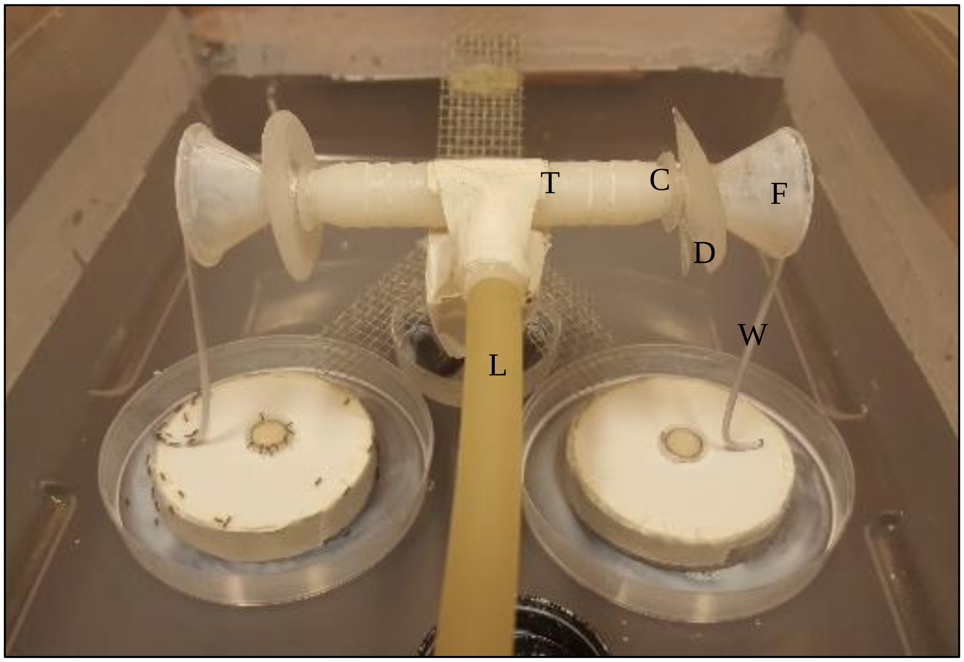

Figure 1.2. Close-up of ant centerpiece, composed of: Latex tubing (L) connected to ant micro-colony, T-connector (T) attached with Duct Tape to flexible camera tripod, Fluon-coated glass funnels (F) with control side plugged, micro-centrifuge tube caps (C) as adapters to insert/remove funnels and switch position of the control, Fluon-coated disks of transparency paper (D), and hanger-wires (W) as bridges from funnels to feeders.

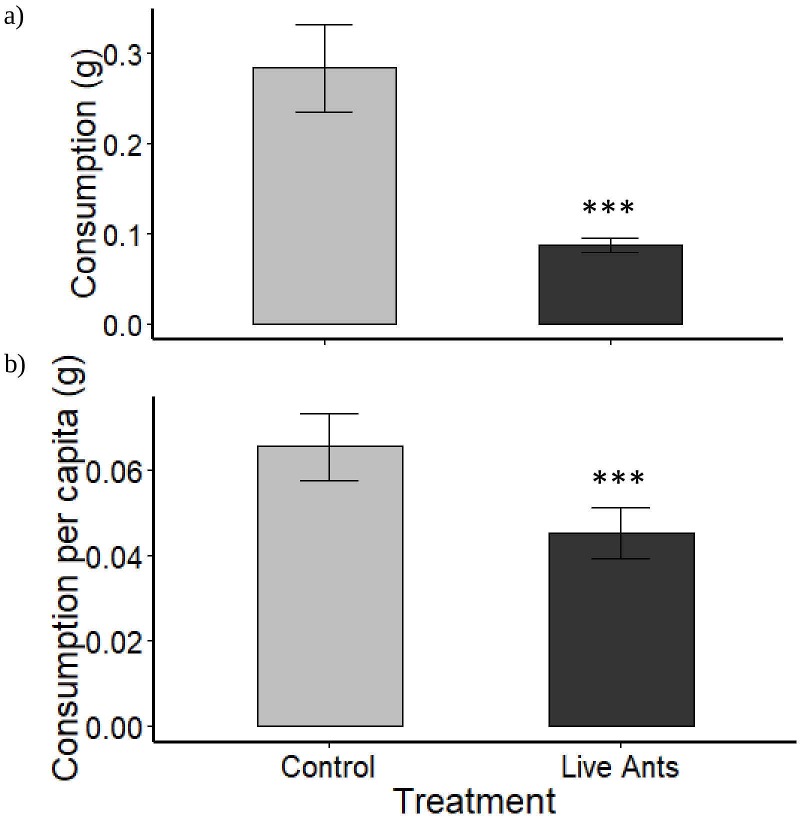

Figure. 1.3. Ant presence. Treatment feeders experienced (a) decreased total nectar consumption by bee colonies (Table 1.3: $F_{1,34} = 53.2$, $p = 1.89e\text{-}8$), and (b) decreased per capita nectar consumption (Table 1.4: $F_{1,34} = 9.07$, $p = 0.00487$) compared to control feeders. Bars represent means, and whiskers represent ± 1 SEM. NS indicates $p > 0.05$, * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

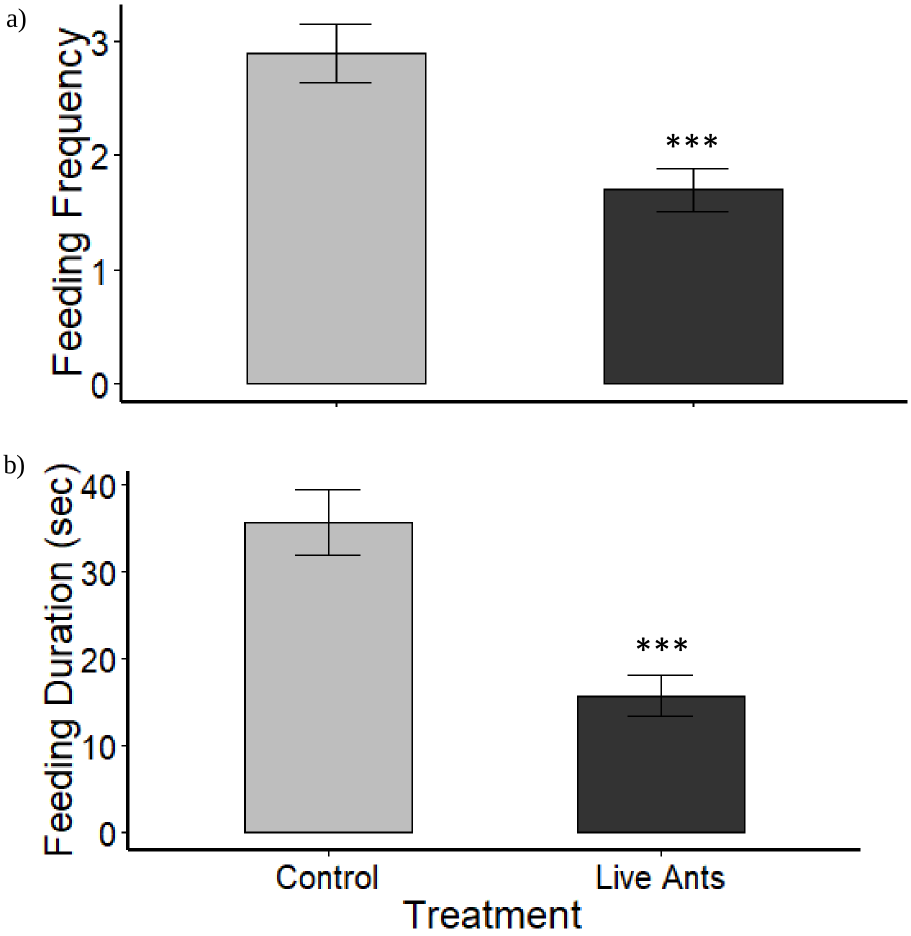

Figure. 1.4. Ant presence. Treatment feeders had (a) decreased forager feeding frequency (Table 1.5: $F_{1,151}$ = 15.68, p = 0.000115), and (b) decreased forager feeding duration (Table 1.6: $F_{1,381}$ = 17.84, *p* = 3.006e-5) compared to control feeders.

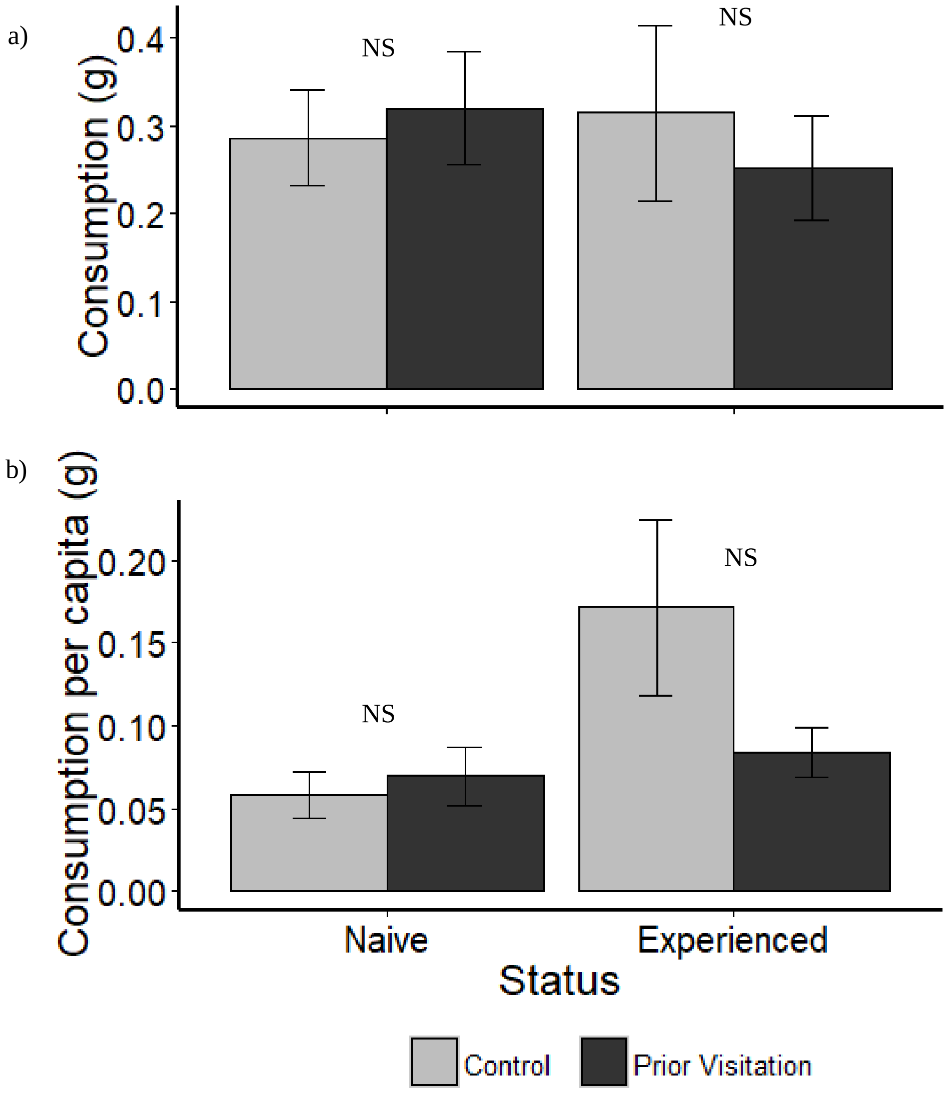

Figure. 1.5. Prior visitation. Treatment feeders and control feeders exhibited (a) no difference in total nectar consumption by bee colonies (Table 1.8: $F_{1,61} = 0.3885$, $p = 0.535$), and (b) no difference in consumption per capita (Table. 1.9: $F_{1,62} = 3.2838$, $p = 0.0748$).

