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The European honey bee, *Apis mellifera*, is a vital species for agriculture, providing pollination for crops all around the world. Recent declines in honey bee health have been concerning, and the spread of the ectoparasitic mite *Varroa destructor* is thought to be one of the leading causes of this decline. Examining how *Varroa destructor* finds its larval host for reproduction is important for understanding how *Varroa destructor* can ultimately lead to the death of the colony. Investigating the factors that guide *Varroa destructor* host seeking is also an important step for developing tools to control *Varroa destructor* within the *Apis mellifera* hive without the use of toxic acaricides. While early studies showed promising results of identifying *Varroa* attractants, an adequate in-hive trap has not been developed. I investigated the influences of three factors on *Varroa destructor* host selection of *Apis mellifera* larvae in two sets of experiments: caste, nurse bee visitation rate, and larval weight. I also investigated gene expression and virus titers as possible consequences to mite invasion. Overall, we found complex interaction among the tested factors. My comparison among worker and drone cells showed that with increasing nurse bee visitation rates there is an increased chance of cell invasion by *Varroa*. However, drone larvae did not have a significantly higher chance of invasion compared to worker larvae, despite higher visitation rates. Worker larvae manipulated through starving and feeding did not exhibit altered nurse bee visitation rates, although some weight changes were observed. Larvae with increased weight did not to have a higher chance of cell invasion. Interactions between visitation

rates and molecular variables, such as immune activity and virus titers were studied. The immune gene Dicer-Like and deformed wing virus-A were both associated with *Varroa* cell invasion. These results provide insight into how physical, behavioral, and chemical factors influence *Varroa destructor.* This study shows how *Varroa destructor* acts and interacts within *Apis mellifera* hives, that can be used to develop methods of control in the future.

Key words: Honey bee, *Apis mellifera, Varroa destructor,* visitation rates, nurse bees

EXAMINING THE FACTORS INFLUENCING *VARROA DESTRUCTOR* HOST SELCTION OF *APIS MELLIFERA* LARVAE

by

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CHAPTER I

INTRODUCTION

Apis mellifera, the European honey bee, is an insect of the Apidae family. *Apis mellifera* lives in large colonies, which can contain as many as 30,000 individuals (Seeley 1985). A colony consists of three castes: queen, workers, and drones (Winston 1987). Each colony contains one queen, whose main role is to lay eggs, of which she can lay up to 2,000 per day (Winston 1987). Queens are reared when workers feed an abundance of royal jelly to fertilized larvae. Queens are only produced when the colony needs a queen, such as during a supersedure, queen replacement, or a reproductive swarm (Winston 1987). Worker bees are entirely female and are produced from fertilized eggs that are fed a diet poor in royal jelly (Seeley 1985). Workers perform most tasks of the colony, including feeding larvae, building wax, and collecting pollen and nectar (Winston 1987). The behavior of workers depends on their age, with young workers acting as nurse bees and older workers acting as foragers. The male drones develop from unfertilized eggs and their main task in the hive is to search for virgin queens and mate (Seeley 1985). At the end of the summer, the drones are removed from the hive by workers (Winston 1987).

In addition to the adult bees, the hive of *A. mellifera* contains brood, nectar, pollen, and honey. Brood is usually found in the center of the hive. The queen typically lays eggs in a circular pattern, starting in the middle of the frame. A solid brood pattern without empty cells intermixed is considered a sign of a healthy queen (Seeley 1985).

Most of the brood is destined to become a worker, up to 95% (Seeley 1985). Drone brood is laid in larger cells than worker brood. Young adult worker bees feed the brood and cap the cells with wax at the ninth and tenth day, for worker and drone brood respectively (Winston 1987). Nurse bees spend almost three times more time tending to drone brood than worker brood (Calderone and Kuenen 2003). Worker and drone brood emerge from their cells after 21 and 24 days respectively. Queens are the least commonly reared and emerge 16 days after capping (Winston 1987). The honey, nectar, and pollen are typically stored in the top part of the hive. Nectar is collected from plants, and enzymes within the workers' stomachs are used to break down sugar within the nectar. Nectar is then placed in cells, where the water within the nectar evaporates. The nectar is considered ripened when sugars are converted and most of the water has evaporated, at which point it is called honey (Winston 1987). Pollen is also collected from plants and is the main source of protein for the colony (Winston 1987). Older adult workers act as foragers and collect pollen, water, propolis, and nectar, searching as far as a two-mile radius from the hive (Seeley 1985).

Apis mellifera is very important to the world economy, providing annually $207 billion in pollination and other services around the world, and over $15 billion in the United States (Wilson-Rich *et al*. 2014, Ellis *et al.* 2010). The European honey bee pollinates over 130 different crops, many of which rely heavily on insect pollination (Wilson-Rich *et al.* 2014). *Apis mellifera* also produces honey, wax, and other products such as propolis (Wilson-Rich *et al.* 2014). These products are valuable and are sold worldwide for a variety of purposes. Honey is sold, on average, at five dollars per pound

within the United States (Wilson-Rich *et al.* 2014). Beeswax is sold and used for multiple purposes, such as preservatives, cosmetics, pharmaceuticals, and lubricants for various products (Wilson-Rich *et al.* 2014). The European honey bee is also important for various cultural and religious beliefs (Wilson-Rich *et al.* 2014). Since *A. mellifera* is important on an ecological, economical, and cultural level, research is needed to fully understand the problems facing the species.

Apis mellifera health has been declining over the last several decades and has been monitored for several years (Lee *et al.* 2015). For example, the annual loss of honey bee hives in 2014 was 40.6%, with a summer and winter loss of 25.3% and 22.3%, respectively (Seitz *et al.* 2016). The decline in honey bee health is presumably caused by a combination of factors, including pests, pesticides, pathogens, and poor nutrition (Seitz *et al.* 2016). The multiple factors influencing the decline of *A. mellifera* health make it very difficult to fully understand the problem but it's not a lost cause.

One of the main causes for the decline in health of *A. mellifera* is *Varroa destructor* (Rosenkranz *et al.* 2010). *Varroa destructor* feeds off the honey bee during the adult and larval stage. The severe symptoms caused by heavy *V. destructor* infestation are known as varroosis (Francis *et al.* 2013). Symptoms of varroosis include visible injury of brood and adult bees and decline of the overall hive population (Boecking and Genersch 2008). Bees that were fed on during the pupae stage may start foraging prematurely (Rosenkranz *et al.* 2010). This feeding during the bee's development also makes the bees significantly lighter, with drones losing up to 10% of their body weight

when parasitized by one mite in the brood cell (Duay *et al.* 2003). The decreased weight can cause problems for the bee later in life in the form of decreased flight performance, which can lead to a decreased lifespan (Rosenkranz *et al.* 2010). Bees parasitized as pupae may fail to fully develop characteristics of winter bees, which may cause them not to survive to the spring (Amdam *et al.* 2004). Parasitized bees in a *V. destructor* infested hive show decreased learning capacity and are two times more likely to not return to the hive after foraging (Kralj and Fuchs 2006).

Importantly, *V. destructor* is a vector several honey bee viruses, including deformed wing virus (DWV) and Israeli acute paralysis virus (IAPV) (Rosenkranz *et al*. 2010). *Varroa destructor* and DWV are symbiotic. DWV enters *A. mellifera* larvae through *V. destructor* feeding sites. Feeding by *V. destructor* can cause stress, which combined with a wound helps the replication of the virus (Kuster *et al.* 2014). DWV has a potential immunosuppressive effect on *A. mellifera,* which weakens the bees and allows *V. destructor* to feed more easily (Prisco *et al.* 2016). These effects create a positive feedback loop, where *V. destructor* and DWV benefit from each other at the expense of *A. mellifera* (Prisco *et al.* 2016).

The original host of *V. destructor* is the Asian honey bee, *Apis cerana*. *Varroa destructor* gained the ability to infect *A. mellifera* approximately sixty years ago, and has since spread worldwide, reaching the United States in the 1980's (Wilfert *et al.* 2016). *Varroa destructor* is an obligatory parasite, meaning it relies completely on its *Api*s host (Nazzi and Le Conte 2016). In *A. mellifera* hives, *V. destructor* can reproduce in both

worker and drone brood cells (Dietemann *et al*. 2012). In contrast, *V. destructor* inside *A. cerana* worker cells are more likely to be detected and removed by nurse bees, known as hygienic behavior (Page *et al.* 2016). In *A. mellifera* and *A. cerana* have defense mechanisms against pests and pathogens, such as the mentioned hygienic behavior as well as grooming behavior. Grooming involves the cleaning of itself or other bees with the use of the mouthparts and the mesothoracic legs (Boecking and Spivak 1999). Hygienic behavior is the ability of workers to detect and remove infected or damaged larvae or pupae. *A. cerana* evolved along with *V. destructor*, which explains why it is better adapted to deal with *V. destructor* than *A. mellifera* (Boecking and Spivak 1999).

The *V. destructor* population in a hive follow the brood pattern of *A. mellifera*, increasing as the hive produces larvae, usually from spring through the fall (Francis *et al*. 2013). *Varroa destructor* has two life history stages, a phoretic phase during which it attaches to adult bees and a reproductive phase inside a brood cell (Nazzi and Le Conte 2016). Chemical signals are important in the dark hive during all *Varroa* life cycle stages. *Varroa destructor* is able to passively mimic chemical signals of *A. mellifera* to stay hidden within the hive. (Kather *et al.* 2015). The ability of *V. destructor* to hide in the hive makes it more difficult for *A. mellifera* to suppress mite population growth. *Varroa* uses nurse bees to reach brood cells and are rarely seen walking on comb to find a host (Boot *et al.* 1994). *Varroa destructor* will begin its reproductive phase by invading a 5^{th} instar worker or drone cell, just before the cell is capped and the larva transforms into a pupa. In a closed cell, chemical cues from the larva trigger reproduction in *Varroa* females (Frey *et al.* 2013). Mites are rarely found in queen larval cells and this is possibly

caused by shorter capping time of queens and/or the orientation of the cells (Harizanis 1991, Calderone *et al.* 2002). *Varroa destructor* will begin laying eggs after the cell is capped. The first egg laid is unfertilized and will develop into a male, while all others are fertilized female eggs (Martin 1994, Rehm and Ritter 1989). The siblings will mate with each other and then the females molt to maturity and leave the cell when the bee emerges (Rosenkranz *et al.* 2010).

