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The parasitic mite *Varroa destructor* is one of the biggest health problems of the Western Honey Bee, *Apis mellifera*. It feeds from the bees' hemolymph and vectors several honey bee pathogens. *V. destructor* has also been reported to compromise honey bee immunity but available data are insufficient to support this claim. This study was designed to assess the effect of mite infestation on honey bee immune-gene expression during the biologically relevant host developmental stages.

In my experiment, mites were manually introduced into honey bee larval cells at three different levels. Control groups were either left unmanipulated or wounded. Developing bees were collected with any retrievable mites daily from the experimental cells for ten days. Mite reproduction was assessed and bee hosts were analyzed for expression levels of ten immune genes using quantitative RT-PCR.

This experiment showed effects of developmental time and experimental treatment on gene expression that generally contradict the previously hypothesized immunosuppression of bees by *V. destructor*. However, mites might temporarily suppress the honey bees' normal response to cuticle wounding based on reproductive timing. The artificial wounding group exhibited an increased viral load, suggesting that wounding may trigger or enable virus replication. Overall, my results indicate the importance of physical trauma caused by wounding and suggest complex temporal dynamics in the relationships between bee host, mite parasite, and vectored pathogens.

EXPRESSION LEVELS OF IMMUNE-GENES IN DEVELOPING WORKERS OF *APIS MELLIFERA* IN RESPONSE TO REPRODUCTIVE TIMING AND INFESTATION LEVEL OF THE PARASITIC MITE *VARROA DESTRUCTOR*

by

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APPROVAL PAGE

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CHAPTER I

INTRODUCTION

Concerns over honey bee (*Apis mellifera*) health have triggered increased research on the parasitic mite, *Varroa destructor*, as a potential candidate for the worldwide decline in bee colony numbers. Honey bees rely on group-level defenses and individual immunity to combat pathogens and parasites. Once mites have overcome group-level defenses, the individual defense of bee hosts becomes important. Common patterns of cellular and humoral immune-gene responses across invertebrates provide a framework for understanding honey bee responses. Based on this knowledge, and the hypothesis that *Varroa* may manipulate individual host immunity, investigations into the bee-mite relationship are necessary at the molecular level.

Immunity and Immune Function

Every organism that faces fitness costs by direct interaction with another organism will require certain defenses. A primary defense for dealing with parasites is seen in the form of removal behavior, prophylaxis, or external morphological barriers, all attempting to prevent the entry of foreign molecules into an organism (Schmid-Hempel 2005). In an organism the purpose of immunity is to prevent damage by an invading or non-self agent once it has breached initial defenses (Playfair and Bancroft 2004). The main functions of immunity are to recognize invading agents as non-self, communicate

the threat to other necessary components, and to destroy or dispose of the elements with as little damage to the host as possible (Playfair and Bancroft 2004). These responses exist at the molecular level and respond specifically to antagonistic molecules.

Components of Parasite Avoidance and Coevolution

Although life is diverse, the core processes of immunity remain conserved across many multicellular organisms. The first step in immunity is to actually detect the foreign particle and determine whether or not it is a threat, as benign and even beneficial microflora exist (Playfair and Bancroft 2004, Bowman and Hultmark 1987). Circulating proteins that are expressed constitutively and exist free-floating in the circulation system or attached to cell surfaces called pathogen recognition receptors (PRRs) perform this vital task (Medzhikov and Janeway1997). PRRs bind to structures that are common to many groups of pathogens such as components of bacterial cell walls and double-stranded RNA produced by viruses. A wide variety of these recognition proteins exist to detect many types of threats, and upon recognition will initiate a series of subsequent reactions that reaches the nuclear DNA and expresses a pathogen-specific response to destroy the invading bodies. This form of immunity is known as innate immunity (Medzhikov and Janeway 1997). In vertebrates there is a form of immunity often referred to as adaptive immunity, which can produce novel recognition molecules once a foreign body is detected. These molecules are remembered by the immune system, but are also energetically costly to produce. Additionally, this adaptive system initially relies on the innate immune response for pathogen detection prior to communication with

specialized cell types such as T and B cells. As a result of this hierarchy, the innate response is the first line in defense and is highly conserved (Medzhikov and Janeway 1997).

Parasitism is not a new feature of life, therefore approaches to handling foreign molecules are rooted deep in the evolutionary tree and common features can be found in many of the major branches of life (Schmid-Hempel 2005). Innate immunity is an ancient feature that is found in vertebrates, invertebrates, and potentially even in plants. A form of the response pathway known as the nuclear factor kappa-B (NFκB) path is present in humans as well as the fruit fly *Drosophila* (Medzhikov and Janeway 1997). Just as a host organism's immune system is molded over generations to handle dangerous pathogens, the pathogens themselves are also undergoing selection to overcome host defenses. Pathogens can evade host recognition in a variety of ways including, among others, blocking PRRs, mimicking host molecules, producing protective barriers, or even suppressing or attacking factors of the host immune system (Playfair and Bancroft 2004).

Insect Immunity

There are common themes of immune response throughout life, but within the animal kingdom, the most successful class in numbers, Insecta, has a relatively simple system of response when compared to the more familiar vertebrate immune system. This is due to a trade-off associated with high reproductive rates and short lifespan. Insects

benefit from fast immune-gene defenses expressed in RNA and protein structures rather than the costly proliferation of specialized cell types found in vertebrates (Boman and Hultmark 1987). The non-specific, innate system includes cellular responses in insects that are very similar to those in vertebrates and other organisms. Foreign particles are recognized and either encapsulated or phagocytized by specialized hemocytes called granulocytes and plasmatocytes (Wilson-Rich et al. 2009, Boman and Hultmark 1987). Plasmatocytes form nodules around large regions to stop invading cells (Boman and Hultmark 1987). This process is similar to coagulation in vertebrates.

The non-cellular, or humoral, responses to microbial infection set insects apart, as insects are not believed to have adaptive immunity and yet they are able to survive in an impressive range of habitats (Boman and Hultmark 1987). This humoral response is comparable only to the early, innate responses in vertebrates (Christophides et al. 2004). Most induced responses in insects are secreted from the fat body in response to specific foreign molecules, while acute responses consist of preemptively expressed immune factors already circulating in the hemolymph (Christophides et al. 2004). For a response to occur, pathogenic molecules must first be recognized. Bacterial structures such as lipopolysaccharides and peptidoglycan are common triggers of acute PRRs that are found in the hemolymph or bound to cell membranes (Christophides et al. 2004). These communicate with serine proteases which modulate a cascade of many types of responses including melanization by prophenoloxidases to synthesis of antimicrobial peptides (AMPs). AMPs are understood to be broadly expressed in response to groups of

pathogens. For example, recognition of fungal or Gram-positive bacteria activate the Toll pathway, which is responsible for a certain set of AMPs whereas the immune deficiency (Imd) pathway responds to Gram-negative bacteria (Christophides et al. 2004). The mechanism of regulating such induced responses is not known in depth but the presence of AMP isoforms suggests that protein responses can be modulated to respond specifically to various threats (Schmid-Hempel 2005).