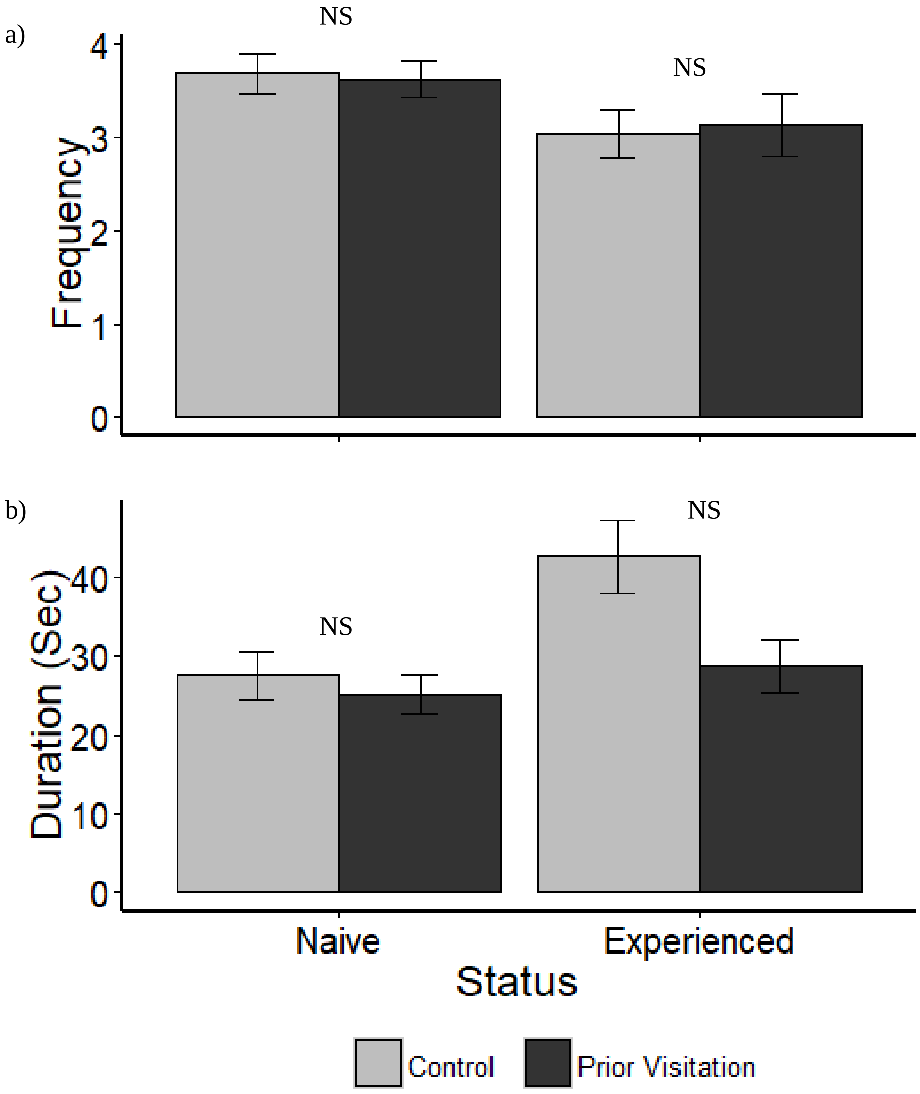

Figure. 1.6. Prior visitation. Treatment and control feeders exhibited (a) no difference in forager feeding frequency (Table 1.10: $F_{1,494} = 0.002$, p = 0.9643), and (b) no difference in forager feeding duration (Table 1.11: $F_{1,974} = 0.9202$, p = 0.3377).

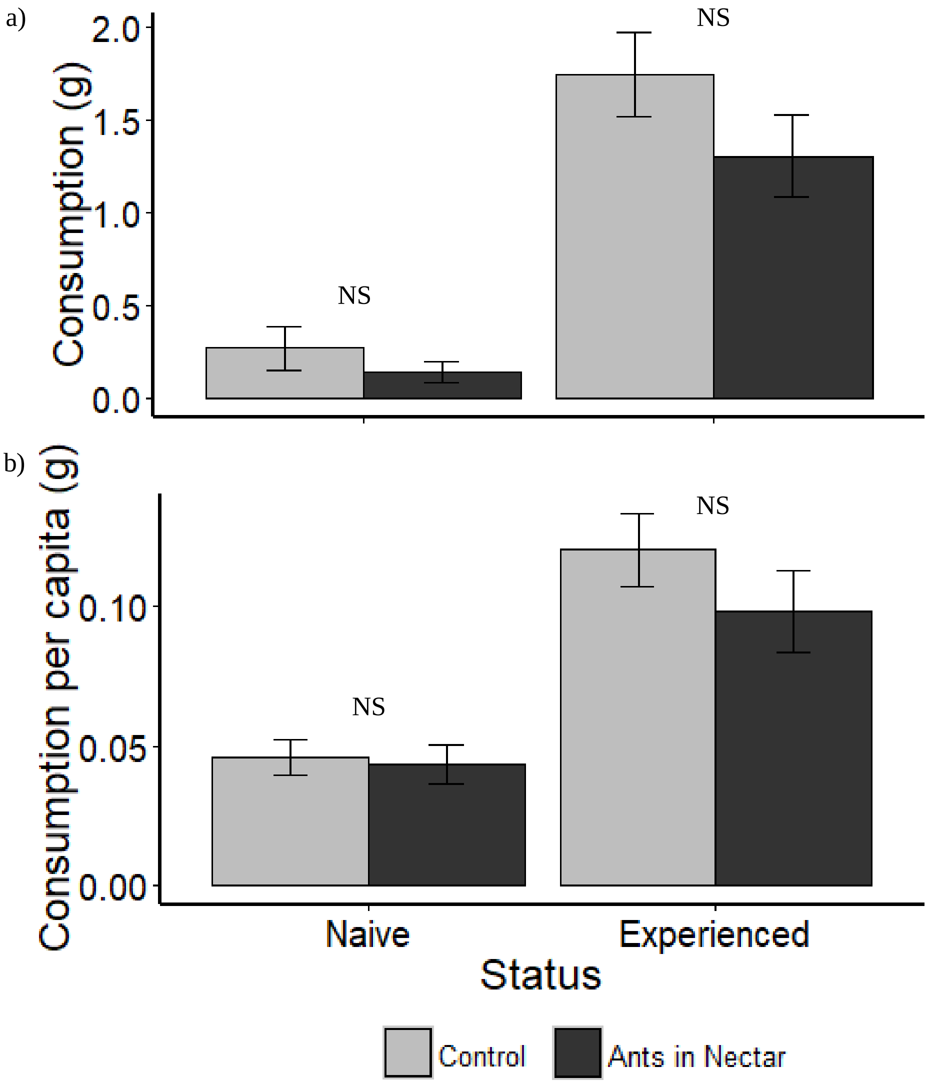

Figure. 1.7. Ants in nectar. Treatment feeders and control feeders exhibited (a) no statistical difference in total nectar consumption by bee colonies (Table 1.13: $F_{1,64} = 2.2089$, $p = 0.1420$), and (b) no statistical difference in consumption per capita (Table. 1.14: $F_{1,61} = 1.5094$, $p = 0.2239$).