Varroa destructor shows host preferences during both life stages. While in the phoretic phase, it favors nurse bees over the older forager bees (Kuenen and Calderone 1997, Kraus 1994), when mite populations are low. This preference for nurse bees gives the mite better access to brood cells, where the mites can start their reproductive phase. However, when mite populations increase *V. destructor* does not always discriminate between nurse and foragers bees (Cervo *et al.* 2014). Attaching to foragers may allow the mites to access other hives and can alleviate competition between mites.

During the reproductive stage, *V. destructor* is more likely to invade the cell of a drone than that of a worker, and drone larvae are 11 times more frequently invaded by *Varroa* than worker larvae (Boot *et al.* 1995, Calderone and Kuenen 2001). It might be advantageous for *V. destructor* to invade the cells of drone brood rather than worker brood because *V. destructor* females on average have 3.0 offspring while in a drone cell and 1.8 offspring within a worker cell (Donze *et al*. 1996). Worker cells can be invaded 15-20 hours before capping, while drone cells can be invaded up to 40-50 hours before capping, which was determined by the time at which the bottom of the cell is completely

covered by the larva (Boot *et al.* 1992). This allows the mite more time to invade drone cells than worker cells. The overall time invested in tending to drone brood by nurse bees is 2.78 greater than worker brood (Calderone and Kuenen 2003). Since *V. destructor* uses nurse bees to find its larval host, mites may choose drone larvae because they have more access to these cells. Alternatively, *V. destructor* may actively prefer to enter drone cells over worker cells based on chemical or other cues.

The hypothesis that *V. destructor* actively chooses drone cell over worker cells by detecting chemical cues from larvae has been studied by Nazzi and Le Conte (2016). They found that simple aliphatic esters released from brood, attract *V. destructor* during their reproductive phase, especially methyl palmitate. At the time, the discovery of these esters, primarily methyl palmitate, was considered a viable method for *V. destructor* control. However, field studies using the identified esters failed to show a response, and further studies have shown that the esters alone do not act as an attractant for *V. destructor*, and that particularly methyl palmitate does not cause a response by *V. destructor* (Boot 1994, Nazzi *et al.* 2001).

As described above, the chemical choice hypothesis states that *V. destructor* uses chemical cues to actively choose drone cells over worker cells. In contrast, I hypothesize that nurse bee visitation to cells determines *V. destructor* cell invasion and overrides any potential the choice by *V. destructor* whether it be by chemical cues or any other mechanisms. I predict that nurse bee visitation rate explains the majority of variation in mite cell invasion under hive conditions, and that the almost threefold higher visitation

rate of nurse bees to drone brood cells relative to worker cells is sufficient to explain the higher mite infestation of drone cells. I suggest that visitation rates of nurse bees is not a factor that is detected and selected by *V. destructor*, but rather controlled by the nurse bees and indirectly influences *V. destructor* infestation. Higher visitation rates of nurse bees to drone cells has been insinuated as a factor for *V. destructor* host selection in many studies (e.g, Boot *et al.*1992, Calderone and Kuenen 2003, Calderone and Lin 2001, Rosenkranz *et al.* 2010, and Diller *et al*. 2006). However, to date, none have focused on if and how much visitation rates influence host selection by *V. destructor*.

Hypothesis and Predictions

Hypothesis: Visitation rate is the dominate factor influencing *Varroa destructor* larval cell invasion of *Apis mellifera* larvae.

The chemical choice hypothesis does not adequately explain *V. destructor* cell invasion patterns under hive conditions, which may be due to the complex olfactory hive environment. Therefore, other factors need to explain the higher infestation rate of drone cells. Nurse bee visitation is a plausible factor because nurse bees invest more time tending drones than workers, giving *V. destructor* more opportunities to descend into drone cells than worker cells.

To test this hypothesis, I make the following predictions:

1. Cells with higher visitation rates are more likely to be invaded by *V. destructor*.

2. Differences in visitation rates of drones and workers are sufficient to explain increased *V. destructor* invasion of drone cells compared to worker cells.

3. Experimental manipulation of food status of larvae will change visitation rates of nurse bees and consequently *V. destructor* cell invasion probability.

4. Physiological variables of worker larvae, including weight, immune status, and DWV infection level, that could also make brood more or less attractive to *V. destructor* do not correlate with mite invasion probability.

CHAPTER II

MATERIALS AND METHODS

Overview

Two experiments were conducted to test the predictions about this relationship of *V. destructor* invasion of honey bee brood cells and nurse bee visitation and some alternative factors. I explored the natural variation in visitation rate in the first experiment (caste experiment) and experimentally attempted to manipulated visitation rate in worker brood in the second (starvation experiment). Both caste and starvation experiments had similar basic experimental designs. For each experiment, a frame of larvae was prepared and transferred into an observation hive, along with nurse bees, *V. destructor* mites, and a frame of honey. All hives used during the experiment were owned by UNCG. The observation hive was kept in a shed at the UNCG bee station, which was kept at the temperature and relative humidity similar to hive conditions. Visitation of cells by nurse bees were quantified using scan sampling. A nurse bee visit was defined as the act of the nurse bee inserting her head or head and part or all of the thorax into the larval cell. Once it was the appropriate time, the frame was placed in a freezer at -20°C. The frame was then removed from the freezer, and the mite load for each cell was recorded. Mite load was defined as the presence or one or more mites in the cell. Visitation rate was defined as the total number of nurse bee visits per larva.

To have access to an adequate number of mites for the study, mites were collected from various hives and added into chosen “mite hives”. *Varroa destructor* mites were collected from hives using the sugar shake method. To collect mites during the sugar shake method, approximately 300 bees were placed in a clean quart jar with a mesh lid. Two tablespoons of sugar were added to the jar. The jar was shaken and then allowed to sit for 1-2 minutes. Afterwards, the jar was shaken upside down onto a damp tissue, allowing mites to fall out. Bees were returned to the hive when possible. After mites were collected, they were added into the mite hives.

Caste Experiment

Frames containing foundation drone and worker wax were inserted into UNCG hives at the beginning of the spring and were allowed to be built up by the colony. The queen from a hive was caged on a drone frame for two days, and then caged on a worker frame for two days. This staggering of queen caging on the drone frame and then worker frame allowed the resulting worker and drone larvae to be capped at the same time. After eggs were laid, the two frames were combined by cutting and melting wax together. Strips of the drone frame were cut out using a soldering iron, and placed in slots of the worker frame, which also had strips of wax removed. Wax strips with the highest amount of eggs were chosen. The frame, labeled now as the experimental frame, was then placed back in the source hive, to allow the adult bees to fully attach the wax strips to each other and the rear the larvae.

During the 2nd instar stage the larvae were culled to a number of larvae for both drone and workers that would be possible to accurately monitor. When the larvae reached 4th instar, the frame was inserted into an observation hive. Adult nurse bees were sugar shaken to remove any mites and added to the observation hive. *Varroa destructor* mites were collected from mite hives using the sugar shake method, and were added to the observation hive, with the total number mites equaling the number of larval cells, between 80-100 mites. The *V. destructor* mites were added to the observational hive by adding them to a jar of bees, which gave the mites an easy way to be introduced to the observation hive. The observational hive was left alone overnight to allow the colony to adjust to the environment. Observations began three days before the cells were to be capped. Visitation rates for cells were quantified for three days, eight hours each day.

Once all the cells were capped, the frame was removed from the hive and placed in a lab incubator, which was kept at 34°C and 55% relative humidity. The nurse bees were returned to their original hive. The experimental frame was kept in the incubator for 2-3 days to allow the larvae to develop further, and it was then placed in a freezer at -20°C. The frame was then removed from the freezer, and the mite load for each cell was recorded. Mite load is defined as the presence or one or more mites in the cell. Visitation rate defined as the total number of nurse bee visits per larva. The caste experiment had two replicates, however the first replicate contained half the number of mites than the second replicate. This is because we realized the experiment would require more *V. destructor* mites, and after the first replicate we increased number of mites that were

introduced into the observation hive. Since only the second replicate contained the correct number of mites, it was the only replicate that was used for data analysis.

Analysis of Caste Experiment

Data was tested for normality using the skewness normality test. Data was shown to be non-normally distributed with a p-value of 0.046. Because of this we used a non-parametric Kruskal-Wallis test to assess difference in visitation rates of workers and drones. We made graphs showing medians and standard errors.

Starvation Experiment

Because the starvation experiment was conducted in the second half of the summer, only worker larvae were used. The treatment groups of this experiment were "control", "starved", "fed", and "starved/fed". During this experiment, data on the visitation rate, weight and mite load of brood were collected.