Social Insects Immunity

Social insects have some advantages in defense, which have made their immune response somewhat different than in solitary insects. A specific type of sociality, called eusociality, consists of differing reproductive castes working cooperatively within a single colony. One of the defining features of most eusocial insects is a centralized nest or hive structure that allows for raising large amounts of brood and storing foods in a protected environment (Wilson-Rich et al. 2009). The potential for introduction of disease is often reduced by a division of labor within workers that includes foragers that seek food sources free of parasites and guards that physically stop outside invaders, or diseased members of the colony, from entering the nest (Cremer et al. 2007). Within the nest, castes specialized in cleaning will often line the hive with various substances, either secreted or collected from plant compounds, containing antimicrobial properties. Cleaning tasks can also include grooming to detect ectoparasites and removal of diseased colony members, an emergent pattern that mimics tasks performed by specialized

immune cells (Wilson-Rich et al. 2009, Cremer and Sixt 2008). Some social insects will tend closely to their brood, and provide it with food sources rich in antimicrobial compounds (Wilson-Rich et al. 2009).

Social Insects Susceptibility

The evolution of eusociality and its benefits at the colony level could have a detrimental effect on individual immunity and trade-offs exist in immune response. The evolution of sociality in insects provides many beneficial, emergent features but group living imposes a new set of risks not experienced by solitary insects. Social insects face especially high risks from parasites and pathogens, due to crowded living conditions and the potential that the similar genetic makeup of nestmates will render them vulnerable against specific pathogens (Schmid-Hempel 2005, Evans and Spivak 2010). This can allow for a small subset of parasites to coevolve and specialize to a great degree, because the nest provides a stable environment and numerous hosts (Schmid-Hempel 2005).

Honey Bee Immune System

One of the well-understood immune systems within social insects is that of the honey bee. Regardless, the way in which induced immunity in honey bees works is not as well-characterized as in *Drosophila* or *Anopheles*, and the studies that do exist look at a narrow group of responses and have used microbial challenges such as *Escherichia coli*

which do not represent natural honey bee pathogens (Evans 2004). One of the earliest studies on honey bee AMPs, by Casteels et al. (1989), discovered a family of peptide structures called apidaecins that responded non-specifically to bacteria and other injected foreign molecules. The key finding was that these peptides responded immediately upon challenge. Three additional AMPs were also characterized; hymenoptaecin, abaecin, and defensin (Casteels-Josson et al. l994). Several of these AMPs have corresponding precursor structures which might be processed differently to respond to specific pathogens and may even involve combining distinctly expressed peptides to create a mature peptide. It is still not well-known how these AMPs respond; however, they are induced following pathogen recognition and can be continuously expressed for long periods of time greater than 36 hours (Casteels-Josson et al. 1994). A study on early larval development of worker honey bees in response to the bacterial disease American foulbrood, *Paenibacillus larvae*, shows the expression of abaecin and defensin in control larvae in stages as early as the first larval instar (Evans 2004). Therefore, these humoral responses exist prior to the adult life stage in bees. Cellular response in brood is lacking and a crucial enzyme precursor involved in cellular responses, pro-phenoloxidase (ProPO), is greatly under-expressed in larvae compared to adults (Wilson-Rich et al. 2009). In comparison to *Anopheles* and *Drosophila*, honey bees have very similar immune-gene pathways. The common insect immune pathways of Toll, Imd, JAK/STAT, and JNK remain intact. Honey bees are adapted to a more narrow set of pathogens and lack diversity in both targeting receptors and in the expressed AMP paralogs that can be found in other organisms (Wilson-Rich et al. 2009, Evans and

Spivak 2010). It is hypothesized that honey bee immunity to viruses involves dsRNA interference, but the response of AMPs to viruses is unknown (Evans and Spivak 2010).

Honey Bees and the Consequences of Domestication

As domesticated animals, the evolution of honey bee immunity experiences unnatural conditions such as the high density placement of hives and introduction of parasites that increase pathogen exposure and contribute to loss of colony number. Given the valuable services these insects perform, managed beekeeping operations have worked in many ways to maximize productivity of honey bee hives for their products as well as crop pollination. In some instances, these practices incorporate the transportation of hives across large geographic regions in order to follow a nectar flow (Boecking and Genersch 2008). The nature of this practice also allows for pathogens to be easily transmitted between colonies due to the great number and close proximity of hives. It has also allowed for inter and intra-specific transmission of novel pathogens (Oldroyd 1999).

Honey Bee Importance

The importance of honey bees as natural pollinators has long been recognized. Many agricultural crops rely heavily on the pollination provided by foraging honey bee workers. Honey bees are the most important source of animal pollination and are responsible for 35% of global food production, producing fruits, vegetables, and

stimulant crops (Genersch 2010). For human health they are invaluable, accounting for the crops that contain micro-nutrients that are necessary for a nutritionally balanced and complete diet (Klein et al. 2011). In the US alone, the added crop value of honey bee pollination is estimated to be $15 billion, making them an economically influential insect (Morse and Calderone 2000). Honey bees are also beneficial pollinators of non-agricultural crops as a contributor to wild flower biodiversity (Genersch 2010). Approximately 80% of all wild flowers rely on insect pollination, and a decline in the honey bee population would no doubt have consequences for plant diversity (Potts et al. 2010).

Honey Bees as Model Organisms

In addition to the valuable pollination services they perform, honey bees are also important as model organisms for study. Experimentally, honey bees can be studied with relative ease in the field or indoors due to their domestication. With tens of thousands of workers present in a stationary hive large amounts of data can be collected with relative ease. Their differentiation into different castes as well as their haplodiploid sex-determination allows comparisons of honey bees to be made, answering many questions about behavioral and developmental mechanisms (HBGSC 2006). Extensive genome work and discovery of many candidate genes exists, which provides a crucial framework for understanding insect immunity (Evans 2006). The genome of the common honey bee, *Apis mellifera*, was released in 2006 and has been updated and annotated in

subsequent years. *A. mellifera* is a particularly useful insect model when compared to either *Drosophila* or *Anopheles* because it is evolving more slowly (HBGSC 2006). This should make evolution of its immune-gene responses easier to pursue, as well as providing for homologous traits with other distantly related organisms.