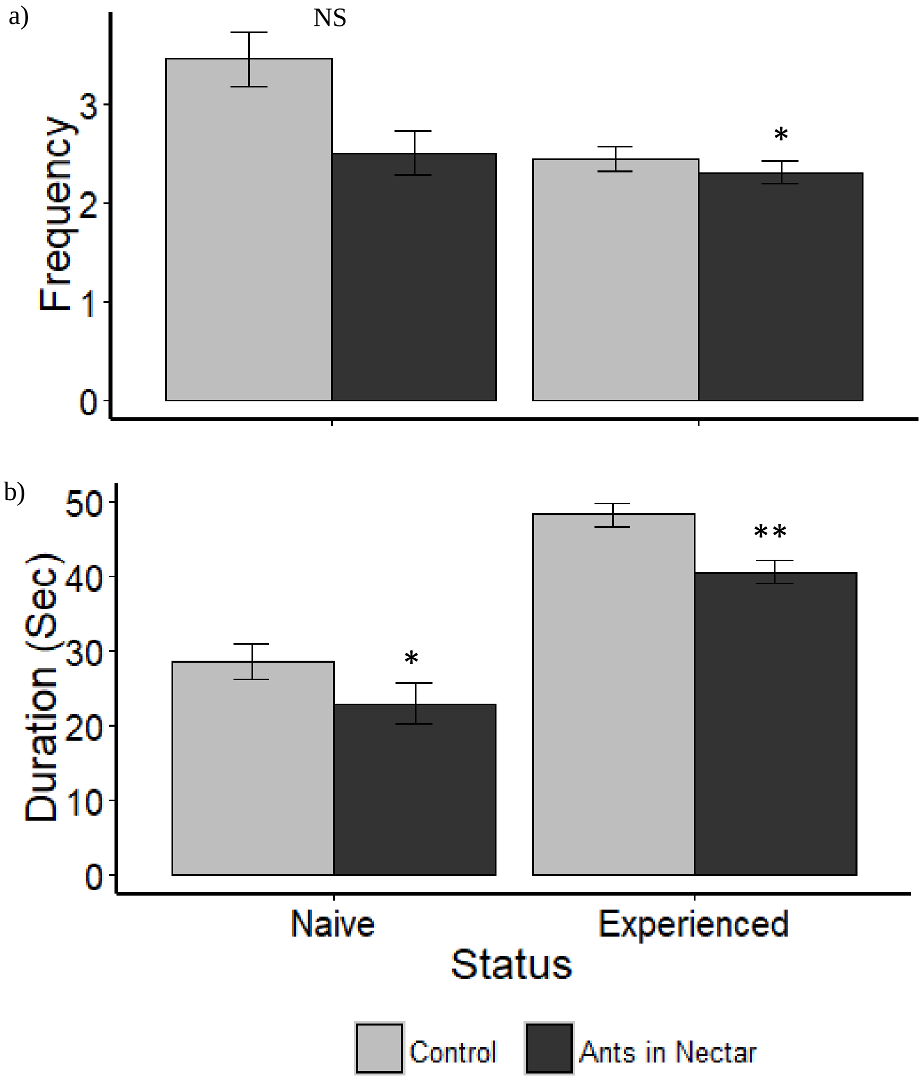

Figure. 1.8. Ants in nectar. Treatment feeders had (a) decreased forager feeding frequency in naïve colonies (Table 1.16: *Z* = 2.68, *p* = 0.037), and (b) decreased forager feeding duration in naïve and experienced colonies (Table 1.17: $F_{1,1832}$ = 18.792, *p* = 1.537e-5) compared to the control feeder.

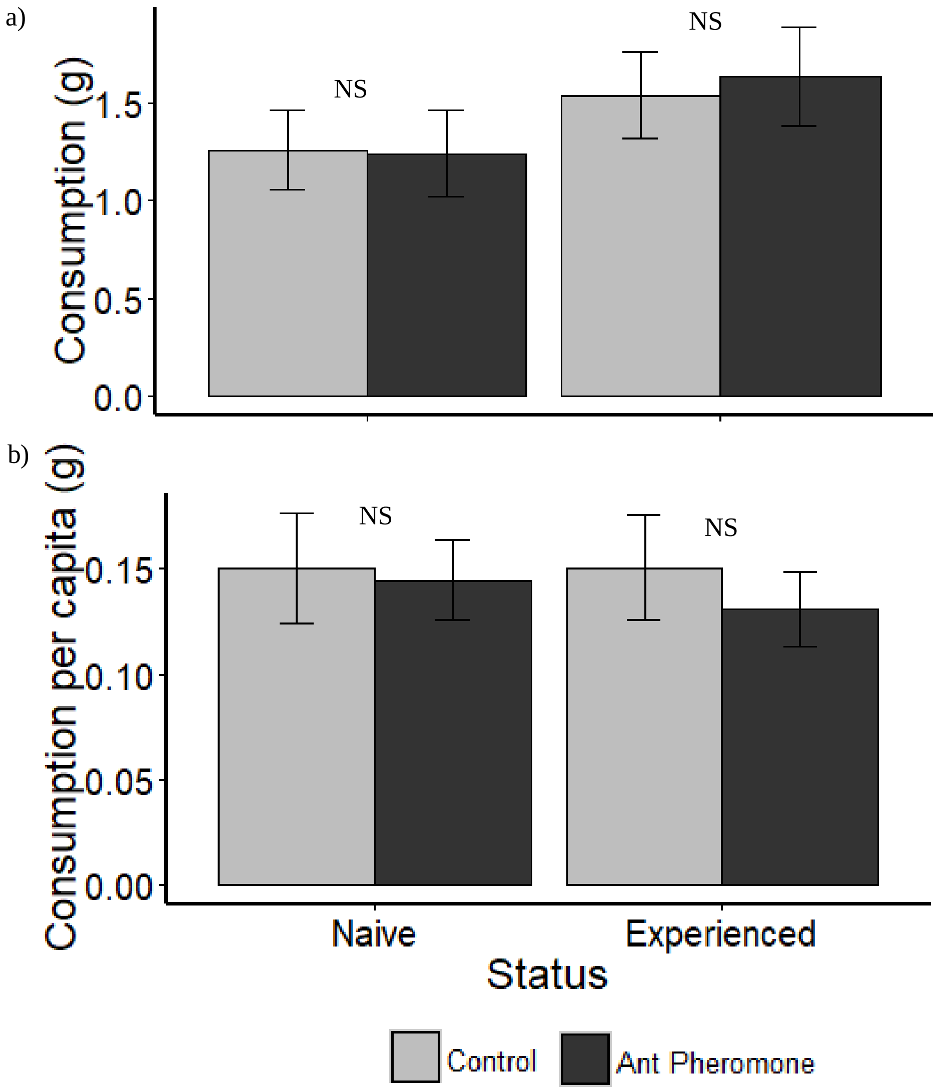

Figure. 1.9. Ant pheromone (Z)-9-hexadecenal. Treatment feeders and control feeders exhibited (a) no difference in total nectar consumption by bee colonies (Table 1.19: $F_{1,67} = 0.0650$, $p = 0.7995$), and (b) no difference in consumption per capita (Table. 1.20: $F_{1,67} = 0.3323$, $p = 0.8559$).

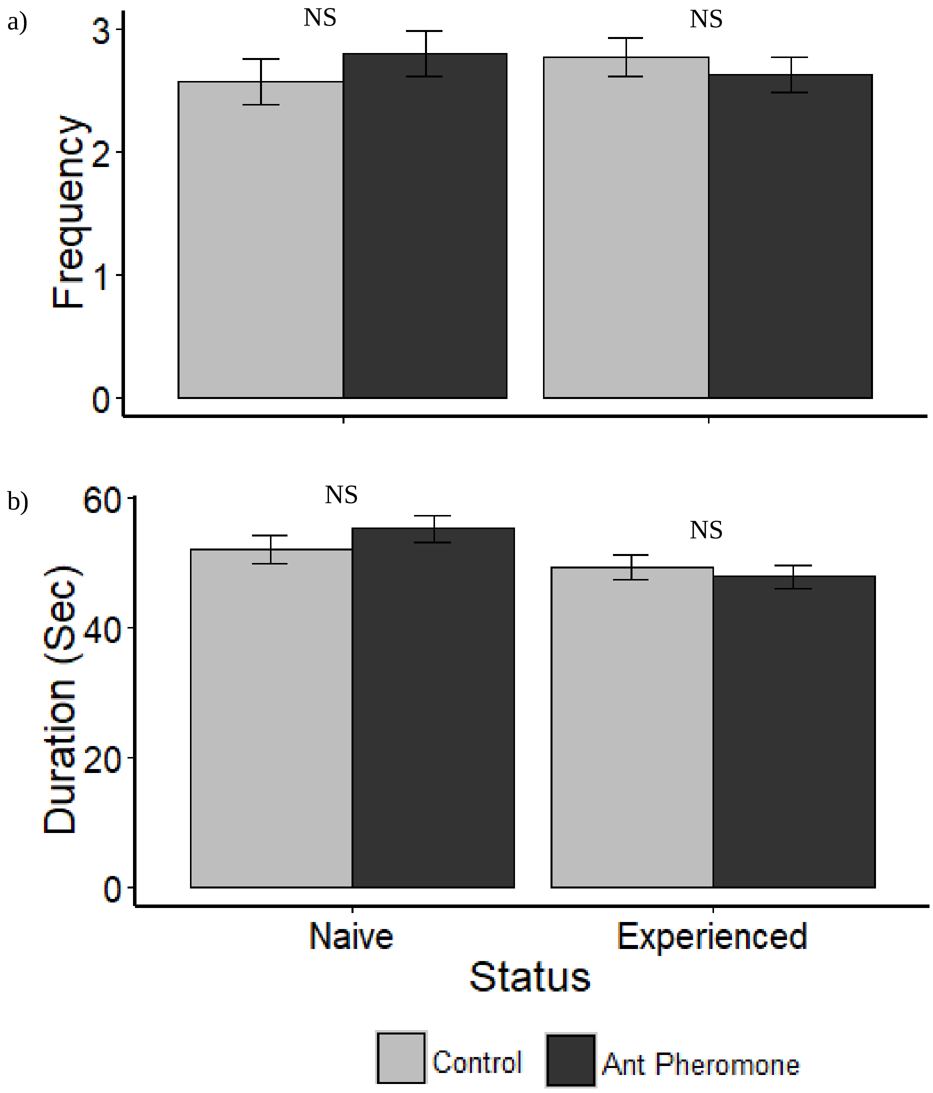

Figure. 1.10. Ant pheromone (Z)-9-hexadecenal. Treatment and control feeders exhibited (a) no difference in forager feeding frequency (Table 1.21: $F_{1,740}$ = 0.2000, p = 0.6549), and (b) no difference in forager feeding duration (Table 1.22: $F_{1,2083}$ = 0.0590, *p* = 0.8081).

Chapter 2 Ant-bee interactions and behaviors during nectar foraging

Introduction

Bees are known to minimize risk while foraging (Dukas 2005, 2008, Li et al. 2014, Wang et al. 2016), including the risk of attack by ants at a flower (Ness 2006). Changes in, or impediments to, bee foraging have implications for bees' ability to acquire necessary food resources. In turn, challenges to resource acquisition could impact the success and stability of pollinator populations (Llandres et al. 2012) upon which natural and agricultural ecosystems rely (Klein et al. 2007, Hudewenz and Klein 2015). Thus, it is important to understand competition between ants and bees at a mutual food resource, particularly as damaging invasive ants continue to spread across the world.

In Chapter 1, we established that the common eastern bumble bee (*Bombus impatiens*) does, indeed, avoid invasive Argentine ants (*Linepithema humile*) at a shared nectar resource. Aggressiveness by ants has been implicated as a deterrent to some bees (Ness 2006, Hanna et al. 2015). However, the interactions that take place between bees and Argentine ants, and the effects of these interactions on bee foraging behavior, have not been directly investigated.