The wax on the opposite side of the frame was removed to prevent the queen from laying eggs on that side. The queen from the source hive was caged on one side of a hive body frame containing empty cells. To assure all larvae were within the same age, the queen was caged for up to 24 hours. Originally, after 24 hours, the queen was released, and the cage remained on frame to prevent the queen from retuning and laying more eggs. During later replicates, we removed the cage completely after the initial 24 hours to prevent cannibalization of larvae. The experimental frame was kept in the source hive until the larvae were seven days old, during which the source hive was provided plenty of food. On day seven, we placed the experimental frame in an observation hive. Adult

nurse bees were collected from other university hives and were treated for mites using the sugar shake method. The adult bees were then weighed and added to the observational hive. We added between 300-500 adult nurse bees based on weight, to the experimental hive per replicate. We allowed the colony to adjust to the new conditions overnight. The following day (day 8) the frame was removed, and the bees on the frame were brushed into a jar. We inserted bee excluding cages over two treatment groups (starved and starved/fed), preventing these larvae from being fed by the nurse bees. We returned the frame to the observational hive and reintroduced the bees. The cages remained over the two treatment groups for six hours. During this time, artificial brood food was made from 53% commercial royal jelly, 6% glucose, 6% fructose, 1% yeast extract, and 34% deionized water (Kaftanoglu *et al.* 2005). We also collected *V. destructor* mites using the sugar shake method from mite hives during the starving period. After collection, we kept mites in glass petri dishes with damp tissue to prevent desiccation.

After the six hours, the experimental frame was again removed from the observational hive, and nurse bees on the frame were brushed into a jar. The starvation cages were removed, and the laboratory brood food was fed 30µl of laboratory brood food to the treatment groups “fed” and “starved/fed”. After feeding, we allowed the larvae at least 15 minutes to consume the food, and then returned the experimental frame to the hive. The collected mites were introduced to the bees in the jar, and after the mites were given time to attach themselves to the bees, the bees were reintroduced into the hive. The hive was allowed at least 30 minutes to resettle before observations began. Visitation rates were recorded using scan sampling. After cells were capped, the

experimental frame was removed from the observational hive and placed in a laboratory incubator, which was kept at 34°C and 55% humidity. The experimental frame was kept in the incubator until the larvae reached the pupa stage. Once the larvae reached the pupae stage, the experimental frame was placed in the laboratory freezer at -20°C. The experimental frame was removed within a week, and the mite load for each cell was recorded, and each pupa was weighed on a Mettler Toledo ™ PL202-S Classic Balance scale in micrograms. After mite load was determined and pupae were weighed, samples were stored in individual microcentrifuge tubes and kept at -80ºC until used for molecular analysis. During the starvation experiment, weight the larvae at the same time the mite presence was measured. This was decided because we were manipulating the larvae through starving and feeding, and we wanted to know if this had any long-term impact on the larvae/pupae. To test for a correlation between variation in visitation rates and probability of cell invasion, the relationship between these two variables were evaluated within and between treatment groups.

Molecular Analysis of Starvation Experiment

To gain a better understanding about the physiological consequences of the treatments of the starvation experiment, which may influence mite host selection, the frozen pupae were studied with respect to the expression of key immune genes and titers of DWV. For a later, additional analysis of the cuticular chemicals that is not included in my study, the individual pupae were rinsed with hexane before further processing. To remove any residual hexane, each sample was carefully placed on a Kimwipe™, and dabbed lightly. After all hexane was evaporated, the pupa was placed in an

microcenterfuge tube. RNA was extracted from each sample using the MasterPure™ Complete DNA & RNA Purification Kit (Epicentre ®) following the manufacturer's recommendations. Briefly, samples were homogenized for 30 seconds individually using pre-sterilized pestles (Axygen®). The RNA was resuspended in 20µl of molecular grade water and stored at -80°C. We measured the concentration of the RNA using a NanoDrop ND-1000 Spectrophotometer and created diluted RNA of 20ng/µl using molecular grade water, with a total volume of 100µl.We made cDNA from the diluted RNA using Mastermix (Applied Biosystems), which consisted of 2µl 10X RT buffer, 0.8µl 25X dNTP mix, 2.0µl 10X RT random primers, 1.0µl multiscribe RT, and 4.2µl molecular grade water per sample. Each cDNA sample was made with 10µl of mastermix and 10µl of diluted RNA. The cDNA was created using an Eppendorf® Mastercycler, running at 23°C for 10 minutes, 37°C for 60 minutes, 37°C for 60 minutes, and 85°C for 5 minutes. The cDNA was kept at -80ºC until use. cDNA was diluted using 10µl of concentrated cDNA and 90µl of molecular grade water. Samples were tested for the expression of six genes and titer of DWV. The genes were two references genes: Actin and RPS5, and three immune genes: Toll-6 (Toll/TLR pathway), Defensin-2 (AMP pathway), and Dicer-Like (RNAi pathway), and Vitellogenin. (Brutscher *et al.* 2015). These immune genes were chosen because they are associated with deformed wing virus and all work on different immune pathways. Two different strains of Deformed Wing Virus, DWV-A and DWV-B, were tested (Cornman 2017). To measure the relative gene expression and relative virus titers, RT-qPCR was performed. Eighteen microliters of mastermix consisting of 10µl 2X SYBR Green mix (Applied Biosystems), 0.5µl of each forward and

reverse primers (Table 1), and 7µl molecular grade water.was mixed with 2µl of diluted cDNA. Reactions were performed in 0.1ml MicroAmp Fast Optical 96-well reaction plate. Plates were run on a StepOnePlus™ Real-Time qPCR machine ran at 95°C for 10 minutes, 95°C for 15 seconds, 60°C for 1 minute, 95°C for 15 seconds, 60°C for 1 minute, and 95°C for 15 seconds, with 40 cycles, including melt curve. Each sample for each target gene or virus was ran in duplicates. CT values were determined with a threshold for all runs set at 0.06 ΔRN for all samples and genes, and the duplicate values for each sample were averaged to be used in subsequent analyses. ΔCT values were calculated by subtracting the reference gene average CT from the average CT of the target gene, meaning the lower the ΔCT value, the higher relative expression.

Statistics

All statistics were performed with "R" (R Core Team 2017). Because assumptions of parametric tests were violated for most variable, non-parametric Kruskal-Wallis ANOVA, Chi-square tests, and logistic regressions were used. Otherwise, parametric tests were employed (see results for details).

CHAPTER III

RESULTS

Caste Experiment

For the analysis, only the first 8 hours of the original 3-day observation period were included because some cells were beginning to be capped at the time, which might confound the analysis of later time periods. The visitation rates were significantly different between worker and drones (Kruskal-Wallis X^2= 48.4, $p<0.001$). Drone larvae were visited 2.5 times more often than worker larvae based on median (Figure 1). The number of invaded drone cells was twice as high as invaded worker cells, although the difference not significant (figure 2) (X^2= 2.3, p=0.133).

Across drone and worker cells, visitation rate exhibited a significant positive effect on the probability of mite invasion (figure 3) (logistic regression: R^2=0.12, df=87, p=0.026). The full-factorial logistic regression simultaneously assessing caste, visitation rate, and their interaction (R^2=0.218) revealed a suggestive interaction effect (Z=-1.9, df=87, p=0.057), while visitation rate significantly influenced mite invasion probability (Z=2.1, p=0.033), and caste type did not (Z=1.8, p=0.073).

Starvation Experiment

Across both replicates, 110 total cells in the four treatment groups ($N_{Control}$=26, N_{Fed}=31, $N_{Starved}$=22, $N_{Starved/Fed}$=31) were successfully monitored for visitation rates, mite

invasion, and weight of resulting pupae. The treatment groups did not have significantly different visitation rates 0.685 (figure 4) (ANOVA F_3= 0.5, p=0.685) but differed significantly in wet weights (figure 5) (ANOVA F_3=10.4, p<0.001). The weight of the starved/fed group was significantly higher than the other three groups (starved/fed vs. control p< 0.001, starved/fed vs. fed p<0.001, starved/fed vs. starved p< 0.001).

The overall invasion of cells through the experiment was low, with only 8 cells invaded by *Varroa* (figure 6). The Starved/fed group had no cells invaded by *Varroa*. There were no significant differences in the mite presence between treatment groups (starved/fed vs. control X^2=1.9, df=1, p=0.166, starved vs. control X^2= .1.3e-31, df=1, p=1.00, fed vs. control X^2=0.19, df=1, p=0.663).

Our results did not indicate a significant influence of visitation rate on the probability of invasion of a brood cell during the starvation experiment (figure 7) (R^2= 0.00, Z= -0.06, df= 105, p=0.949). Weight did not significantly affect mite presence (figure 8) (R^2= 0.04, Z= -1.33, df=105, p=0.183).

The average relative expression levels of the two reference genes, Actin and RPS5, were stable across treatment groups (Actin: Chi^2=5.00, df=3, p=0.172, RPS5: Chi^2= 3.38, df=3, p=0.337), and therefore used to quantify relative levels of the targets genes and viruses. The average expression levels of the two reference genes were stable when compared to invaded and non-invaded cells (Actin: Z=1.4, df=46, p=0.158, RPS5: Z=0.96, df=46, p=0.336). The CT values of tested Actin and RPS5 were averaged to produce one more stable reference (figure 9). The average relative expression of the

combined reference genes was stable across treatment groups (table 9) (Chi2=3.76, df=3, p=0.288). The average relative expression of the combined reference genes was stable when comparing invaded and non-invaded cells (R^2=0.05, Z= 1.21, df=46, p=0.227).

Relative Toll-6 expression was increased for the fed, starved, and starved/fed treatment groups when compared to the control group (figure 10) while the fed, starved, and starved/fed groups were not significant from one another (Chi^2=16.10, df=3, p=0.001). Pairwise comparisons using Wilcoxon rank sum test showed the control group was significantly different than the fed (p=0.003) starved (p=0.004) and starved/fed (p=0.02) groups Toll-6 relative expression was not correlated with the chance of cell invasion (Z= -1.1, R^2= 0.05, df=46, p=0.288). Toll-6 relative expression and visitation rate were tested together as factors of cell invasion, and were both found to be not significant, overall R^2 =0.05, (Toll-6: Z = -1.1, df=46, p=0.290, visitation rate Z= 0.3, df=46, p=0.806).