Health Crisis and Decline

In recent years, there has been a sharp decrease in the number of US colonies, with loss estimates of 30 percent or more occurring during overwintering periods (Pettis and Delaplane 2010, vanEngelsdorp et al. 2011). Colony losses in managed honey bees are prevalent in not only the United States, but in Canada as well as in Europe (Pettis and Delaplane 2010, Guzman-Novoa 2009). USDA estimates show a drop in nearly two thirds of all managed colonies ranging from around 6 million in the late 1940s to a little over 2 million in 2008 (Pettis and Delaplane 2010).

In an attempt to pinpoint a single candidate for the decline in honey bee health, recent literature tends to converge on the parasitic, invasive mite species, *Varroa destructor*, as the pathogen with the most considerable impact (Rosenkranz et al. 2010). *Varroa*, a genus of mite native to the eastern honey bee *Apis cerana*, is estimated to have been transmitted to the western honey bee, *A. mellifera*, only within the last 50 to 60 years by proximity of the two species' hives as well as shared pollination sources (Oldyoyd 1999). This effect is believed to be connected to a rise in the increasing

commercialization of beekeeping globally (Sammataro et al. 2000, Genersch 2010, Rosenkranz et al. 2010). *Varroa* parasitism can increase honey bee mortality by physical damage. However, it is thought that the damage caused by *Varroa* comes from its ability to effectively vector multiple honey bee viruses (Rosenkranz et al. 2010).

Varroa Biology

Varroa mites are obligatory haematophagic mites that have two life stages, a reproductive phase and a phoretic phase. During the reproductive phase a fertile mother mite, often referred to as a foundress, will find a larval host and enter into its cell in the wax comb. The timing of host infestation varies, but typically mites will invade worker brood or drone brood of *A. mellifera* between 15 to 20 hours or 40 to 50 hours prior to cell-capping, respectively (Boot et al. 1992). In as little as 5 hours after cell-capping by workers, the foundress will begin feeding from the abdomen of the developing larva (Rosenkranz et al. 2010). During this period of capped larval bee metamorphosis and development into an adult bee, the mite produces offspring at regular intervals, which then sib-mate within the cell such that reproductive females can emerge with the adult bee host. This begins the phoretic life stage, in which a fertile mite attaches to another adult worker and seeks a new brood cell for invasion. This relatively short life cycle of offspring allows *Varroa* to expand in numbers quickly within a hive. The phoretic life stage allows the mite to enter other beehives through host transport. Common honey bee behaviors such as resource foraging at common sites or robbing from competing hives

permit mites to be transferred from bees of one colony to bees of another (Rosenkranz et al. 2010).

Adaptation in honey bees in response to mites is not as evident as in the mites themselves. This occurs for several reasons. Primarily, the mites are obligate on the honey bee hosts and given their protection from many external forces such as predators, adapting to the host is the primary evolutionary pressure (Oldroyd 1999). In the arms race between mites and bees, mites have a potential upper hand given their relatively short generation time.

Typically, parasitic relations between mites and bees are rare and brood parasitism is even rarer (Oldroyd 1999). From a very general viewpoint, many ticks and mites typically adopt parasitism when their host provides a nest habitat. These associations might actually start out as commensalistic (Evans 1992). If the mites begin by feeding on small host ectoparasites found in proximity and on the nest-making arthropod, the transition to feeding on the arthropod's hemolymph is plausible. This might explain how mites were able to get into close enough contact with bees to form the close symbiosis we see today.

Honey Bee Defenses Against *Varroa* and What They Contribute in Resistance/Tolerance

At the colony level of defense there are some adaptations in honey bees specific to defense from *Varroa* that extend beyond individual immunity. One of the best known

defenses observed in honey bees to protect themselves from mites is hygienic behavior. One type of behavioral response to disease in brood cells is for workers with highly-tuned senses to detect and remove the bee larvae from the hive altogether (Boecking and Spivak 1999). This involves first detecting injured or infested pupae and then opening their capped cell, and this detection is known to vary between colonies. There is also a grooming behavior that bees can perform on themselves or on other colony members that removes loose particles originating from outside of the nest that could contain harmful microbial life. In some lineages of bees, grooming can effectively remove mites. Even though mites have adapted to cling to spots on the thorax or abdomen where "auto-grooming" would be difficult, workers can perform a dance to signal the other workers to assist in grooming. Swarming behavior to abandon compromised hives, seasonal brood allocation, and post cell-capping time are examples of several additional lines of defense thought to combat the *Varroa* life cycle, but they have not been studied heavily (Rosenkranz et al. 2010). This is likely due to difficulty involved in tracking colonies as well as issues involved in creating accurate models. Another poorly-understood trait of bees thought to influence mite infestation is the variable sizes of the wax cells (Piccirillo and De Jong 2003). Finally, bees do have a chitinous cuticle as well as intestinal microflora that provide resistance to some pathogens, although it does not directly defend against *Varroa* (Boecking and Spivak 1999). Additionally, mites vector pathogens, have a short reproductive cycle compared to that of their honey bee host, and possibly even suppresses their host's specific immune responses.

Immunosuppression by *Varroa*

There have been several studies, namely those by Gregory et al. (2005) and Yang and Cox-Foster (2005), which suggest that mites reduce transcript levels of specific AMPs in their hosts, suggesting a mechanism of immune-gene suppression in *Varroa* dependent on mite number as well as pathogen presence. Gregory et al. (2005) performed quantitative PCR on collected pupae naturally infested with mites to observe the relationship between mite number and expression of the antibacterial peptides defensin and abaecin expressed in their hosts. A significant decrease in abaecin expression was measured in bees infested with a single mite relative to non-infested bees. Interestingly, as the number of mites increased from 1 to 6, this trend of abaecin suppression diminished. It was determined that bees infested with lower mite numbers of 1 to 4 showed decreased AMP expression while heavily infested bees containing 5 or 6 mites did not differ from non-infested controls. Defensin transcripts followed a similar pattern with mildly infested cells containing 2 to 4 mites having significant AMP suppression. A second major study about immune-gene suppression by *Varroa* measured a wide range of cellular and humoral responses in honey bees as well as virus titers (Yang and Cox-Foster 2005). Pathogens are a growing area of concern in comparative expression of immune targets (Evans 2004). Yang and Cox-Foster (2005) discovered several novel instances of down-regulated immunity-related responses at both the cellular (enzymatic) and humoral levels. Additionally, the study injected nearly-emerged adult bees with *E. coli* to see if interaction between pathogens and *Varroa* infestation existed.

Expression of the AMP hymenoptaecin was significantly lowered by the presence of mite feeding. Defensin and abaecin expression levels were also lowered with mite infestation, but only in host bees the authors had grouped by degree of overt physical deformities caused by deformed wing virus (DWV). In these bees, immunosuppression was also dependent on the presence of a bacterial challenge, suggesting that the mites alone might not be affecting immunity. Enzymes required for cellular immune responses, glucose dehydrogenase, glucose oxidase, phenol oxidase (PO), and lysozyme, were suppressed to some degree by mite infestation. The degree of suppression was again dependent upon viral titers and bacterial challenge. This underlines the necessity for pathogen screening when investigating immunity.