In this study, we examined ant-bee interactions and behaviors to investigate the hypothesis that Argentine ants interfere with bumble bee nectar foraging at a shared resource, with aggression by live ants as the basis of interference. We predicted that (a) bees are less likely to feed on resources where ants are present, (b) the number of ants on a shared resource is positively correlated with ant aggression and negatively correlated with whether or not a bee feeds at that resource, and (c) ant aggression in the form of biting is negatively correlated with bee feeding frequency. We recorded and characterized the specific actions (i.e. behaviors and heterospecific interactions) that took place on the shared feeder and assessed the transitions between actions that occurred when individual bee foragers were in the presence of Argentine ants. By doing so, we have helped clarify the manner in which Argentine ants modify bumble bee behavior and foraging success.

Materials and Methods

We used the same experimental set-up for ant exposure trials as described in Chapter 1, with relevant details summarized here. Behavioral observations with six queenless colonies of the common eastern bumble bee (*B. impatiens*, Biobest) were carried out as foraging choice tests between experimental feeders. Each feeder was made using a plastic jar lid 5.9 cm in diameter, a 1.5 mL micro-centrifuge tube, a shortened 1 oz polypropylene portion container (Dart Container Corporation), a two-inch piece of cotton dental wick (Johnson & Johnson), and a 5.8 cm diameter disk of filter paper (Grade 1, Whatman) covering the face of the feeder. Each feeder contained 3.5 mL of 40.6% (w/w) sucrose solution as experimental nectar.

Ant Exposure Experiments

During each 30-minute trial, the treatment feeder was accessible to a queenless micro-colony of Argentine ants, comprising approximately 200 workers and at least five larvae. Ants moved freely between their colony and the treatment feeder, and Insect-a-Slip Insect Barrier — Fluon (PTFE-30,DISP30, BioQuip Products, Inc.) prevented ants from going elsewhere in the experimental arena (42.5L x 30.2W x 17.8H cm, Sterilite Corporation). The control feeder was ant-free, and each bumble bee colony's entire workforce could freely enter and exit the experimental arena.

Each of six *B. impatiens* colonies received three consecutive half-hour sessions of foraging while the treatment feeder was open to ants. An online digital coin flip simulator (Google.com, "flip a coin") was used to randomize the placement of treatment and control feeders (left or right) for a colony's first of three ant exposure trials. Feeder locations were switched for the second session and then again for the third. Each colony's 1.5 hours of ant exposure preceded "Ant Cue Experiments" discussed in Chapter 1.

Prior to the start of any ant exposure trials, the treatment feeder was accessible to an Argentine ant micro-colony for at least 15 minutes, starting when the first ant arrived on the feeder. Ants had free range of the treatment feeder during each 30-minute trial, free to feed and interact with visiting bees.

In order to identify individual bee foragers, unique number tags were adhered to each of their thoraces using Super Glue (Loctite). For each individual bee, we recorded entrance into the treatment or control feeder space, whether or not the bee fed while visiting the feeder, and each observed action (i.e. behavior or interaction between the foraging bee and ants) that took place on the treatment feeder (Table 2.1). We also examined the frequency of transitions (i.e. transition from one action to another) for these 8 actions. If a bee fed more than once during a single visit (e.g., “ff”), it was recorded as a single feeding instance unless there was an intervening transition to or from a different action. Thus, we determined the frequency of transitions out of the 63 possible combinations of the eight actions (which excludes “ff”).

Statistical Analyses

As in Chapter 1, all statistical analyses were conducted and figures created using R v. 3.4.1 (R Core Team, 2017). We used the function chisq.test in the stats package (R Core Team, 2017) to conduct Chi-square tests assessing counts of foragers for differences between feeders and whether the bees fed or chose not to feed. Standardized residuals were calculated and values <-1.96 or >1.96 were considered to be significantly different from expectation. To test if the number of ants present on the feeder influenced bee feeding, we also used the glm function in the stats package to conduct logistic regressions, where the y-response in logistic regression was whether a bee fed or not during a feeder visit and the independent variable was the number of ants. To test if the number of ants present on the feeder influenced whether a bee is bitten during a visit, the y-response in logistic regression was whether a bee was bitten or not and the independent variable was the number of ants. To test if being bitten by ants influenced bee feeding frequency, the y-response in logistic regression was whether a bee was bitten or not during a feeder visit and the independent variable was the frequency of feeding.

Results

The presence of ants had a significant effect on the feeding behavior of foraging bumblebees ($X_1^2 = 58.45$, $p = 2.089\text{e-}14$). Feeding frequency was 11.2% higher than expected on control feeders and 10.9% lower than expected on the ant feeders during these ant exposure trials. We also observed that 17.5% fewer bees than expected chose not to feed during a visit to the control feeder, while 16.9% more bees than expected chose to feed during a visit to the treatment feeder.

The most frequently observed actions that took place on the treatment feeder were “Bee Fed” (Figure 2.1: 32.6% of all behaviors; 1,413 observations), “Bee Bitten” (29.8%; 1,291 observations), and “Gape Mandibles at Ant” (16%; 693 observations). Much less commonly observed actions were “Attack Ant” (9.6%; 415 observations), “Step on Ant” (8.9%; 385 observations), and “Ant Chemical” (1.9%; 81 observations). The least common actions were “Touch” (0.7%; 31 observations) and “Antennation” (0.5%; 22 observations), and these low frequency actions were excluded from analysis. Overall, two-thirds of the observed actions were non-feeding (2918/4331). All of these non-feeding actions described interactions (physical or non-physical) between bumble bees and Argentine ants, and the majority (85%) of these actions were aggressive (i.e. bite, gape, attack, or chemical).

Out of 63 possible transitions between the eight actions (excluding “ff”), 56 combinations were observed in a total of 2,295 behavioral transitions (Table 2.2, Figure 2.1). Seven combinations occurred frequently (100-362 total observations): bb, fb, bg, gc, gb, gg, and bc. Interestingly, 29 combinations were observed less than 10 times, and 7 were never observed at all (Table 2.2).

Of these transitions, 1612 (70%) did not involve feeding; of the non-feeding transitions, 82.3% (1326/1612) represented transitions among aggressive actions. An aggressive action with the bee as the recipient or actor led to another aggressive action 13-fold more often than to a non-aggressive action. Interestingly, some of these transitions were more common in one direction than the other. For example, a transition from a bee feeding to being bitten by ants was seven-fold more common than a transition from being bitten to feeding. Bees also appeared to respond in sequence to ant bites: first gaping and then often transitioning to attacking the ants in return (Figure 2.1).

Bee foraging behavior was dependent on the abundance of ants occupying the feeder at that time. Using logistic regression, we found that there is a negative correlation between the number of ants on the feeder and the probability that a bee fed during a feeder visit (Figure 2.2a, odds ratio = 0.834, $Z = -17.09$, $P < 0.001$). We also found that the probability of a bee being bitten increased with the number of ants on the feeder (Figure 2.2b, odds ratio = 1.127, $Z = 13.88$, $P < 0.001$). Additionally, we found that feeding frequency by a bee decreased as the probability that the bee was bitten increased (Figure 2.2c, odds ratio = 0.879, $Z = -3.829$, $P < 0.001$).

Discussion

When we examined feeding rates between the control and treatment feeders during these trials, we observed higher successful feeding rates on control feeders and higher failure to feed rates on the ant treatment feeders than we would expect if ants had no impact on the bees' foraging. These results are consistent with bees deciding not to feed after interacting with the ants and provide another line of evidence that interference competition is occurring. We also observed the behaviors and interactions between foraging bumble bees and ants on a shared resource. Previous studies on bumble bees (Chapter 1) and honey bees (Sidhu and Wilson Rankin 2016, Sinu et al. 2017) have documented that bees avoid feeding on resources occupied by invasive ants. By examining the individual interactions between ants and bumble bees at a feeder, we gained a better understanding of what experience may lead to such avoidance at a subsequent resource.

It is important to note that the bees did feed from feeders with ants, albeit at lower rates than we would expect if ants have no effect on feeding. Although the threat of risk is known to reduce bee visitation to floral resources, risk rarely eliminates all visitation (Llandres and Rodríguez-Gironés 2011, Tan et al. 2013). Aggressive interactions at the floral resource may decrease bee visit duration (LeVan et al. 2014) and, thus, resource collection (Cembrowski et al. 2014). Therefore, bees experiencing aggressive or disruptive interactions may be less efficient and collect less resources than if they fed on the control feeder. This could have important implications for the health and fitness of the bee colony in the long term.

Our study looked at the intensity of the competition by analyzing the response of bees in relation to the number of ants and whether the bees are bitten. We found that the more ants on a feeder, the higher the likelihood that bees will be bitten by ants, and the lower the likelihood that bees will feed. Further analysis of these data on the treatment feeder demonstrated that bees that are bitten by ants feed less frequently than bees that are not bitten by ants. This clearly demonstrates that the number of ants is predictive of ant aggression and has a strong impact on bee behavior. Recent studies have shown that different pollinator species, which are highly susceptible to predators, preferred rewarding, predator-free patches and avoided predator infested patches even when they provided rich rewards (Llandres et al. 2012). Bumble bees, like honey bees, are at risk of predation and harassment by ants and other predators at flowers. By ceasing foraging in response to increased ant abundance at a resource, bumble bees may be facing a trade-off between resource acquisition and predator avoidance.

Aggression begets aggression in many species, including humans (Rocque et al. 2015) and honey bees (Rittschof et al. 2015). Here, we observed that bumble bee foragers that were involved in an aggressive interaction with ants were 13-fold more likely to continue engaging in aggressive interactions than switching to non-aggressive interactions, and they were 8-fold more likely to continue with aggressive interactions than feeding. Interacting with ants in any capacity appears to affect the subsequent feeding behavior of bees. If bees were engaged in non-aggressive interactions with ants, they were 10 times more likely to continue interacting with ants than to begin feeding. These aggressive interactions with ants may have lasting effects on the recipient bees, as ant encounters can be traumatic or physically damaging for the bee (Cembrowski et al. 2014). By examining the behaviors, interactions, and the transitions between them, we have gained additional insight into what experiences influence future foraging decisions by bees. Future studies should examine how aggressive interactions influence foraging behavior in the long-term and what effects they have on foraging ability and pollinating ability.