Relative Defensin-2 expression was significantly different between treatment groups (figure 11) (Chi^2= 17.08, df=3, p<0.001). The control group was significantly different than the fed (p=0.002), starved (p-value=0.002), and starved/fed (p=0.012) groups, while the relative expression of Defensin-2 was not significantly different among the fed, starved, and starved/fed groups (starved vs. fed: p= 1.0, starved vs. starved/fed: p= 1.0, fed vs. starved/fed: p= 1.0). Defensin-2 was not correlated with a higher chance of cell invasion (Z= -0.83, R^2= 0.03 df=46, p=0.407). Defensin-2 and visitation rate were

tested together as factors of cell invasion, and were both not significant, with an R^2= 0.03, Defensin-2 (Z= -0.8, df=46, p=0.410), visitation rate: Z= 0.28, df= 46, p=0.778).

Relative Dicer-Like expression was significantly different between treatment groups (figure 12) (Chi^2= 13.71, df=3, p=0.002). The control group was significantly different from the starved (p=0.012) and starved/fed (p=0.043) groups but was not significant from the fed group (p= 0.199). The starved group was not significant from the fed (p= 0.274) and starved/fed (p=1.0) groups, and the fed and starved/fed groups were not significantly different (p=1.0) (figure 13). Dicer-Like was correlated with cell invasion, (R^2=0.19, Z=-2.04, df=46, p=0.041). Dicer-Like and visitation rate were tested together as factors of cell invasion, with Dicer-Like being significant (Z= -1.96, df=46, p=0.049), and visitation rate not being significant (, Z= -0.12, df=46, p= 0.902).

The DWV-A titers were significantly different between treatment groups (figure 13) (Chi^2=10.43, df=3, p=0.015). The starved/fed group was significantly different than the fed group (p= 0.034), and near significantly different than the starved group (p=0.059). The control group was not significantly different than the fed (p=0.410), starved (p=1.0), or the starved/fed (p= 0.180) groups. The starved and fed groups were not significantly different from one another (p=1.0). DWV-A titers were correlated with cell invasion (Z= -2.8, R^2= 0.49, df=46, p=0.005). DWV-A titers and visitation rate were tested together as factors and DWV-A titers were correlated (Z= -2.66, df= 46, p=0.008),

but visitation rate was not (Z= 0.71, df=46, p=0.478). The overall R^2=0.50. We also tested for DWV-B but saw no expression in any samples.

The relative expression of Vitellogenin was significantly different between treatment groups (Figure 14) (Chi^2= 17.01, df=3, p<0.001). The control group was significantly different than the fed (p= 0.002), starved (p=0.002), and starved/fed (p= 0.016) groups. Vitellogenin relative expression was not correlated with cell invasion (R^2= 0.02, Z= -0.69, df=46, p=0.494). Vitellogenin relative expression and visitation rate were tested together as factors of cell invasion and were found not significant (R^2=0.02), (Vitellogenin: Z=-0.69, df=46, p=0.490), (visitation rate: Z= 0.31, df=46, p=0.756).

CHAPTER IV

DISCUSSION

My data support my hypothesis that visitation rate is a factor in *V. destructor* host selection. My data showed that the natural differences in visitation rates range enough to influence the chance of cell invasion by *V. destructor*, confirming previous suggestions about visitation rates (Boot *et al.* 1992, Calderone and Kuenen 2003, Calderone and Lin 2001, Rosenkranz *et al.* 2010, Diller *et al.* 2006). This could mean that *V. destructor* cell invasion depends on the exposure to the larval cells, and cells that *V. destructor* are exposed to more have a higher chance of cell invasion. If this is the case, and *V. destructor* does not depend solely on chemical signals, in-hive traps may not be able to realistically work correctly or may only work when no brood is present in the hive. Mites do not move around the hive on their own, so in-hive traps would require nurse bees to visit them very often for them to be effective (Boot *et al.* 1994).

My study shows that drone larvae are more visited than worker larvae by adult nurse bees, with drone larvae being visited 2.5x more frequently than worker larvae. My study results are similar to those of Calderone and Kuenen (2003), which showed drone larvae had higher visitation frequency of 2.78 by nurse bees than worker larvae. Their study focused on 12 total cells, and broke nurse bee visits down into four acts, while our study had 80-100 cells per replicate and used scan sampling which may explain the small differences in visitation rates.

One possible reason for increased visits to drone larvae could be the size of the larvae, and consequently the amount of food the larvae need and signal for. The distance between larva and cell opening has been shown to be a factor in cell invasion, and it is possible that the larger size of drone larvae makes this distance small enough to elicit invasion (Goetz and Koeniger 1993). Because this distance is smaller, the signal strength the larvae are sending to the nurse bees may be stronger, thus resulting in more visits to those cells. It is thought that the larvae send signals to the nurse bees, telling the nurse bees to come to the cell and feed the larva (Huang and Otis 1991a). Inspection visits are non-random and most likely determined by nurse bee detecting signals from larval cells (Huang and Otis 1991b). Of the overall inspection visits, a percentage are also feeding visits, when nurse bees determine that the larval cell is in need of food. It is clear that nurse bee visits are non-random, and that drone larvae are being tended at a higher rate than worker larvae. Past studies have defined feeding visits as nurse bees inserting its head and thorax inside a larval cell for more than 10 seconds, and inspection visits as nurse bees inserting itself for less than 10 seconds (Huang and Otis 1991a). Differentiating between inspection and feeding visits in future studies could explain if only one of these types of visits influences cell invasion, or if increases in both feeding and inspection visits lead to higher chance of cell invasion.

My results followed the overall pattern of drone cells being invaded more than worker cells, however the analysis found that neither caste type had a higher chance of cell invasion. This is surprising since drone cells are invaded by *V. destructor* up to 11 times more frequently than worker cells, even to the point of beekeepers using drone

larval frames to remove *V. destructor* from hives (Boot *et al.* 1995, Fuch 1990). It is possible that the insignificant data was caused by the overall low invasion rate. This low invasion rate we noted through both our caste and starvation experiment could have been caused by the high amount of disturbance that the experiments required. Future studies of similar nature with modifications are needed to reduce the overall disturbance. My drone and worker invasion data could also be explained by cell invasion periods. Worker larval cells are invaded 15-20 hours prior to capping, while drone larval cells are invaded 40-50 hours prior to capping (Boot *et al.* 1992). The invasion period, which was determined by the number of invading mites present at the bottom of larval cells over 60 hours, was two to three times longer for drones than workers (Boot *et al*.1992). Boot *et al.* (1992) also showed the rate of invasion was constant over the invasion period, but drone brood invasion decreased when approaching cell capping. My experiment inadvertently effected the cell invasion periods. It was noted in that drone cells were beginning to be caused as early as day two. This means that *V. destructor* in the observation hive had about 48 and 72 hours to invade drone and worker cells, respectively. My experiment reduced the natural invasion period for both castes. Since mites had a longer period to invade worker larval cells than drone larval cells, this could explain the surprisingly insignificant difference in caste cell invasion.

Data show that *V. destructor* reacted similarly to both worker and drone cuticle extracts, implying *V. destructor* does not use these cuticle compounds to detect the different larvae castes (Calderone and Lin 2001). It is possible that our study gives support to the later idea, that *V. destructor* uses cuticle cues to detect if the cell contains a

certain aged larva, but the cells that mites ultimately invade is based on which cells mites have more exposure.

Contrary to my third prediction, experimental starvation and feeding of larvae did not alter subsequent nurse bee the visitation rates. In the second experiment, I expected to see significantly different visitation rates of the four treatment groups, with starved larvae increasing signaling to nurse bees, resulting in higher visitation rates, and fed larvae decreasing signaling, resulting in lower visitation rates, and the control and starved/fed having similar visitation rates. However, my data showed that all four treatment groups had similar visitation rates. My data contradicts data from Huang and Otis (1991a), who found that starved larvae had both increased inspection and feeding visits from nurse bees. Differences in sample size and/or visitation rate measuring could account explain contradictory results.

Visitation rate was not shown to have an effect of cell invasion. This could mean that, while there could still be variation in visitation rates within groups, variation was not sufficient to influence cell invasion. The visitation rates recorded in the caste experiment ranged from 1 to 20, while the visitation rates in the starvation experiment ranged from 3 to 19. This slightly smaller range in visitation rates along with smaller samples sizes could account for the lack of cell invasion significance. For further studies of the relationship between nurse bee visitation rates and *V. destructor* invasion, alternative methods to manipulate visitation rates are needed. I also tested the nutritional stress of starving/feeding as a factor of cell invasion. No treatment group had a higher chance of

cell invasion than other. This could mean nutritional stress does not increase the chance of cell invasion by *V. destructor*.

The average weight of the starved/fed larvae were significantly higher compared to the other three groups. I expected the visitation rates and weight of treatment groups to follow the same pattern. Since the visitation rates were not significantly different between treatment groups, we expected the weights to also not be significant. The starved/fed group could have over-compensated because of the starving period. Larvae starved during 5^{th} instar have higher glucose storage when faced with the same stressor as adults (Wang *et al.* 2016, Wang *et al.* 2015). The compensation of the starved/fed group could have led to more consumption of the extra food and caused them to be heavier, while the fed group may not have eaten all food given.

Weight did not have influence on cell invasion. The starved/fed group, despite having significantly higher weights than the other three treatment groups, did not have a higher or lower chance of cell invasion by *V. destructor*. This could mean that within castes, higher weight larvae do not have a higher chance of cell invasion than lower weight larvae. Since drone larvae weigh more than worker larvae, it has been suggested that this is one of the factors for increased drone cell invasion. Based on our data, weight and/or body size may not be a factor of *V. destructor* cell invasion. However, my data suggests weight is not a factor in cell invasion, further supporting my hypothesis.