Typically, a wound piercing both the cuticle and body wall of a honey bee elicits immune responses for pathogen defense as well as clotting to prevent loss of hemolymph (Richards et al. 2011, Dushay 2009). A recent study found that salivary gland extracts from adult *V. destructor* mites damage haemocytes by disintegration and can also inhibit aggregation and pseudopod formation required for wound healing (Richards et al. 2011). This study was unable to separate various constituents of the salivary gland extract that might also aid in digestion or other lysosomal enzymes that are not actively excreted into the host as a means of host immune-gene manipulation. If the immune response of honey bees is shut off such that no response is possible, then adaptation that would normally select for advantageous forms of innate defense would be nearly ineffective. One interesting aspect of the host-parasite balance is that the mites must not kill their pupal

host because it is required for the mites to escape from the sealed wax cell upon emergence (Oldroyd 1999). In this same line of reasoning, parasites that cause overly severe diseases in their hosts can increase selection within the host's immune system due to increased selection pressure (Ewald 1983).

Considerations Given Immune-gene Suppression Studies

The two studies that show the immunosuppressive ability of mites do not capture a biologically meaningful or realistic way of accounting for mite number and fail to control for the developmental age of the honey bee hosts (Gregory et al. 2005, Yang and Cox-Foster 2005). Gregory et al. (2005) looked at the effect of increasing mite number on host immune-gene response using the total number of mites collected from cells. Thus, adult and nymphal mites were considered in the same way, even though their nutritional demands are vastly different and nymphal offspring will require less hemolymph than adult mites (Garedew et al. 2004). Both previous studies on *Varroa* immunosuppression looked at pupae near emergence (Gregory et al. 2005, Yang and Cox-Foster 2005). Consideration of the interactive effects of AMPs under varying microbial challenges makes it difficult to ascertain a direct causal relationship between the challenge and the response measured (Evans 2004). Therefore, pathogen presence is an important factor to control for. Yang and Cox-Foster (2005) considered DWV titers and found no correlation with the expression of the AMPs they found to be immunosuppressed, leaving the suppressive effects contributed by mites and pathogens

undetermined. The groups of mite-infested bees they found to have lowered AMP expression were sorted based on their visible level of wing deformity rather than level of mite infestation alone. These studies don't provide a direct link between mites and immune-gene suppression. Induced responses, requiring activation of a signal transduction pathway, might respond in a manner specific to mite feeding whereas acute responses utilize immune molecules that have already been expressed and are circulating in the hemolymph preemptively to infection (Christophides et al. 2004). It is important to know if humoral factors in the bees infested by the mites are induced or acute. Understanding what elements are normally produced for acute responses as well as the interaction with cellular defenses during normal and infested cells could help shed light on the interaction between mites and their hosts at the gene level. These responses are expected to change with bee development and increase as the developing bee's immune system matures (Laughton et al. 2011). Thus, it is important to understand the process of honey bee immune-gene response during the times when developing brood is most susceptible to mite infestation, which is immediately after the brood cell is capped.

Hypotheses

My study attempts to uncover what effect varying degrees of mite infestation have on the expression patterns of several immune-genes of honey bees over the course of host development.

- I hypothesize:
    - Expression of innate immune responses in honey bees will vary with mite number
    - Expression of innate immune responses in honey bees will vary with the age of the larval or pupal host

- I predict:
    - Increasing mite number will correspond to a decrease in some innate responses if immune suppression exists, namely abaecin, defensin, hymenoptaecin, and apidaecin.
    - Early larval hosts will express less induced responses to mite infestation than later pupal hosts

To clarify issues arising in the literature stemming from sampling host bees near emergence, my study includes the biologically relevant time point immediately after cell-capping. Ideally, this captures the beginning, rather than the end, of the mite-bee interaction within capped cells. Additionally, I use a controlled system of mite introduction and collection to see what effect adult foundress number and nymphal offspring have on host immune response. An additional aim of my study is to see if any expression levels of innate responses correspond to other intermediate products expressed in the Toll and Imd pathways while also considering the levels of commonly present pathogens such as DWV.

CHAPTER II

METHODS

A primary goal of the experiment was to effectively mimic the interaction between mites and honey bees in nature. As such, all experiments were carried out in hives in Greensboro, NC. The project involved manually introducing *Varroa* mites into randomized cohorts of honey bee brood along with controls so as to avoid the potentially confounding variables of colony or seasonal variation. Introductions and sampling took place between June and August 2011 to capture the peak production of honey bee worker brood (Winston 1987).

Brood Considerations

For a controlled study of mite-bee interactions it is crucial to artificially introduce mature mites into honey bee brood cells that contain larvae of a specific age. To successfully monitor the developmental progress of the brood before initiation into the study, a transparency sheet was attached to the experimental frames using tacks, and open cells containing fourth instar larvae were marked on this sheet (de Ruijter 1987). After marking, the frames were immediately returned to their hive and six hours later all freshly-capped cells could be identified and used in the experiment. This ensured that all experimental brood was approximately the same age. All experimental brood frames were transferred quickly into a temperature controlled shed (28°C) and all cells were

covered with damp paper towels to keep brood from losing moisture. Varroa mites are not known to feed on or affect their larval host in any manner prior to cell-capping, therefore this time period was not of interest to my study (Garedew et al. 2004). Cell-capping provides a straightforward, distinct life-stage at which to begin studying the species interaction between *Varroa* and the bee host. Previous studies have successfully used this methodology as a repeatable way to manually introduce mites *in situ* (de Ruijter 1987, Spivak unpublished).

Mite Introduction

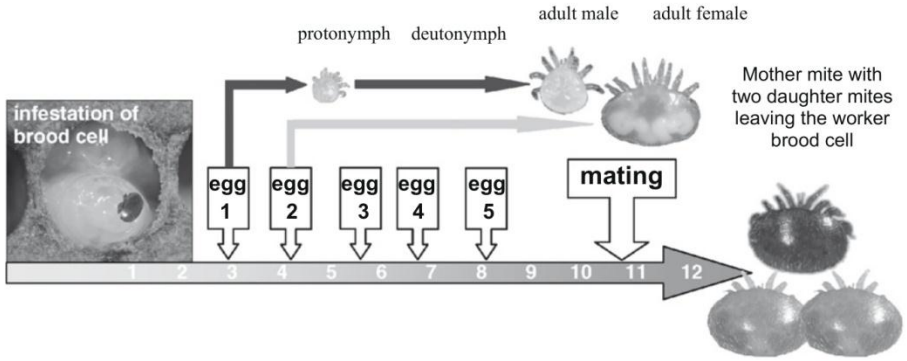
Mites were extracted from two existing donor colonies using the powdered sugar shake method as described in Boecking and Ritter (1993). Donor colonies were established in late April 2011 by inserting two frames of highly mite-infested drone brood into two productive colonies receiving no mite control treatments. For each introduction, approximately 300 to 600 worker bees were collected from brood frames into a 500mL mason jar using a bee brush (Brushy Mountain Bee Farm). Brood-tending workers were collected because mated female mites preferentially choose nurse hosts for transport to new brood-containing cells (Kraus 1993). The collection of adult phoretic mites ensures that mites are in the correct reproductive state, which cannot be guaranteed in mites that are collected from within cells. Mites that are collected from within cells may be in the midst of a reproductive phase or may have not yet mated, thus this approach was avoided (Steiner et al. 1994). Directly after adult mites were collected, they were quickly introduced into experimental cells to avoid physiological harm to the

mites that could arise from environmental stress (e.g. temperature, desiccation), or starvation.