Foraging bees will avoid foraging or forage at lower rates when ants are present (Chapter 1, Sidhu and Wilson Rankin 2016, Sinu et al. 2017). However, other studies do not examine what may cause this avoidance by bees. In this study, we examined how bumble bee foragers interacted with Argentine ants on nectar feeders. Ants are some of the most common antagonists that bees encounter at floral resources (Lach 2008, Junker et al. 2010) and have been shown to decrease bee visitation to ant-occupied flowers (LeVan et al. 2014, Sidhu and Wilson Rankin 2016). Here we documented how bumble bees interact with Argentine ants and examined patterns in behavioral transitions. Increases in the abundance of Argentine ants negatively affected bee feeding frequency and led to increased likelihood of the bee being bitten. If bitten, bees were less likely to feed and more likely to engage in aggressive responses, such as gaping mandibles and attacking the ant. Decreased visitation and collection of nectar resources have serious implications for sustaining healthy pollinator populations, particularly in areas invaded by Argentine ants. Consequently, ants are a serious pest to consider when developing strategies to promote pollinators and their pollination services.

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Tables

Table 2.1. Recorded actions (behaviors and interactions) between bees and ants on the live ant treatment feeder. The letter in parentheses indicates the one letter code used to reference the specific behavior.

Behavior	**Description**
Bee Fed (f)	Bee fed during visit to the live ant feeder
Step on Ant (s)	Bee stepped on ant
Antennation (a)	Bee's antenna made contact with ant
Touch (t)	Bee's body came into contact with ant's body (other than with a step or antenna)
Gape Mandibles at Ant (g)	Bee gaped mandibles at ant
Attack Ant (c)	Bee lunged at ant to bite it using mandibles (often fatal to ant)
Bee Bitten (b)	Bee bitten by ant
Ant Chemical (i)	Ant's gaster was intentionally oriented toward or touching bee (possible use of iridoid compounds as ant chemical offense/defense)

Table 2.2. Transition frequency matrix for transitions from actions shown in rows to actions shown in columns. Action codes are the same as Table 2.1. f = Bee Fed. s = Step on Ant. a = Antennation. t = Touch. g = Gape Mandibles at Ant. c = Attack Ant. b = Bee Bitten. i = Ant Chemical.

From / ***To***	**f**	**b**	**g**	**c**	**s**	**i**	**a**	**t**
f	----	309	95	24	68	13	7	17
b	43	363	185	104	47	26	3	2
g	81	119	106	145	24	8	2	3
c	29	86	70	60	13	7	0	1
s	16	28	24	9	88	5	1	1
i	4	25	7	4	2	11	0	0
a	2	4	5	2	2	0	2	1
t	0	5	5	2	1	2	0	2

Figures

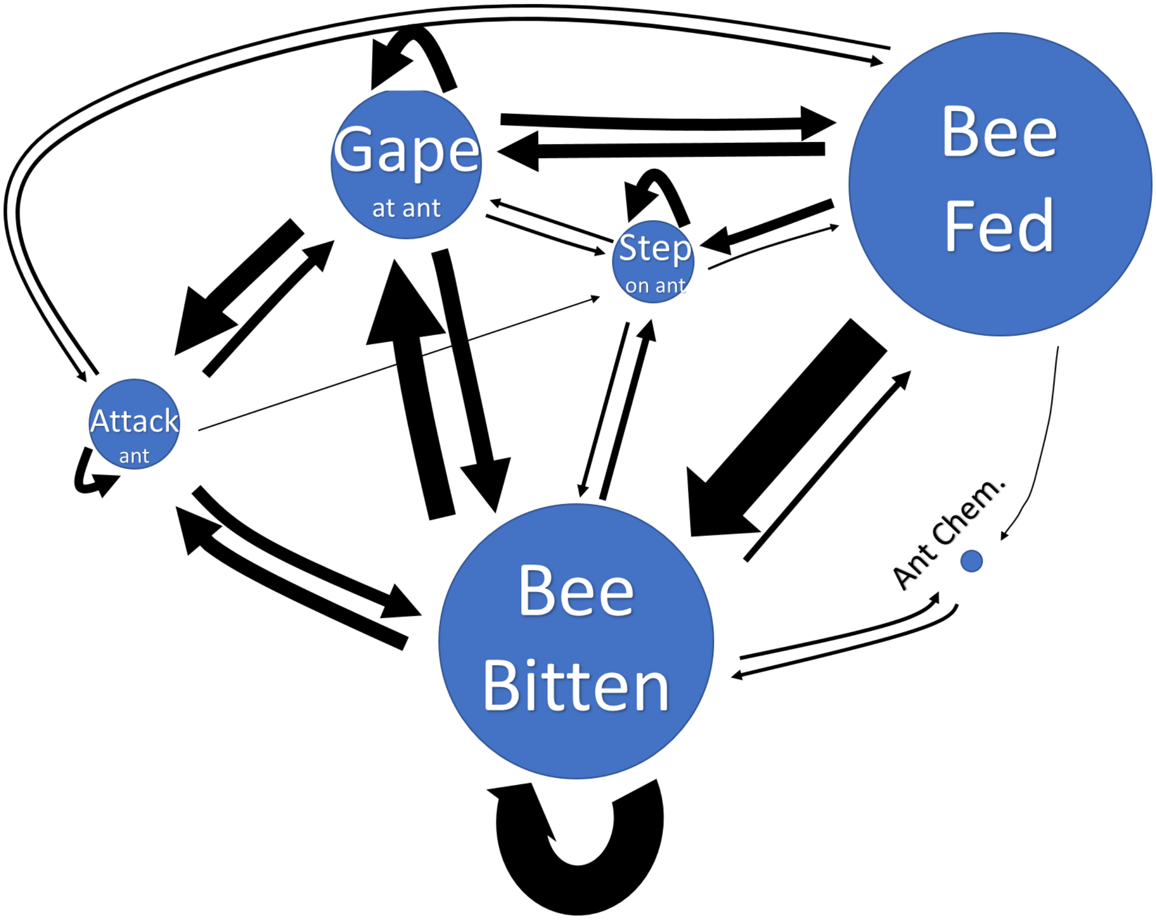

Figure 2.1. Transition frequency diagram for actions (behaviors and interactions) on the live ant treatment feeder. Each circle represents an action listed in Table 2.1, and circle diameter illustrates the relative frequency of occurrence. N = 4,331 total observed actions . Each arrow indicates a transition from one action to another, and arrow width illustrates the relative frequency of occurrence. N = 2,295 transitions. Only actions that were observed at least 40 times are depicted here; only transitions that occurred more than 10 times are included in the diagram.

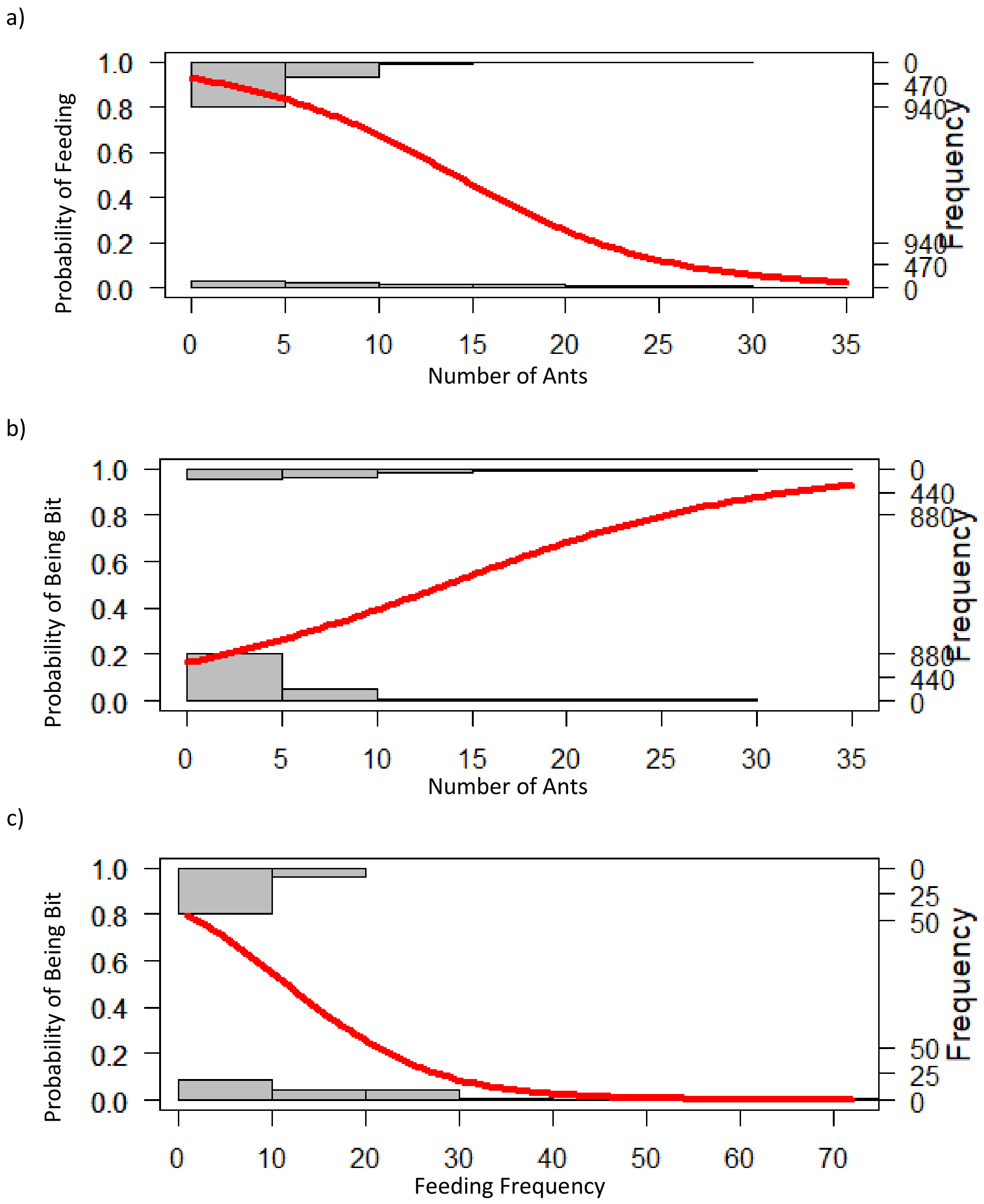

Figure 2.2. Logistic regression plots of the relationships between (a) if a bee fed (1) or not (0) and the number of ants on the feeder, (b) if a bee was bitten (1) or not bitten (0) and number of ants on the feeder, and (c) if a bee was bitten (1) or not bitten (0) and bee feeding frequency. The probability curve values are plotted on a line (left y-axis), while the frequency distribution values are plotted using bars (right y-axis).