The power to assess influence of visitation rates on cell invasion was limited and therefore I studied further factors that could influence *V. destructor* host selection in spite of my hypothesis. I expected the reference gene expression levels to remain constant

throughout all treatment groups, which is what the data showed. This means that the RNA extraction, cDNA synthesis, and qPCR were completed correctly. If the reference genes had been significantly different from one another, this could have meant the samples had different concentrations of RNA, which would have misled the possible differences in relative expression. I also tested whether the average CT of the reference gene influenced cell invasion and saw no effect.

For the three immune genes that we studied Toll-6, Defensin-2, and Dicer-Like, there was relative expression difference between the control group and some or all of the experimental groups. My data argues that increase in expression of immune genes is likely because the larvae were being stressed. The fed, starved, and starved/fed groups all had increased relative expression of Toll-6 and Defensin-2 compared to the control group. The starved and starved/fed groups both had increased relative expression of Dicer-Like compared to the control group. All three starved and/or fed treatment groups had increased expression of at least 2 of the immune genes when compared to the control group, which means all three groups were stressed by our food manipulation. This was not what was expected, since we predicted the fed group would not be stressed by an extra feeding and would not have increased immune gene expression. I concluded that both the feeding and starving that we used during our starvation experiment resulted in stressed larvae. The lack of differences in invasion probability among groups could mean that nutritional stress does not make larvae more attractive to *V. destructor*. DWV has been shown to have an immunosuppressive effect on larvae, benefitting *V. destructor* (Prisco *et al.* 2016). Based on this, nutritional stress, leading to an upregulation of

immunity would have a deterrent effect on *V. destructor*. To study more directly the relation between the expression of our target genes and mite invasion, I compared immune gene expression between larvae of invaded and non-invaded cells. The only immune gene that was significantly different was Dicer-Like, which was up-regulated. I tested the larvae for the expression of the hormone Vitellogenin, because it is associated with immune expression. Based on previous studies (Bordier *et al.* 2017, Koleoglu *et al.* 2017) I predicted that with increasing stress, the Vitellogenin expression would decrease. The results showed the opposite, with larvae with increasing stress showing increasing Vitellogenin expression. Bordier *et al.* (2017) showed decrease in Vitellogenin expression after exposure to immune challenges but increases in Vitellogenin expression when exposed to heat challenges. Koleoglu *et al.* (2017) showed decreases in Vitellogenin expression of adult bees when exposed to *V. destructor*, but no significant difference of Vitellogenin expression of brood exposed to *V. destructor*. Vitellogenin expression may vary on the type of stressor and life stage of the bee at the time of exposure. Vitellogenin was not associated with a higher chance of cell invasion.

Since *V. destructor* and DWV have a symbiotic relationship, we decided to test two strains of the virus, A and B. DWV-A is known to be more prevalent than DWV-B (Brettell *et al.* 2017). We did not find any DWV-B in any samples, presumably because DWV-B is not as prevalent as DWV-A (Brettell *et al.* 2017). I found DWV-A in all groups except the control group. The virus titers of DWV-A were significantly higher in the starved/fed group compared to the other three groups. DWV had evolved along with *A. mellifera* and is found at low levels within the colony (Wilfert *et al.* 2016). I predicted

that levels of DWV would increase as larvae were stressed, because the normally low virus titers would be able to increase, and we did see that nutritional stress led to increase in DWV titers. I compared the DWV-A virus titers of larvae of invaded and non-invaded cells. Virus titers of larvae of invaded cells were significantly higher than larvae of non-invaded cells, which is not surprising since DWV is associated with *V. destructor*. This could also mean larvae with higher levels of DWV are selected more by *V. destructor* or that *V. destructor* increased DWV titers.

CHAPTER V

CONCLUSION

Varroa destructor is one of the biggest problems honey bees and their keepers are currently facing. Many studies have been conducted focusing on the chemical choice hypothesis, which states that *V. destructor* primarily uses chemical cues from larval cuticles to choose a host for its reproductive phase. However, the literature lacks data on *V. destructor* behavior within the natural hive settings, and the influence that physical factors could have on *V. destructor* cell invasion. This study focused on the influence of nurse bee visitation rates, and the hypothesis that these visits to larval cells is the main factor of *V. destructor* cell invasion. I found that with increasing visitation rate, there was an increasing chance of cell invasion. This experiment is the first to show that nurse bees are indirectly involved in *V. destructor* cell invasion, which has been inferred many times in the literature (Boot *et al.* 1992, Calderone and Kuenen 2003, Calderone and Lin 2001, Rosenkranz *et al.* 2010, Diller *et al.* 2006). Neither caste type or weight were a factor in cell invasion, contrary to what was expected. The molecular analysis of the starvation experiment did not support our prediction that immune genes and virus titers alone are not associated with cell invasion. Both the immune gene Dicer-Like and DWV-A were associated with larval cell invasion, alone and with the factor of visitation rate. It is possible that both of these factors were associated with cell invasion because DWV-A could have been spread by the mites, and Dicer-Like is an RNAi-component and may

have been elevated because of the increase in virus. Through this study, we have learned how *V. destructor* acts within natural hive environments. This study could mean the ideal method of control fo*r V. destructor* would need to not only act as an attractant for the mites, but also attractant nurse bees, which act as the main transportation for *V. destructor*.

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APPENDIX A

TABLES AND FIGURES

Table 1. Table of Primers

Table is showing the forward and reverse primers used for the targets genes and virus strains.

Target	Forward Primer	Reverse Primer
Actin	CCTAGCACCATCCACCATGAA	GAAGCAAGAATTGACCCACCAA
RPS5	AATTATTTGGTCGCTGGAATTG	TAACGTCCAGCAGAATGTGGTA
Toll-6	TCCGAGGCGTCAACAGGAATCGACC	GACAGGTCGAACGTCTCCAG
Defensin-2	GCAACTACCGCCTTTACGTC	GGGTAACGTGCGCGTTTTA
Dicer-Like	CCAACAGGAGCTGGAAAAAC	TCTCCACTAAGTGCTGCACAA
DWV-A	TACTAGTGCTGGTTTTCCTTT	CTCATTAACTGTGTCGTTGAT
DWV-B	TACTAGTGCTGGTTTTCCTTT	CTCATTAACTGAGTTGTTGTC
Vitellogenin	GTTGGAGAGCAACATGCAGA	TCGATCCATTCCTTGATGGT

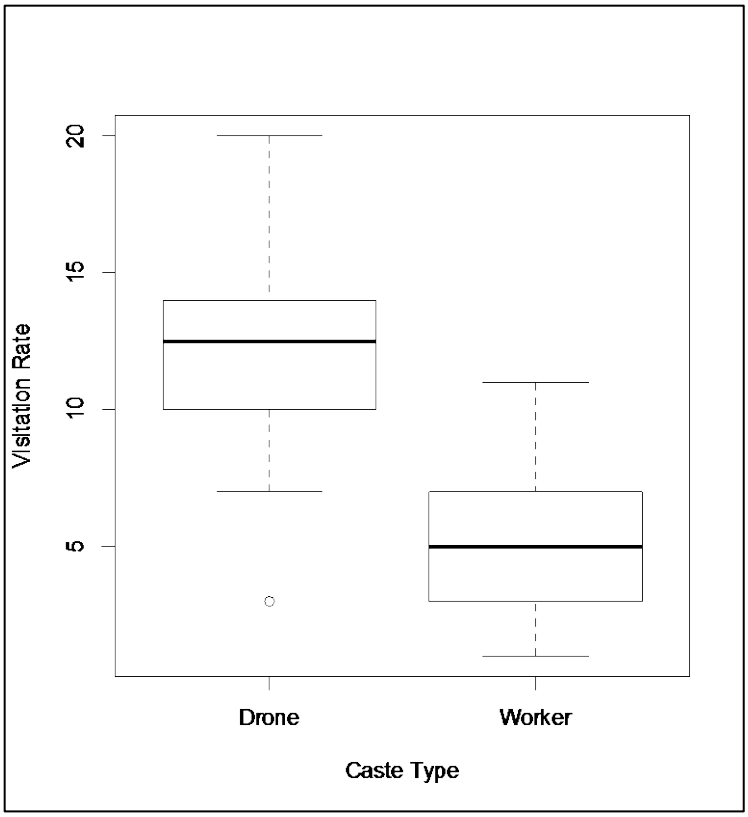

Figure 1. Differences in Visitation Rate between Drone and Worker Castes
Differences in visitation rates (defined as number of nurse bee visits per cell) of drone and worker larvae (median ± quartiles) over an 8-hour observation period. Drone larvae had significantly higher visitation rates than worker larvae (Kruskal-Wallis, $p<0.001$).