Six hours post-capping (HPC) was the target time for selecting experimental cells in my study. It was crucial that mites be introduced within 24 HPC so that their oocyte development occurred normally (Boot et al. 1999, Steiner et al. 1994). Unpublished data from Marla Spivak suggest that the optimal time for mite introduction is within the first six HPC. Mites were introduced into brood cells as described in Boot et al. (1999). Newly-capped worker cells were cut open with a razor blade and live mites were carefully inserted before pushing the wax back into place. Mites were cleaned of powder sugar using a small paintbrush and water, and were then transferred into cells using ethanol-washed insect pinning needles. Aumeier and Rosenkranz (2001) used solvent-washed forceps to make a very small (approximately 2.5mm) incision on one side of the cap, and therefore 95% ethanol was used to clean all introduction tools between cell manipulations to prevent accidental introduction of various, confounding pathogens. The cell was sealed by pressing the wax of the cell's wall and cap together using forceps and the handle of a small paintbrush. This step is necessary to prevent introduced mites from escaping.

To test for effects of foundress number and help disentangle the effect of the mite age and number that were confounded in Gregory et al. (2005), three mite introduction treatments were performed. Cells received one mite in the first group, two mites in the second group, and three to four mites in the third treatment group. In addition to the treatment groups, two control groups were established (Figure 1). The first, negative control group received no mites or manipulation aside from cutting of the cell cap to simulate potentially confounding variables caused by the cell disturbance of the mite

introduction procedure. The control bees also provided a necessary timeline of naturally expressed immune-gene levels in mite-free brood. The second, positive control group was established to mimic the physical wounding of the bee host made by the mite. The contrast between positive control and treatment groups was performed to provide a more accurate understanding of the degree of immunosuppression caused by specific elements of the mite's feeding (e.g. salivary extracts), as opposed to general immune responses to wounding at the feeding site.



NTC	8	10	10	9	9	10	8	9	12	15
Wound	10	10	10	12	11	10	10	10	10	15
1 Mite	10	10	11	10	10	11	10	11	10	16
2 Mites	11	10	9	11	10	10	10	10	14	11
3+ Mites	10	10	10	11	11	10	10	10	8	9
HPC	24		72		120			192		240

Figure 1. Schematic diagram of the experimental setup and its relation to mite life cycle. The shaded cells in the table indicate samples investigated in quantitative PCR. Cell numbers indicate the number of cells (n) collected within each time and treatment. Non-treatment controls (NTC) and positive controls are indicated in the first two rows. Picture modified from Rosenkranz et al. 2010.

All thorax wounding was performed on fifth larval instars used for introductions as described above. Preliminary tests proved that these wounds could be made on larvae without noticeable effect on their development or mortality, in accordance with previous studies that pierced the cuticle of white, red, and brown-eyed pupae at the second

abdominal sternite (Herrmann et al. 2005). Based on this study, a 50um diameter capillary needle washed in ethanol was inserted no more than 0.3mm deep into larvae. Wounds were made on the dorsal side between the second thoracic segment (T2) to the first abdominal segment (A1) of larvae with special care to avoid puncture of the visible midgut or the tracheal or nervous systems along the dorsomedial region of the larva (Dade 1994).

Sample Collection

Random subsets of experimental cells that randomly received either negative control, positive control, single, dual, or highly mite-infested treatments were collected regularly at 24 hour time intervals over the course of ten days following cell-capping (Figure 1). Cells were dissected using standard forceps. The larval or pupal hosts were immediately removed using soft forceps and were carefully cleaned of any attached mites to avoid genetic contamination of the samples. Bees were placed in 1.5mL TRIzol reagent (Invitrogen), briefly homogenized by hand using a sterilized pipette tip, and immediately stored on dry ice.

Adult mites and all mite offspring, ranging from egg to pre-adult nymphal stages, were recorded. Identification of mite developmental stage was determined visually based on morphological references (Ifantidis 1983, Rosenkranz 2010). Mites were considered to be egg/larva, protomale/protonymph, deutonymph, light adult, or dark adult. The youngest category of nymphal mites, protonymphs, was classified based on overall body size, idiosomal shape, and positioning of the legs. At this age, the sex of the mites could not be visually determined (Steiner 1988). It has been repeatedly observed that only one

male is produced in the beginning of the mite reproductive cycle. Separation of male and female offspring number could be approximated in singly-infested cells under this assumption (Martin 1994, 1995). Deutonymphs were classified based on a larger body size and a more ellipsoidal dorsal shield. At this developmental stage, male mites could be distinguished based on their teardrop body shape, longer leg length, and moderate darkening of both the leg and idiosoma coloration (Rosenkranz et al. 2010). The immobile deutochrysalis form was also classified as deutonymphal. Given the limited developmental time within the cell for female offspring, it was also possible to distinguish freshly moulted adult females from foundress females based on the degree of sclerotization and darkness of body coloration. Mites were considered dead only if they were immobile as adults. All mites collected within a single cell were stored together in approximately 0.5mL TRIzol and immediately stored on dry ice. All samples were subsequently stored at -80°C to prevent degradation prior to RNA extraction.

Sample Preparation

The experimental design resulted in samples collected from 500 cells (5 experimental groups × 10 time points). An exhaustive molecular characterization of all samples would have been prohibitively expensive. Therefore, quantitative molecular data were collected from select groups and time points across the sample set. Those groups analyzed include the negative and positive controls as well as singly and highly mite-infested cells. For each of these four experimental groups the samples collected 24, 72, 120, 192, and 240 HPC were studied to capture the beginning, middle, and end of host development. The 72 and 192 hour time points were included to capture the time

immediately after the initiation of the foundress mite laying her first male offspring and the deposition of the final female offspring, respectively (Ifantidis 1983).