Chapter 3: Participation and foraging success in the context of interference competition

Introduction

Social insect colonies are typically described as hubs of activity, but for many social species there is a high rate of inactivity within colonies. This has been described in ants (Charbonneau et al. 2017), bumble bees (Jandt et al. 2012), honey bees (Tenczar et al. 2014), and wasps (Hurd et al. 2003). There is substantial individual variation in activity level such that not all workers forage, and, further, not all individuals are equally adept at foraging (Oster and Wilson 1979). For example, Goulson et al. (2002) found that larger bumble bees (*Bombus terrestris*) tended to be the better nectar foragers. These productive or "elite" foragers are defined here as those that are most successful at collecting food resources. Tenczar et al. (2014) point out that elitism has not been well-studied in bees because of the relative difficultly of observation. Compared to honey bees, however, bumble bees' larger body size and smaller colony size make them a good study model for behavioral observation. While previous studies have examined intrinsic properties of individuals as predictors of forager success, the influence of antagonistic interspecific interactions on forager success has remained largely unresolved.

The relationship between bee forager success and interference from non-pollinator competitors has implications for pollination services. In order to properly manage for pollination, there may also be a need to properly manage for pests (i.e. integrated pest and pollinator management *sensu* Biddinger and Rajotte 2015). The better we understand the relationship between pests and pollinators, the better we can develop management practices to manage pollinators and maximize pollination services. For example, in the context of widespread invasion by the Argentine ant (*Linepithema humile*), pest eradication may be a better solution than increasing the number of bee colonies in an agricultural field. The better able we are to maximize bee foraging in the face of biological invasion, the better we can maximize pollination services in disturbed areas.

The ability to predict colony foraging participation and success in this context could translate into the ability to predict pollination services to crops in which interactions with ants are common. If we can forecast how successful a colony's foragers will be, then we can better manage for pollination services, for example, in food production. Further, if we know what behavioral indicators (e.g. retaliation toward ants) to look for in a colony, then we might selectively breed bumble bees for traits that are correlated with success of individual foragers and entire colonies.

In Chapter 2, we focused in on the character and sequence of specific ant-bee interactions and the transitions taking place at a shared nectar resource only. Here, we explored the possibility of forager elitism in *Bombus impatiens* by (a) taking a broader view of participation (i.e. visitation to a feeder) and foraging success (i.e. feeding during a visit to a feeder) by individual bees of a colony and (b) looking at individual bees' movement and feeding between ant-occupied and ant-free nectar resources. We hypothesized that there is foraging specialization among *B. impatiens* workers, particularly in the context of interference competition with Argentine ants. We expected to (a) find a core group of individual bees that were the most effective foragers and (b) identify one or more behavioral characteristics that are linked with bee foraging activity and success across ant-free and ant-occupied resources. We recorded visitation and feeding of individually marked bees and described how bees that visited one or both (control and live ant) nectar sources differed in their response to ants.

Materials and Methods

Experimental feeders were created as described in Chapters 1 and 2. Briefly, experimental feeders were made using plastic jar lids, micro-centrifuge tubes, polypropylene portion containers, cotton dental wicks, and filter paper. Once prepared, each feeder contained 3.5 mL of sucrose solution (40.6% w/w sucrose) and a piece of cotton wick saturated with the sucrose solution, and was placed in a petri dish lined with Insect-a-Slip Insect Barrier — Fluon (PTFE-30, DISP30, BioQuip Products, Inc.). Each trial was conducted in a 42.5L x 30.2W x 17.8H cm container topped with plexiglass, wherein a treatment and a control feeder were placed.

Ant Exposure Experiments

During each of three trials with each of six queen-less *Bombus impatiens* colonies, we recorded each bee's visit to a feeder, whether she fed while visiting the feeder, and any interactions with ants (Table 2.1): bee fed, step on ant, gape mandibles at ant, attack ant, bee bitten, ant chemical, antennation, and touch. In order to identify individual bees, unique number tags were adhered to each of their thoraces. We recorded the date and ID number each time that a bee was tagged, as well as when it died. Because the majority of bees in a colony had ID tags during every trial, the number of tagged bees provides a good estimate of the colony size at that point in time. With such data, we determined how many bees participated in each trial, the identities of these participating bees, and what behaviors each bee exhibited during foraging.

Statistical Analyses

To assess the degree and type of participation for each colony in foraging experiments, we determined the number of unique bees that, during the ant exposure experiment: participated or not in trials, entered/did not enter the treatment versus control feeders spaces, and fed/did not feed while visiting the feeder. Chi-square tests were performed using function chisq.test in the stats package in R v. 3.4.1 (R Core Team, 2017) to assess the statistical significance of these behavioral differences. Foraging observations were replicated three times with the same six bumble bee colonies used each round. Tests for homogeneity revealed that we could not pool the results of these three rounds of replication so each round was analyzed separately.

To determine behaviors or interactions associated with bee participation during each round of ant exposure, logistic regressions were performed using the stats package. The y-response was whether the bee fed on both the treatment and control feeders, and the independent variables were each of the actions in Table 2.1. As with Chapter 2, we excluded behaviors for which the overall frequency was under 40, comprising two actions ("Touch" and "Antennation") whose combined frequency accounted for only 1.2% of the total. We used a Chi-square test to compare the frequency of a forager's rejection of the treatment versus control feeder. We used the prop.test function in the stats package to conduct a Z-test to compare the proportion of foragers who landed first on the control feeder and fed only from the control, versus the proportion of foragers who landed first on the treatment feeder and fed only from the treatment. A second Z-test compared the proportion of foragers who landed first on the control feeder and then fed from both feeders during the trial, to the proportion of foragers who landed first on the treatment feeder and then fed from both feeders during the trial.

Results

During all three rounds of ant exposure, a significant majority of unique bees did not participate in foraging trials (Round I: $X_1^2 = 187.46$, $p < 0.001$; Round II: $X_1^2 = 187.46$, $p < 0 .001$; Round III: $X_1^2 = 187.46$, $p <0 .001$; Table 3.1).

During Round I (Figure 3.1a; Table 3.1), similar numbers of bees visited the live ant treatment feeder and the control feeder ($X_1^2 = 0.02$, $p = 0.88$), and the number of bees that fed upon arrival to the treatment feeder did not significantly differ from those that did not feed ($X_1^2 = 3.2$, $p = 0.07$). However, significantly more bees fed during a visit to the ant-free control feeder ($X_1^2 = 52.08$, $p < 0.001$), and a larger proportion of bees rejected feeding during a visit to the treatment feeder when compared to rejection of the control feeder ($X_1^2 = 16.65$, $p < 0.001$). Overall, 20% of individual bees fed. The odds that an individual bee would feed on both feeders during a given trial were significantly associated with the following actions: “Bee Bitten” (odds ratio = 0.8734051, $p < 0.001$), “Step on Ant” (odds ratio = 0.7334351, $p < 0.05$), and “Ant Chemical” (odds ratio = 0.5570117, $p < 0.05$).

During Round II, (Figure 3.1b; Table 3.1), we again observed that the number of bees that visited the live ant treatment feeder and the control feeder were similar ($X_1^2 = 0.07$, $p = 0.80$). Unlike Round I, significantly more bees fed than did not feed while visiting either the treatment feeder ($X_1^2 = 59.52$, $p < 0.001$) or the control feeder ($X_1^2 = 71.86$, $p <$

0.001), and there was no relationship between if bees fed and which feeder they were visiting ($X_1^2 = 0.42$, $p = 0.52$). Overall, 33% of individual bees fed. In this round, the likelihood that a unique bee would feed on both feeders was significantly correlated with the action, “Gape Mandibles at Ant” (odds ratio = 1.474396, $p < 0.001$).

Results of Round III are similar to Round II. During Round III, (Figure 3.1c; Table 3.1), the numbers of individual bees that visited the live ant treatment feeder and the control feeder were similar ($X_1^2 = 0.0$, $p = 1$). Significantly more bees fed than did not feed while visiting both the treatment feeder ($X_1^2 = 51.77$, $p < 0.001$) and the control feeder ($X_1^2 = 42.88$, $p < 0.001$), and there was no relationship between if bees fed and which feeder they were visiting ($X_1^2 = 0.17$, $p = 0.68$). Overall, 21% of individual bees fed. Again, we found that “Gape Mandibles at Ant” was significantly correlated with the likelihood that a unique bee would feed on both feeders (odds ratio = 1.522175, $p < 0.05$).

We further examined how the first feeder visited influenced subsequent foraging behavior. For bees that landed on the treatment feeder first during a given trial, 40% did not feed. Rejection of the control feeder (22%) was significantly lower ($X_1^2 = 9.1$, $p = 0.0025$). For bees that fed from only one feeder during a given trial, 91% of the individuals that landed first on the control feeder fed only from that feeder. Significantly fewer bees (77%) fed only on the live ant treatment feeder if it was the first feeder upon which they landed ($Z = -2.23$, $p = 0.0129$). For bees that fed from both feeders during a given trial, there was no statistical difference between the proportions of individuals that had landed on the treatment feeder (30%) versus the control feeder first (22.8%; $Z = 1.04$, $p = 0.1492$).

The majority of feeding visits were carried out by a small proportion of foraging individuals. Across all colonies and trials, approximately 75% of the foraging was done by an average of 34% of bees that fed (range per colony: 27-41%). The colony with the largest number of foragers also had the highest proportion of bees that contributed to 75% of the feeding visits (45/111). However, the colony with the fewest foragers did not have lowest proportion of contributors (16/52).

Discussion

We found that the majority of bees in our colonies did not successfully forage, as only 20-33% of bees ever fed from experimental feeders. Further, across all three rounds, the majority of feeding was carried out by a select minority of bees. Our results are consistent with other studies that find a high degree of inactivity among workers in social insect colonies (Hurd et al. 2003, Jandt et al. 2012, Tenczar et al. 2014, Charbonneau et al. 2017) and are suggestive of an elite group of foragers within bumble bee colonies of this species. A honey bee colony is able to replace its elite foragers due to plasticity of forager activity (Tenczar et al. 2014). However, bumble bees display alloethism with differences in nectar-foraging ability depending on body size (Goulson et al. 2002), so a colony's flexibility in replacing elite foragers may be more complicated for *Bombus* species. More research on this topic is merited.

For all three rounds of ant exposure, visitation rates were similar across feeders. The equal selection of both feeders indicates that there was no barrier to visitation or long-range avoidance of ants. For all three rounds, significantly more bees fed than did not feed upon arrival to a feeder, regardless of which feeder is was. For two out of three rounds, significantly more bees fed successfully on the treatment feeder than failed to feed on it (Fig. 3.1b, c). Round I was the only round during which the difference between the number of bees that did and did not feed was marginal ($P = 0.07$). However, the overall pattern of more total bees feeding during a visit to the treatment feeder is consistent with our findings for the other two rounds.