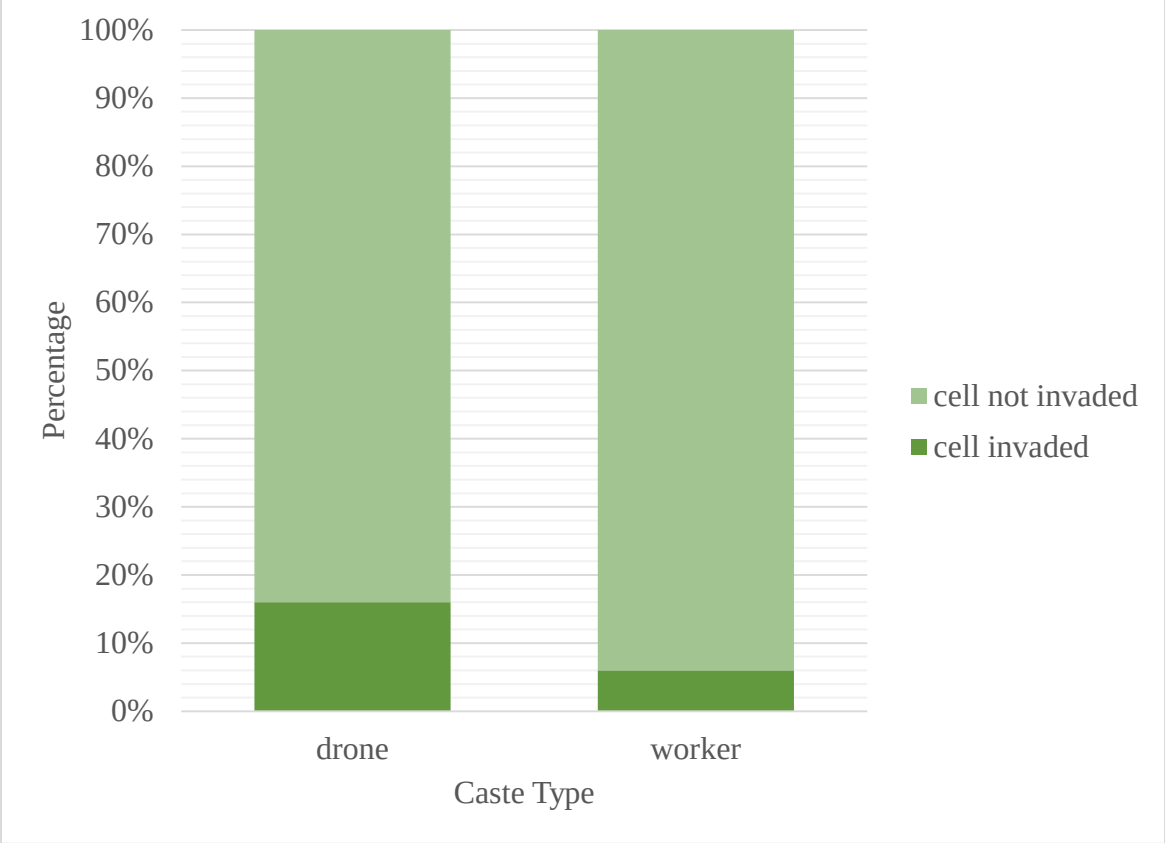

Figure 2. Mite Invasion of Drone and Worker Cells
Percentage of invaded and not invaded by one or more *Varroa* for drone and worker larvae. Drone larvae had higher percentage of cells invaded but not a significant difference based on Chi^2 test ($p=0.133$).

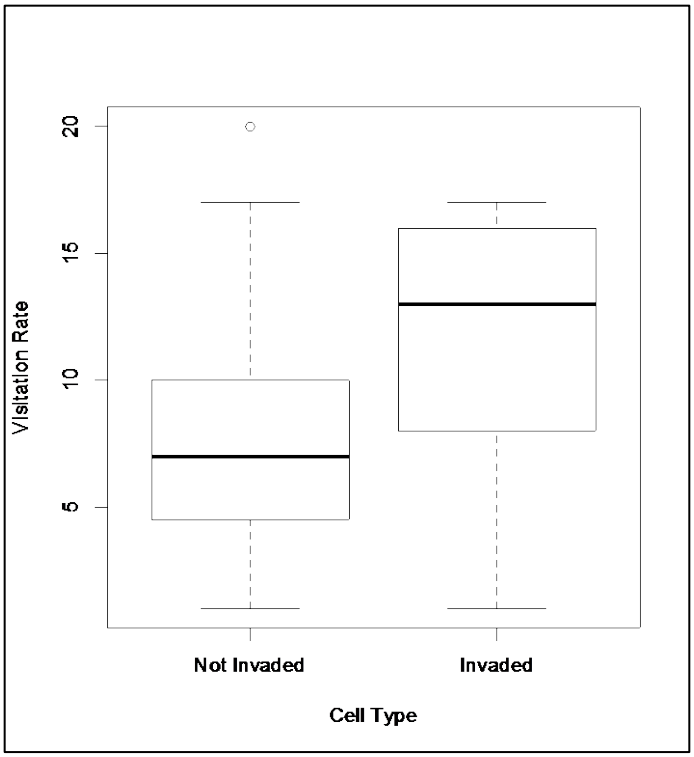

Figure 3. Visitation Rate and Cell Invasion
Average visitation rate of cells that contained a mite compared to the average visitation rate of cells that did not contain a mite revealed the relation between the two variables, which was statistically tested by logistic regression of mite invasion on visitation rate ($p=0.026$).

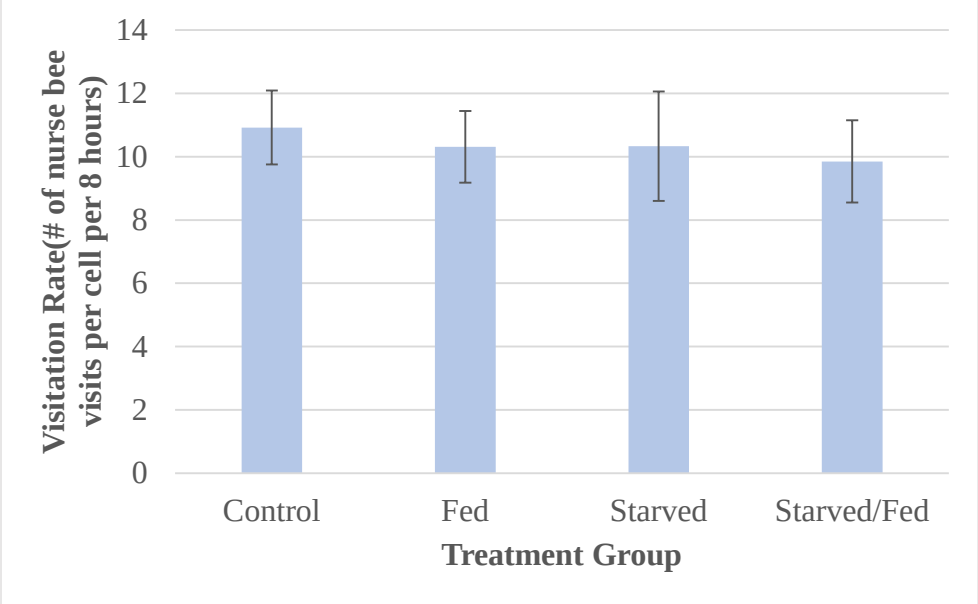

Figure 4. Treatment Groups and Visitation Rates
The visitation rates (defined as the number of nurse bee visits per cell) of the treatment groups of the starvation experiment (average ± 95% confidence interval). Visitations rates were not significantly different between treatment groups using ANOVA (p=0.685). Treatment groups: control, fed, starved, and starved/fed.

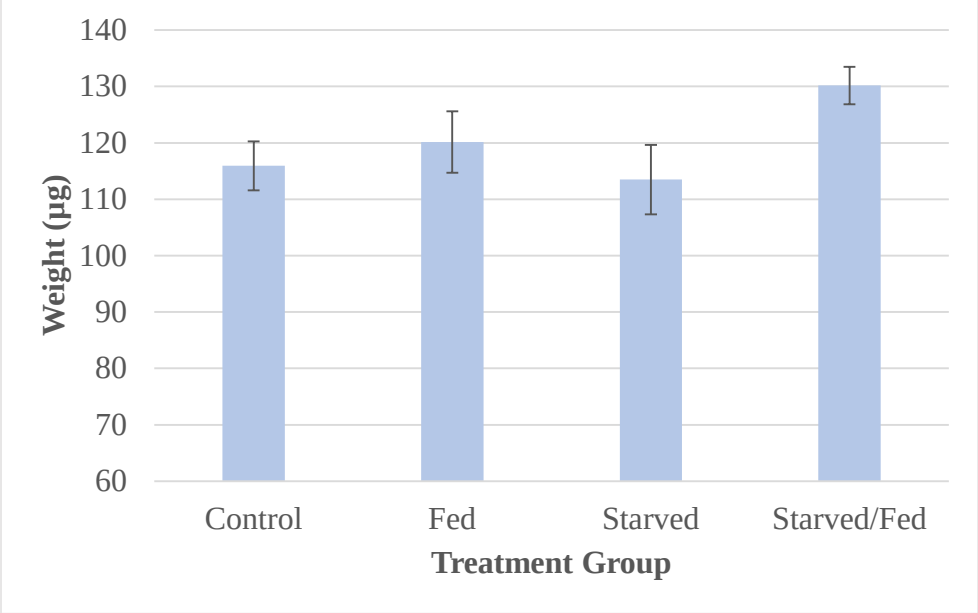

Figure 5. Weight and Treatment Groups
The wet weight (average ± 95% confidence interval) of the treatment groups of the starvation experiment. Treatment groups: control, fed, starved, and starved/fed. Weight of treatment groups was significantly different using ANOVA ($p<0.001$). Post-Hoc Tukey test showed starved/fed group had significantly higher weight than control ($p<0.001$), fed ($p<0.001$), and starved ($p<0.001$) groups. Error bars showing 95% confidence intervals.