All RNA extractions were performed following the standard TRIzol protocol (Life Technologies). Frozen samples were thawed to room temperature for five minutes and subsequently mixed using a vortexer for 20 to 30 seconds and further homogenized using a p1000 pipette tip. 300µL chloroform was added to each sample and shaken by hand for 15 seconds. After two to three minutes of additional incubation at room temperature samples were centrifuged at 12,000G for 15 minutes at 4°C for phase separation. The uppermost, aqueous phase containing the isolated RNA was carefully pipetted into a clean microcentrifuge tube. 750µL 100% isopropanol was added. After ten minutes of incubation at room temperature, samples were again centrifuged at 12,000G for ten minutes at 4°C. The supernatant was carefully decanted and the RNA pellet was washed with 1.5mL 75% ethanol before a final centrifugation at 7,500G for five minutes. The supernatant was removed and once the pellet had sufficiently air-dried, the RNA was resuspended by adding 50µL RNase-free water diluted with 1:100 RNA inhibitor (RNaseOUT, Invitrogen) added. Sample tubes were then placed in a 60°C heat block for 15 minutes to aid in resuspension.

To remove DNA contaminants and prevent sample degradation due to RNases, reactions were performed in a 96-well plate using 1µL DNase buffer, 1µL DNase enzyme, and 0.2µL RNaseOUT (Invitrogen). This solution was added to 8µL aliquots of the extracted RNA quantified using spectrophotometry (Nanodrop) and normalized to 500ng/µL using RNase-free water. Samples were incubated for one hour at 37°C, and another ten minutes at 75°C to inactivate enzymes. To each sample, 0.02µL poly-dT (n=12-18, 0.5µg/µL), 0.5µL random hexamer (50µM), 0.2µL 2mM dNTP, and 0.298µL

H_2O were added. The samples were heated to 65°C for five minutes and then placed on ice for ten minutes to allow for annealing of the random primers. Superscript II (Invitrogen) was used for cDNA synthesis, and 4μL provided buffer, 2μL DTT, 0.5μL enzyme, and 3.5μL H2O were added to each sample and incubated at 42°C for 50 minutes, then at 70°C for 15 minutes to inactivate the enzyme. Finally, samples were diluted 1:5 with molecular grade water (G Biosciences) for a total of 100μL cDNA template for quantitative polymerase chain reaction (qPCR).

Quantitative PCR

Target sequences for amplification of individual samples included immune response genes, constitutively expressed "housekeeping" genes, intermediate detection and transcription factors in the Toll and Imd pathways, a precursor to ProPO, and DWV (Table 2). These transcripts were selected from a larger list of putative immunological and pathogenic primers (Evans 2006, Boncristiani et al. 2011). An initial screen of 27 primers were screened using cDNA templates comprised of samples pooled from each experimental group for the 24 HPC, 120 HPC, and 240 HPC time points. Only screened primers exhibiting a prominent melt-curve peak at the predetermined annealing temperature were used in the study of the full data set. This ensured specific primer binding and thus successful amplification of the desired sequence, if present in the sample.

QPCR was performed using Brilliant SYBR Green Master Mix (Applied Biosystems). 20 μL reactions were created using 10μL master mix, 1μL cDNA template, 0.5μL forward and reverse target primers at a 8μM concentration (Evans 2006). Samples

were run in a StepOnePlus (Applied Biosystems) quantitative PCR thermocycler following Evans' (2006) protocol with a slight adjustment to the initial holding stage and annealing temperature. The following cycling conditions were used: three minutes at 95°C, then 40 cycles of 95°C for 20 seconds, 60°C for 30 seconds, 72°C for one minute, and 72°C for another 20 seconds during which fluorescence measurements were taken. A final melt curve stage was included at 95°C for 15 seconds, 60°C for one minute, and a final ramp at 0.3°C to 95°C for 15 seconds. The fluorescence level for each sample was based on the quantity of SYBR green reporter dye incorporated into the amplified cDNA and allowed for a comparison of the relative concentration of a given target gene between samples (Schmittgen and Livak 2008).

Analyses

The threshold cycle (C_T) is the cycle of PCR amplification at which the target gene reaches a defined level of fluorescence. C_T values were collected based on the default StepOnePlus algorithmic threshold search criteria. Multiple reaction plates were required to account for the total number of samples. In these instances, the Applied Biosystems' threshold value was calculated individually for each plate. To determine a standard threshold level of fluorescence for an individual primer target, all plate-specific threshold levels that were calculated by the internal Applied Biosystems StepOnePlus algorithm were taken into account. A pooled threshold value of all plates was created, weighted by the number of samples contributing to each threshold value on a given 96-well plate. This standard threshold value was then used to recalculate every sample's cycle threshold (C_T) for a given target. Using a standardized threshold allowed for

comparison across different plates which was necessary to simultaneously assess the effects of time and treatment.

For samples exhibiting no expression in a given run, a second reaction was performed. In the event that an individual sample was run twice with undetermined amplification, a C_T value of 45 was used instead of the missing value. This value exceeds the maximum number of cycles in the reaction protocol and was therefore a valid surrogate value.

C_T values determined from qPCR were compared overall and across treatment groups within each of the five time periods using ribosomal protein S5 (RPS5) as an internal control. RPS5 provides a fundamental functional role in gene synthesis and is expected to reflect an organism's capacity for expression even at early larval stages, which makes it a good "housekeeping gene" (Evans and Wheeler 2000). Alpha tubulin and beta actin were also tested as potential housekeeping genes as internal standards but showed many inconsistencies in melt curves and amplification as well as in expression between treatment groups. The C_T values for the housekeeping gene were subtracted from the C_T values of the target gene of interest for each sample to calculate the ΔC_T value. This value was then inversely scaled under the assumption that PCR efficiency was 2, such that the transcript number doubled each cycle of PCR yielding 2^N copies (Schmittegen and Livak 2008). To account for skew in the scaled ΔC_T data, the data was log transformed prior to statistical analysis. Analysis of housekeeping genes was also scaled in the same manner prior to ANOVA in order to more accurately represent mRNA concentrations corresponding to cycle number.

The overall expression levels of the target sequences were tested in response to time and treatment using two-way analysis of variance (ANOVA) using R statistical software. An interaction term was included in the model to discern the individual, main effects of the factors of interest vs. their potential compounded effects.

Additionally, mite reproductive success was compared among the treatment groups and correlated to immune gene expression. Reproductive success was analyzed separately as either total offspring in a cell or a value of individual reproductive success per mite (offspring/foundress mite). All analyses concerning mite reproduction were performed only on mite-infested cells within the 72, 120, 192, and 240 hour time points. For *V. destructor*, the mean number of HPC for the laying of the first observable offspring is approximately 60 HPC (Ifantidis 1983, Martin 1994). It could therefore be assumed that only time points greater than 60 HPC were suitable for analysis of reproductive success. Expression values of target transcripts (ΔC_T) were correlated to reproductive success using Pearson's product-moment correlation coefficient.