These results indicate that most of the foragers that visited a feeder were motivated to feed. This may mean that if bees are able to access a resource, then pollination services may be delivered in spite of ant presence. If so, it may helpful to identify colony- or individual-level traits that would help predict how many and which bees will go to a feeder or flower. We may be able to predict if a bee is likely to feed on a second flower—and, thereby, perhaps more likely to pollinate—depending on the type of heterospecific interactions in which they engage.

We found that when a bee landed first on the treatment feeder, it was more likely to visit both feeders during a trial. In Round I, feeding on both feeders was less likely if a bee was bitten by ants, stepped on ants, or had ant chemical directed at her. In both Rounds II and III, a bee that gaped its mandibles was more likely to visit both feeders rather than just one. Taken together, we might predict that a bee that is attacked by ants (regardless of which feeder it lands on first) is less likely overall to feed on both feeders, unless it is a bee that shows aggression toward ants by gaping its mandibles. This warrants more investigation so that we can predict from behavioral interactions with ants if some bees are more likely to successfully visit more resources before returning to the nest.

Foraging is a risky endeavor for individuals (Schmid-Hempel 1990, Roth et al. 2014), as foragers are exposing themselves to danger, for example predators or parasites. Here, we had the risk of being bitten by competitive ants, and, indeed, 35% of the individuals that did forage on the treatment feeder got bitten. Most of the bees in our colonies did not forage at all, likely conducting lower risk jobs within the nest. Jandt and Dornhaus (2011) showed that *B. impatiens* workers that remained in the nest were more likely to be reproductive than were foragers. *Bombus terrestris* workers show a similar trend: workers inside the nest are less likely to be foragers and are more likely to be reproductive, producing males (van Doorn and Heringa 1986). However, Doorn and Heringa (1986) also showed that these non-foraging elite egg layers tended to exhibit aggression toward the queen. Future studies could examine the links between elite egg-layers, colony cycle, elite foragers, and aggression levels. Since body size could be related to both foraging success (Goulson et al. 2002) and ant interaction (Gonzálvez et al. 2013), future research should also incorporate the relative size of nest mates into interactions with ants and foraging success.

We consistently saw bees gaping in response to ant aggression, and it often preceded bees biting ants (Chapter 2). With the risk involved in foraging, the most aggressive foragers may be the most successful at procuring resources. Furthermore, it is possible that bees that gaped their mandibles were more likely to have visited both feeders because these are the more active foragers. In terrestrial systems, foragers have a higher risk of being attacked as foraging effort increases (Verdolin 2006), so it is also possible that bees that visited both feeders were more likely to gape their mandibles simply because visiting both feeders made them more likely to come into contact with ants. Similarly, a bee that visited both feeders may have been less likely to get attacked by ants than bees that only fed from the treatment feeder simply because it spent part of the time feeding on an ant-free feeder.

While the gaping behavior was primarily observed in bees on the feeder with live ants, bees did rarely gape their mandibles while on the control feeder. During Round I, four different foragers gaped their mandibles on the control feeder during these trials. During Round II, four bees gaped their mandibles on the control. During Round III, five bees exhibited this behavior. All but two of the bees who gaped their mandibles on the control feeder had prior experience being bitten by ants. This could indicate a precautionary aggression being adopted by bees that learn to associate feeder visits in general with ants, regardless of whether live ants are actually present.

It would be reassuring if bees could still deliver pollination services regardless of the presence of ants, or that ant presence might improve pollination by causing bees to make shorter, more frequent visits between flowers. Unfortunately, studies suggest that this is not the case (LeVan et al. 2014, Hanna et al. 2015). It may vary depending on the specific plant and pollinator species involved, as well as the degree of ant invasion. More research is needed to look at the relationship between ant-bee interactions and pollination success for different wild and agricultural plants in invaded areas.

While we did find that a bee is more likely to feed when visiting a nectar resource than not to feed, a bee is almost twice as likely to reject feeding where live ants are present as compared to an ant-free resource. Further, if bees are scared off of ant-occupied resources without the opportunity to consume enough nectar—and nectar consumption ultimately affects bee fitness and population dynamics—then pollination in ant-invaded areas may not be sustainable. Parallel patterns in pollen collection by bees may have consequences for pollination in the plant system in question.

In summary, the majority of *Bombus impatiens* bees do not participate in feeder visits, and the minority of the bees that did feed made the majority of foraging trips. Thus, most bees in a colony do not contribute to colony nectar stores. We saw that bees who did participate visited treatment and control feeders equally overall, and that the majority of bees that landed on a feeder fed. However, fewer bees fed if ants were present. It is possible that bees that show aggression towards ants are the more successful and active foragers. In addition to intrinsic measures such as bee size and age, the type of interactions that a forager has with ants may be predictive of her foraging success and, ultimately, delivery of pollination services in the face of biological invasion by an aggressive, non-pollinating competitor.

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Tables

Table 3.1. Summary and results of chi-square tests and logistic regressions performed to assess bumble bee participation and foraging success. “C” and “T” indicate control and treatment feeders, respectively. “OR” indicates the odds ratio for logistic regression.

	Round I	**Round II**	**Round III**
Num. bees visit vs. do not visit a feeder	Most do not visit $X_1^2 = 187.46$, $p < 0.001$	Most do not visit $X_1^2 = 187.46$, $p < 0 .001$	Most do not visit $X_1^2 = 187.46$, $p <0 .001$
Num. bees visit control vs. treatment	Equal visitation $X_1^2 = 0.02$, $p = 0.88$	Equal visitation $X_1^2 = 0.07$, $p = 0.80$	Equal visitation $X_1^2 = 0.0$, $p = 1$
Num. bees feed vs. do not feed on control	C: more feed $X_1^2 = 52.08$, $p < 0.001$	C: more feed $X_1^2 = 71.86$, $p < 0.001$	C: more feed $X_1^2 = 42.88$, $p < 0.001$
Num. bees feed vs. do not feed on treatment	T: equal feed $X_1^2 = 3.2$, $p = 0.07$	T: more feed $X_1^2 = 59.52$, $p < 0.001$	T: more feed $X_1^2 = 51.77$, $p < 0.001$
Proportion bees reject feeding during visit to control vs. treatment	More reject T $X_1^2 = 16.65$, $p < 0.001$	Equal $X_1^2 = 0.42$, $p = 0.52$	Equal $X_1^2 = 0.17$, $p = 0.68$
Actions significantly associated with feeding on both feeders (Table 2.1)	Negative: Bee Bitten OR = 0.87, $p < 0.001$ Step on Ant OR = 0.73, $p < 0.05$ Ant Chemical OR = 0.56, $p < 0.05$	Positive: Gape Mandibles at Ants OR = 1.474396, $p < 0.001$	Positive: Gape Mandibles at Ants OR = 1.522175, $p < 0.05$

Figures

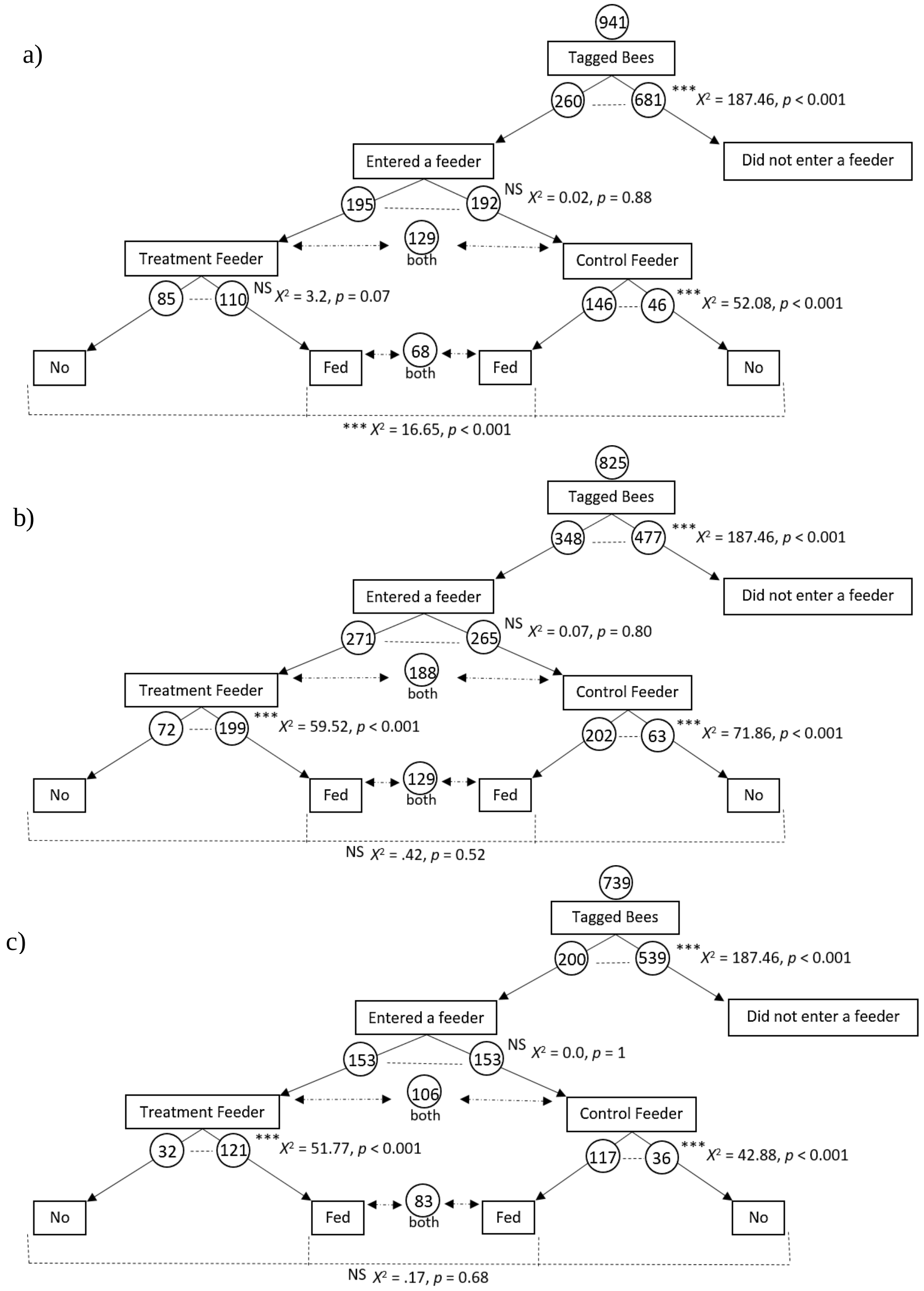

Figure 3.1. Participation and foraging success of bees from six *B. impatiens* colonies during Round I (a), Round II (b), and Round III (c) of Ant Exposure Experiments with *L. humile* on the live ant treatment feeder. The three rounds are depicted in the order in which they occurred. Numbers in circles specify the number of unique bees which behaved as indicated. Significant differences are marked with asterisks (***$p < 0.001$; NS = no significant difference).