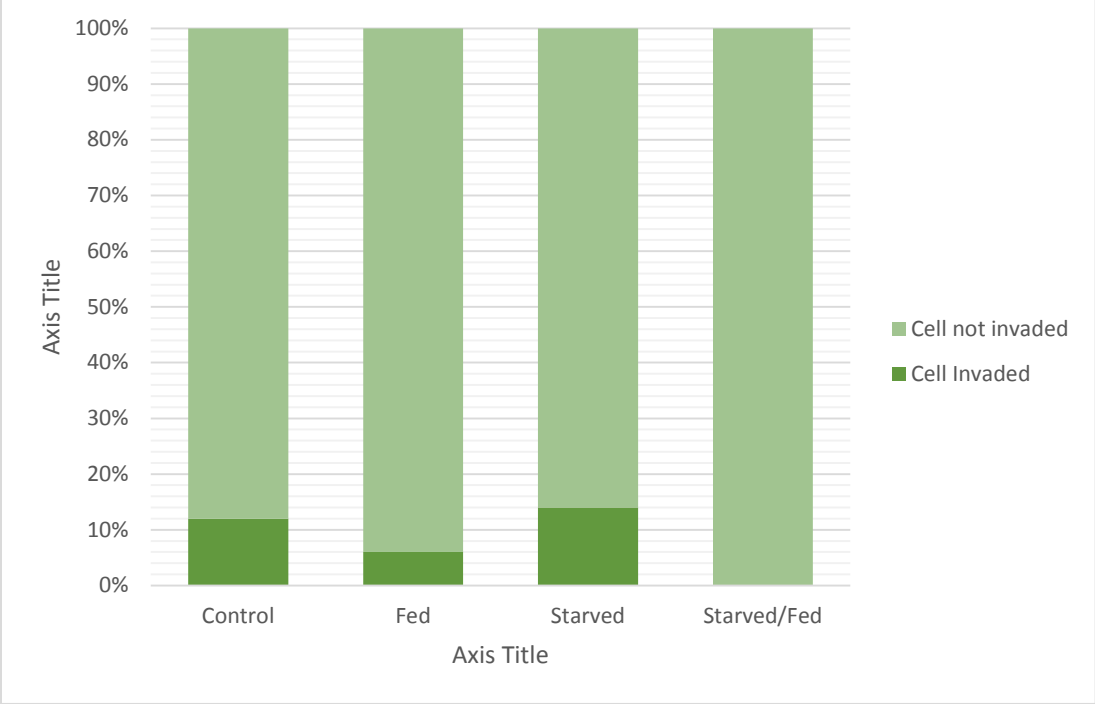

Figure 6. Treatment Group and Cell Invasion
Percentage and total number of cells invaded for each treatment group (starved, fed, starved/fed, and control). The starved, fed, and starved/fed groups were tested against the control using a Chi^2 test, and none were significantly different (starved: p=100, fed: p=0.663, starved/fed: p=0.166).

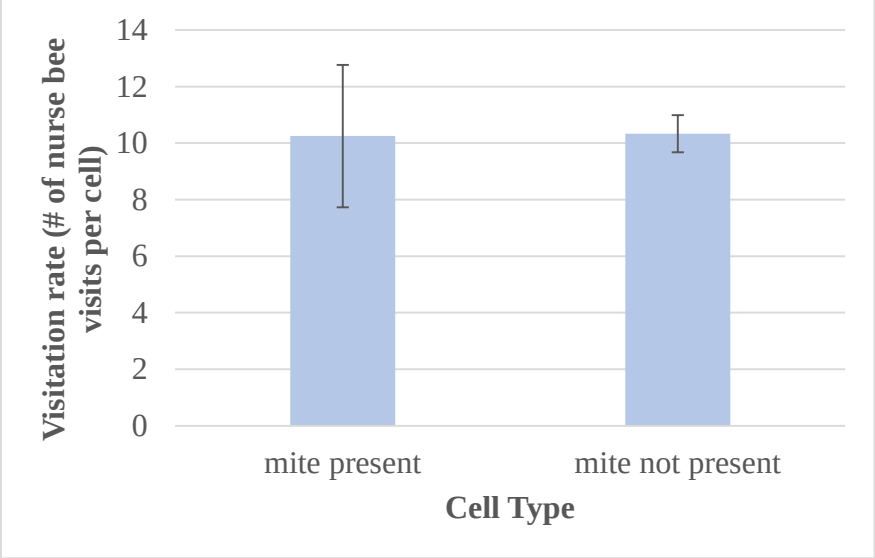

Figure 7. Visitation Rate and Mite Presence
The average visitation rate of cells invaded and not invaded by mites of the starvation experiment revealed that there was not a relation between the two variables, which was statistically tested by logistic regression of mite invasion on visitation rate (p=0.949). These results differed from the caste experiment. Error bars showing 95% confidence intervals.

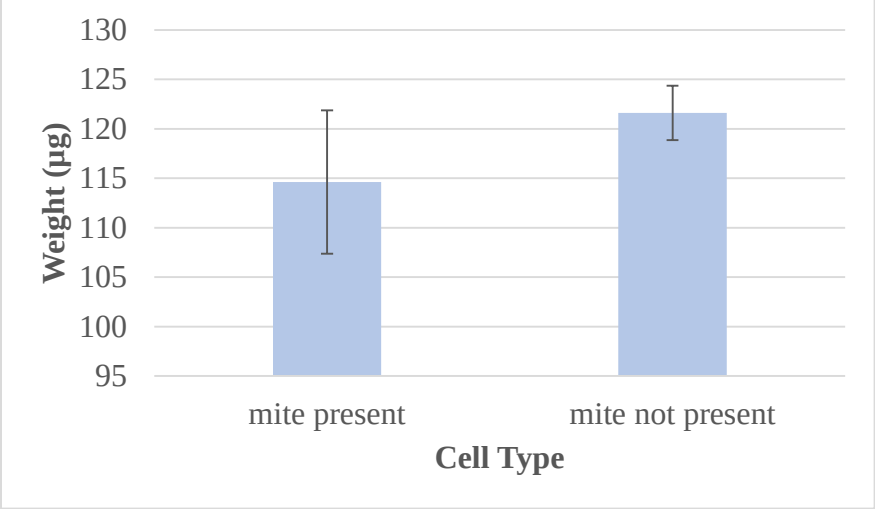

Figure 8. Weight and Cell Invasion
Average weight of cells that contained a mite compared to the average visitation rate of cells that did not contain a mite revealed that there was not a relation between the two variables, which was statistically tested by logistic regression of mite invasion on weight ($p = 0.183$) Error bars showing 95% confidence intervals.

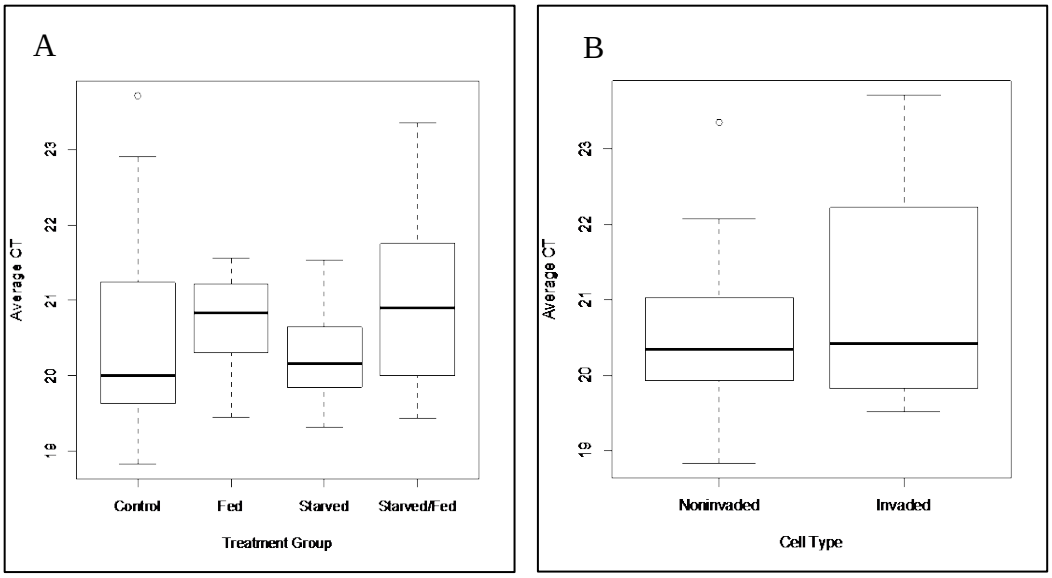

Figure 9. Differences of Combined Reference Gene across Treatment Groups and Cell Type

(A) Median CT values of combined reference gene of treatment groups. Kruskal-Wallis showed no significant difference between treatment groups (p=0.288). (B) Median CT of combined reference gene of invaded cells compared to median CT of combined reference gene of non-invaded cells revealed no relation between the two variables, which was statistically tested by logistic regression of reference gene CT on mite invasion (p=0.227).

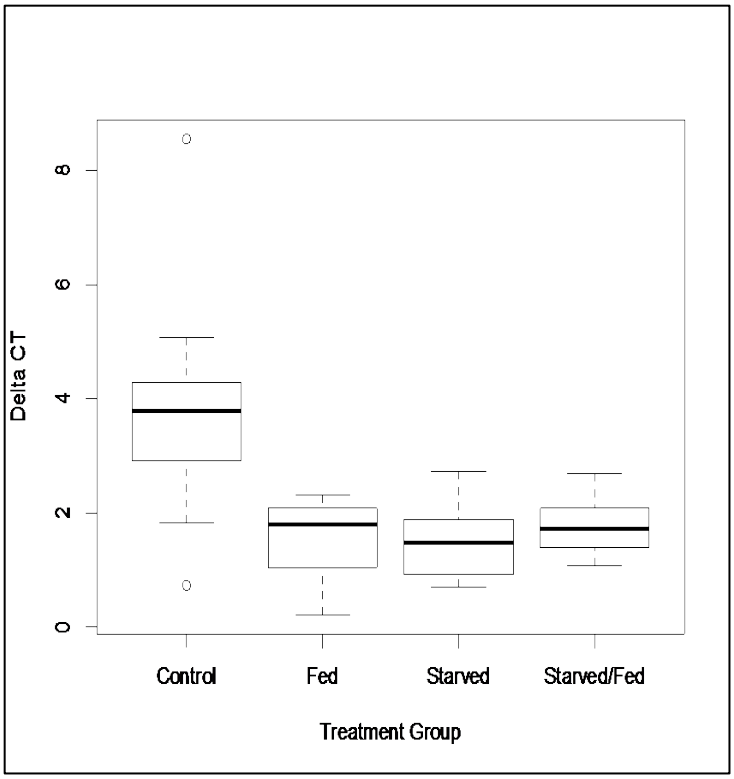

Figure 10. Toll-6 Relative Expression across Treatment Groups
Median ΔCT values of Toll-6 of treatment groups. Kruskal-Wallis showed significant differences between treatment groups (p=0.001). Post-Hoc Tukey test showed control group was significantly higher than fed (p=0.003), starved (p=0.004), and starved/fed (p=0.02) groups. The lower the ΔCT value, the higher relative expression of Toll-6 gene.