To test the association of the wounding and mite-infestation treatments on workers' expression levels, ANOVA was performed within each time interval sampled using the adjusted mRNA concentrations (ΔC_T) in response to the factor of experimental group. A post-hoc analysis (Tukey's HSD) was then performed to investigate pairwise comparisons of each experimental group to further investigate the potential sources of variation.

Pearson's product-moment correlation was performed on all 11 transcripts used in the full data set. The raw C_T data was used without any normalization to ensure that the

effect of the internal control would not influence the target gene's association with the other target genes across treatments and time points.

CHAPTER III

RESULTS

Reproduction and Time

The manual introduction of adult phoretic mites resulted in successful offspring production in nearly all cells where adults survived. The first eggs/larvae were observed at 72 HPC except for a single egg deposited prior to 48 HPC. Based on the assumption that adult mites produce their first offspring within the first 72 HPC, 77.5% of all mite-infested cells containing live adults had at least one successful reproductive event. On average, the number of offspring per foundress produced in each cell increased over the ten days examined (Table 1).

Table 1. Mite offspring reproduction (measured in offspring/adult foundress mite) with increasing time (HPC) and mite-infestation levels. The fraction of cells exhibiting reproduction is indicated by brackets. The lower right portion of the table represents samples appropriate for analyses using reproduction as a variable.

	Hours Post-Capping (HPC)									
	24	**48**	**72**	**96**	**120**	**144**	**168**	**192**	**216**	**240**
NTC	0 [0/8]	0 [0/10]	0 [0/10]	0 [0/9]	0 [0/9]	0 [0/10]	0 [0/8]	0 [0/9]	0 [0/12]	0 [0/15]
Wound	0 [0/10]	0 [0/10]	0 [0/10]	0 [0/12]	0 [0/11]	0 [0/10]	0 [0/10]	0 [0/10]	0 [0/10]	0 [0/15]
1 Mite	0 [0/10]	0 [0/10]	1 [5/11]	2 [6/10]	2.71 [7/10]	2.2 [5/11]	2.29 [7/10]	2.6 [10/11]	3.89 [9/10]	3.64 [11/16]
2 Mites	0 [0/11]	0.5 [1/10]	0.69 [8/9]	1.83 [9/11]	1.72 [9/10]	2.06 [8/10]	2.88 [8/10]	1.67 [6/10]	2.04 [13/14]	2.5 [11/11]
3+ Mites	0 [0/10]	0 [0/10]	0.44 [8/10]	0.67 [7/11]	1.42 [10/11]	1.48 [7/10]	1.55 [8/10]	1 [8/10]	1.25 [7/8]	1.57 [8/9]

Reproduction expected under natural conditions

Reproduction and Mite-infestation

Mite-infested cells exhibited a pattern of decreasing mean offspring per adult mite as infestation increased from one to three or greater mites (Figure 2). Averaging across all time points ≥72 HPC, mites in doubly mite-infested cells produced 79% of the per-mite offspring found in singly infested cells. Mites in highly infested cells were less than half as reproductively successful as in singly infested cells, producing 47% of offspring per adult mite. However, the percentage of success in instances of multiple mite infestation was not constant over the duration of the sampling period. The doubly mite-infested cells varied from 52% to 126% per-mite reproduction with the 144 and 168 HPC time points showing surprisingly high relative success compared to the singly infested cells at 94% and 126%, respectively. Highly mite-infested cells varied from 32% to 68% with the 144 and 168 HPC time points exhibiting the greatest success relative to the singly-infested groups.

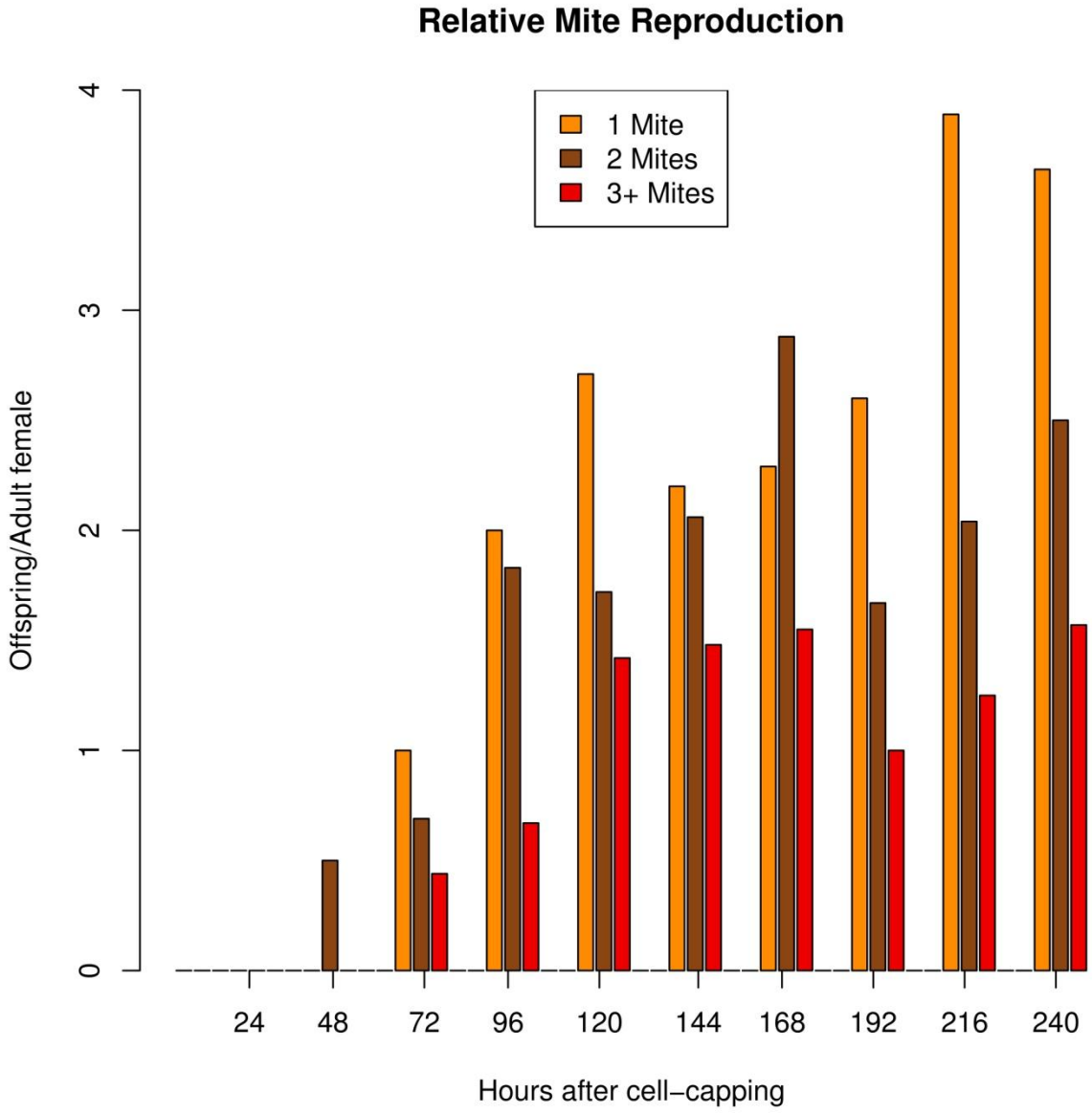


Figure 2. Reproductive success (measured in offspring/adult foundress mite) with increasing time (HPC) and mite-infestation levels.