Appendix 1: Supplemental Protocols

Chemical Rinse Protocol

1) Hexane: Apply hexane from small, dense polyethylene squeeze bottle to areas of feeder where insects may have made contact. Allow hexane to evaporate.
2) dH_2O: Rinse feeders with water and allow it to evaporate.
3) 70% Ethanol: Rinse feeders with ethanol and allow it to evaporate.
4) dH_2O: Same as (2). Once dry, feeders can be stored or prepared for a subsequent trial.
Note: Evaporation is quickened by turning on the air in the fume hood.

Pheromone Solution Protocol

1) Place 1-5μL capillary tube in/on a large weigh boat and tare using a Denver Instrument Company A-250 balance
2) Take up pheromone into capillary tube by quickly dipping tube into pheromone concentrate
3) Place capillary tube back in/onto weigh boat and weigh
4) Add pheromone in tube to a volume of solvent at a ratio of 1ng pheromone to 1μl solvent.

Pheromone Detection Protocol with GC-MS

1) Standard: Add one drop (Z)-9-hexadecanal standard (concentrate) into 2 dram glass vial, expose SPME fiber to vial headspace for 30 sec, and run GC-MS to analyze volatiles absorbed by the SPME fiber. Compound identified by retention time 16.237 (Figure A1).
2) 10 min aging: Apply 100 uL of stock solution (1 ng pheromone per 1 uL 100% ethanol) to filter paper disk, let filter paper sit 10 min fume hood, fold and place filter paper into 2 dram glass vial, seal vial with aluminum foil, let sit 1 hr in sealed vial, puncture foil to insert SPME fiber and expose to vial headspace for 1 hr, and run GC-MS to analyze absorbed volatiles.
3) Blank: Run blank SPME fiber to make sure there is no residual pheromone from previous run.
4) 40 min aging: Add 100uL of stock (1 ng z-9 per 1 uL 100% ethanol) to filter paper disk, let sit 40 min in fume hood, fold and place filter paper into 2 dram glass vial, sealed vial with aluminum foil, let filter paper sit 1 hr in sealed vial, puncture foil to insert SPME fiber and expose to vial headspace for 1 hr, run GCMS to analyze absorbed volatiles.
Equipment specifications: SPME sampler: 100 μm polydimethylsiloxane (PDMS); Supelco, Inc., Bellefonte, PA, USA. Electron impact mass spectra (70 eV) were taken with an Agilent 5975C mass selective detector interfaced to an Agilent 7890A gas chromatograph equipped with a HP-5MS column (30 m × 0.25 mm inner diameter, Agilent Technologies). Samples were injected in splitless mode, with an injection inlet temperature of 250°C. Flow rate in the column was 1.2 mL/min. Temperature program was 50°C for 1 min and then 10°C min^{-1} to 280°C with 5-min hold.

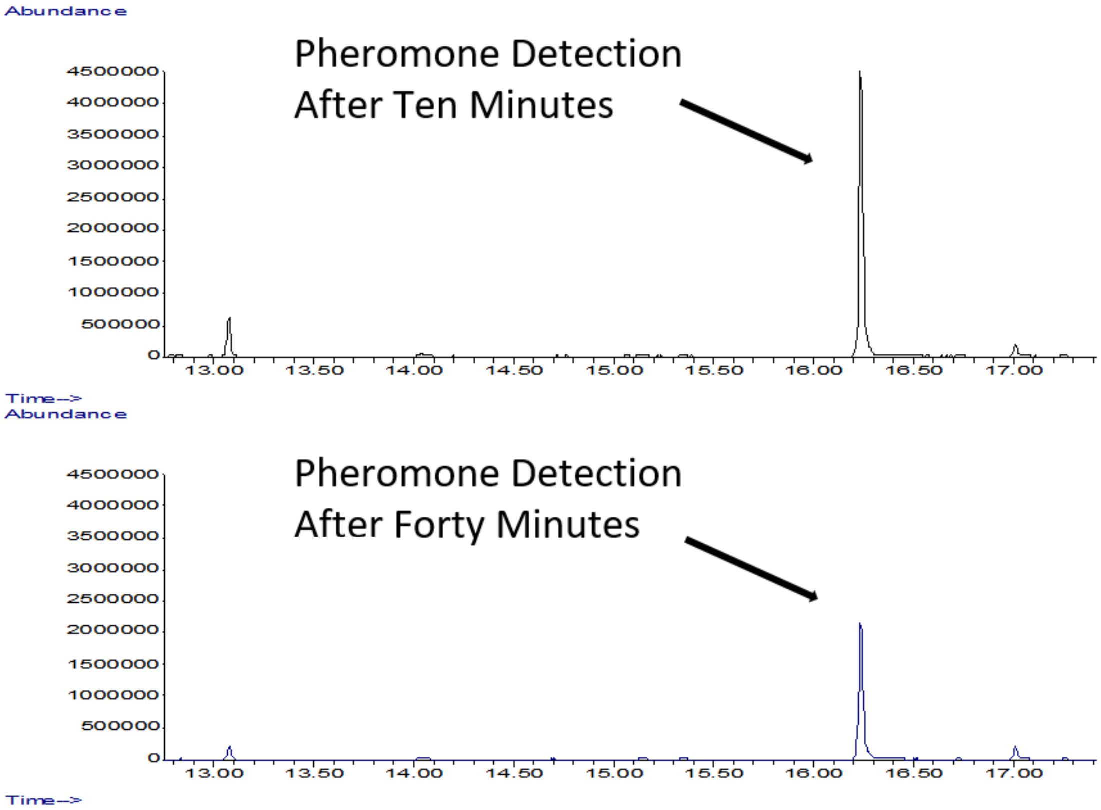

Figure A1. Chromatogram peak integration results show a width area of 72,137,231 after 10min aging and 33,525,453 after 40min aging. The pheromone is detectable when applied at a concentration of 1ng (Z)-9-hexadecanal per 1uL 100% ethanol. Based on width area comparison of pheromone detected at ten versus forty minutes, 46.47% of the pheromone was still available after thirty minutes (the length of an observational trial).

Appendix 2: Ethanol Pilot for Ant Pheromone Experiment

Using in the same “*General experimental set up*” described in Chapter 1, three 30-minute trials were carried out with one bee colony to assess any preference in visitation to the unmanipulated control feeder versus a feeder treated with 100µL of 100% ethanol. Analysis and contingency plot (Table A1, Figure A2) were done using JMP Statistical Software.

Table A1. Contingency table of feeder by trial for the ethanol pilot.

		control	**treatment**	**Total**
Trial 1	Count	27	26	53
	Total %	17.20	16.56	33.76
	Col %	37.50	30.59	
	Row %	50.94	49.06	
Trial 2	Count	24	29	53
	Total %	15.29	18.47	33.76
	Col %	33.33	34.12	
	Row %	45.28	54.72	
Trial 3	Count	21	30	51
	Total %	13.38	19.11	32.48
	Col %	29.17	35.29	
	Row %	41.18	58.82	
Total	Count	72	85	157
	Total %	45.86	54.14	

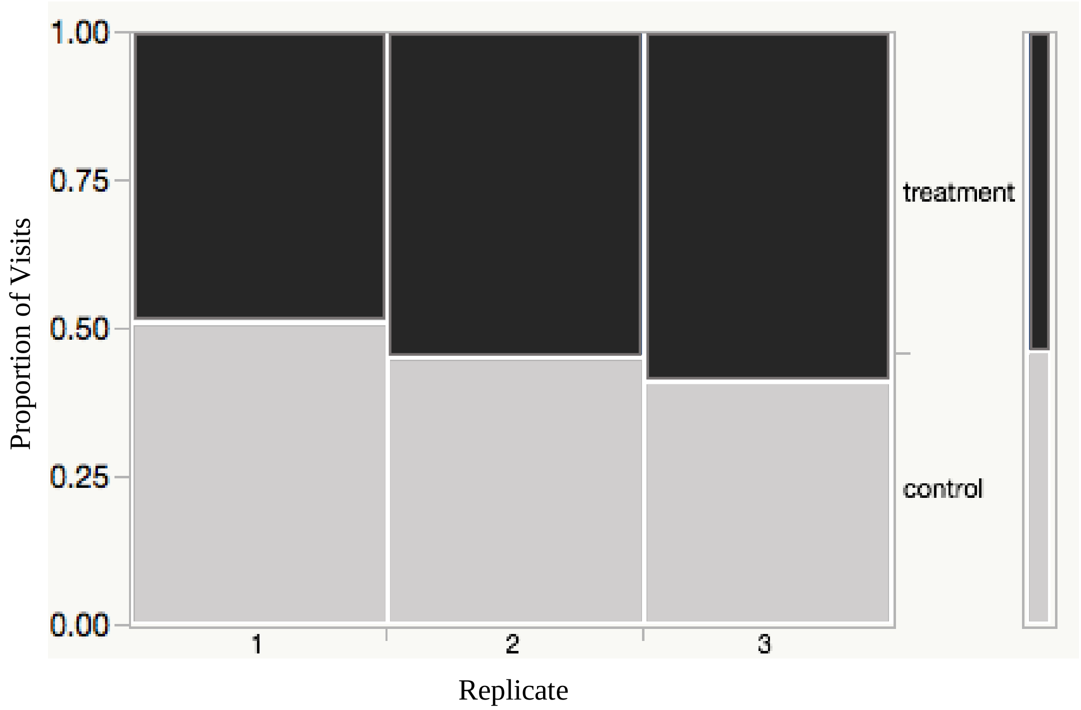

Figure A2. Mosaic plot for contingency analysis of feeder by replicate for the ethanol pilot. Visitation did not differ between the feeder types across any of the three replicates ($X^2_2 = 1.010$, $p = 0.6037$)