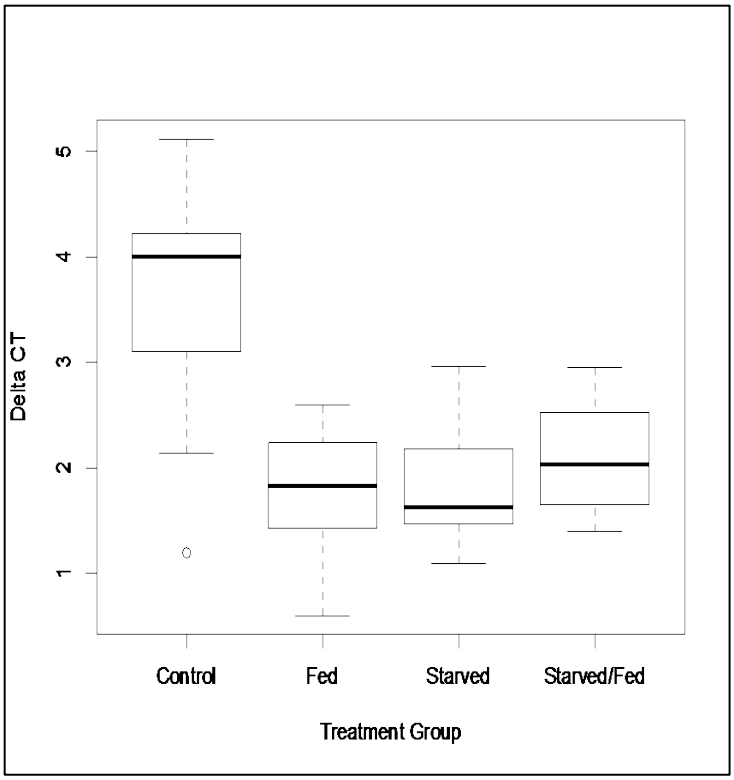

Figure 11. Defensin-2 Relative Expression across Treatment Groups
Median ΔCT values of Defensin-2 gene of treatment groups. Kruskal-Wallis showed significant difference between treatment groups (p=<0.001). Post-Hoc Tukey test showed control group was significantly higher than fed (p=0.002), starved (p=0.002), and starved/fed (p=0.012) groups.

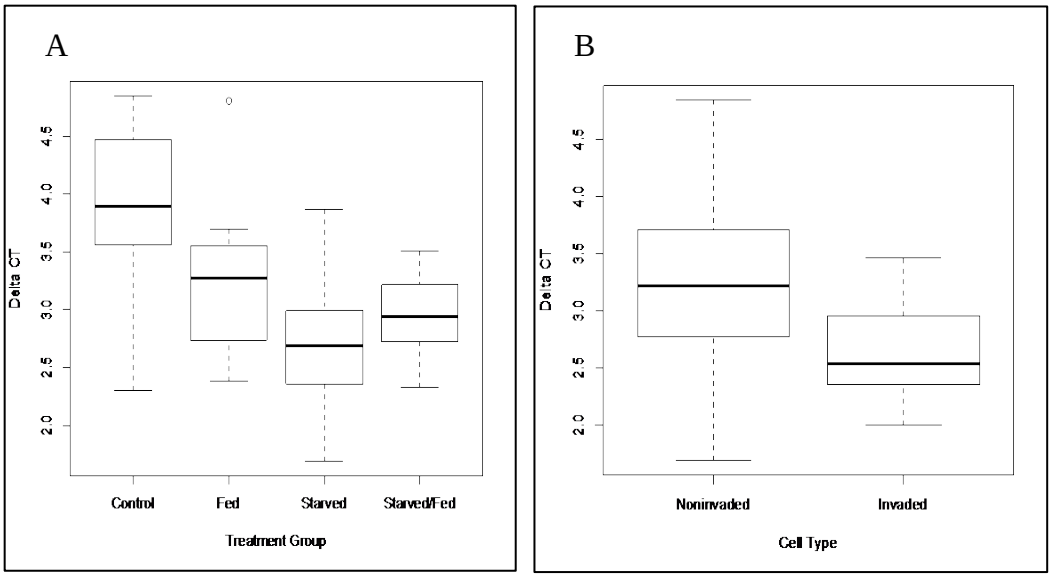

Figure 12. Dicer-Like Relative Expression across Treatment Groups and Cell Type
Median ΔCT values of Dicer-Like gene of treatment groups. Kruskal-Wallis showed significant difference between treatment groups (p=0.003). Post-Hoc Tukey test showed control group was significantly higher than starved (p=0.012) and starved/fed (p=0.043) groups.

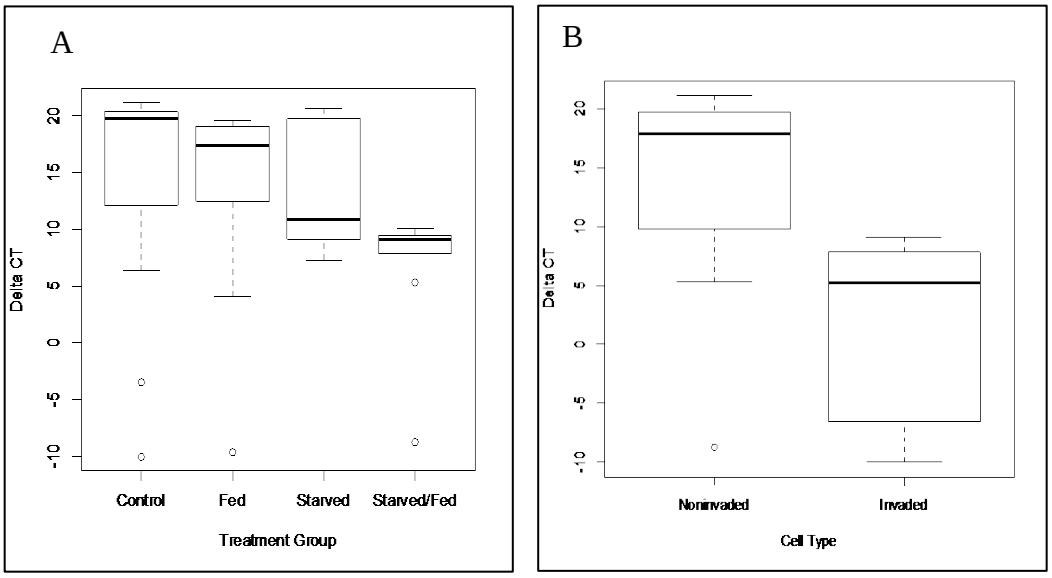

Figure 13. DWV-A Relative Titer Level across Treatment Group and Cell Type
(A) Median ΔCT values of DWV-A of treatment groups. Kruskal-Wallis showed significant differences between treatment groups (p=0.015). Post-Hoc Tukey test showed the starved/fed group was significantly different than the fed group (p=0.034), and nearly significantly different than the starved group (p=0.059). (B) Median ΔCT of DWV-A of invaded cells compared to median ΔCT DWV-A of non-invaded cells revealed there is a relation between the two variables, which was statistically tested by logistic regression of mite invasion on DWV-A relative expression (p=0.005).

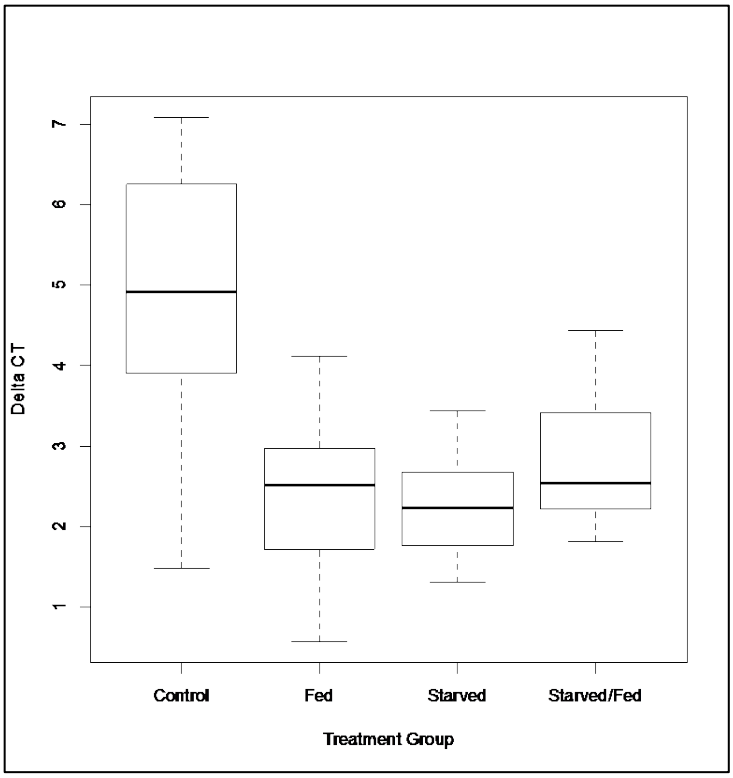

Figure 14. Vitellogenin Relative Expression across Treatment Groups
Median ΔCT values of Vitellogenin of treatment groups. Kruskal-Wallis showed significant differences between treatment groups ($p<0.001$). Post-Hoc Tukey test showed control group was significantly different than the fed ($p=0.002$), starved ($p=0.002$) and starved/fed ($p=0.016$) groups.