Preliminary Screening of Target Genes

An initial panel of primers encompassing immune, housekeeping, and pathogenic sequences was screened using pooled template cDNA. Only primers exhibiting specific amplification, as described in the methods, were employed for further analysis in this study (Table 2). The unused primer sets are listed in Table 7 in the Appendix.

Table 2. Primer sequences of transcriptional targets successfully amplified in this study.

	Forward	Reverse
Alpha Tubulin	GCACGTGAAGATCTAGCAGCTC	GCACCTTCTCCTTCACCTTCAG
RPS5	AATTATTTGGTCGCTGGAATTG	TAACGTCCAGCAGAATGTGGTA
Abaecin	CAGCATTCGCATACGTACCA	GACCAGGAAACGTTGGAAAC
Apidaecin	TTTTGCCTTAGCAATTCTTGTTG	GTAGGTCGAGTAGGCGGATCT
Cactus	CCTGGACTGTCTGGATGGTT	TGGCAAACCCTTTCTCAATC
Defensin	GCAACTACCGCCTTTACGTC	GGGTAACGTGCGACGTTTTA
Hymenoptaecin	CTCTTCTGTGCCGTTGCATA	GCGTCTCCTGTCATTCCATT
PGRPLC710	TCCGTCAGCCGTAGTTTTTC	CGTTTGTGCAAATCGAACAT
PGRPSC4300	GAGGCTGGTACGACATTGGT	TTATAACCAGGTGCGTGTGC
PGRPSCnew	CACAAAATCCTCCGCCATT	ATGTCACCCCAACCCTTCTC
PPOact	GTTTGGTCGACGGAAGAAAA	CCGTCGACTCGAAATCGTAT
Relish	GCAGTGTTGAAGGAGCTGAA	CCAATTCTGAAAAGCGTCCA
Deformed Wing Virus	GAGATTGAAGCGCATGAACA	TGAATTCAGTGTCGCCCATA

Overall Gene Expression Patterns

The age of the host worker bee was associated with a significant change in expression for all 11 cDNA targets studied (Table 3). Treatment was associated with a significant change in expression for all targets (Table 3), expect for abaecin (ANOVA, $p = 0.6$). Additionally, all interaction between treatment and time were significant (Table 3), except for abaecin ($p = 0.073$) and DWV ($p = 0.45$). The mean for each gene in each experimental group is given in Table 8 in the appendix.

Expression and Reproduction

There were few consistent, significant correlations between transcript abundance and mite reproductive success found across the samples (Table 4). At 72 HPC, PGRPsc4300, and PGRPscNew showed a negative correlation to the measure of reproductive success (offspring/foundress mite). PPOact showed a negative correlation to the total offspring number at this time point. PGRPsc4300 showed a negative correlation with offspring number at 120 HPC. Hymenoptaecin was negatively correlated with both per-mite reproductive success, as well as offspring number at 192 HPC, and apidaecin was negatively correlated with per-mite fitness at 240 HPC.

Table 3. Two-way ANOVA analyses for the contribution of treatment, hours post-capping (HPC), and their interaction on the expression of the panel of target sequences.

Defensin2	F value	P-value
Treatment	8.49	<0.0001
HPC	16.92	<0.0001
Treatment*HPC	3.47	<0.001
Abaecin	**F value**	**P-value**
Treatment	0.63	0.6
HPC	13.23	<0.0001
Treatment*HPC	1.69	0.073
Hymenoptaecin	**F value**	**P-value**
Treatment	14.24	<0.0001
HPC	41.69	<0.0001
Treatment*HPC	4.24	<0.0001
Apidaecin	**F value**	**P-value**
Treatment	51.67	<0.0001
HPC	81.84	<0.0001
Treatment*HPC	7.14	<0.0001
Relish	**F value**	**P-value**
Treatment	11.19	<0.0001
HPC	12.24	<0.0001
Treatment*HPC	5.14	<0.0001
Cactus	**F value**	**P-value**
Treatment	15.39	<0.0001
HPC	12.24	<0.0001
Treatment*HPC	2.82	0.0014

PGRPlc710	F value	P-value
Treatment	11.77	<0.0001
HPC	6.86	<0.0001
Treatment*HPC	3.50	<0.001
PGRPsc4300	**F value**	**P-value**
Treatment	5.06	0.0021
HPC	19.99	<0.0001
Treatment*HPC	3.77	<0.0001
PGRPscNEW	**F value**	**P-value**
Treatment	21.50	<0.0001
HPC	38.60	<0.0001
Treatment*HPC	3.66	<0.0001
PPOact	**F value**	**P-value**
Treatment	4.72	0.0034
HPC	24.58	<0.0001
Treatment*HPC	2.51	0.0044
DWV	**F value**	**P-value**
Treatment	31.30	<0.0001
HPC	8.94	<0.0001
Treatment*HPC	1.00	0.45

Table 4. Correlation of cDNA targets and per-mite fitness (offspring/foundress mite) and total offspring number using Pearson product-moment correlation. Associated p-values are indicated as follows: p < 0.05*, p < 0.01.**

	72 HPC		120 HPC		192 HPC		240 HPC	
	offspring/adult	offspring #	offspring/adult	offspring #	offspring/adult	offspring #	offspring/adult	offspring #
Defensin2	-0.478	0.154	0.148	0.008	-0.001	-0.307	-0.396	0.020
Abaecin	-0.107	0.086	0.052	-0.041	0.289	-0.366	-0.115	-0.204
Hymenoptaecin	-0.496	0.390	-0.029	0.210	-0.486 *	-0.456 *	-0.113	-0.248
Apidaecin	-0.472	0.115	-0.236	-0.001	0.076	-0.378	-0.486 *	-0.274
Relish	-0.455	0.084	-0.333	0.183	0.050	-0.289	-0.372	-0.230
Cactus	-0.135	0.341	-0.460	0.337	0.104	-0.077	-0.038	0.051
PGRPlc710	-0.345	0.155	-0.202	0.011	0.159	-0.132	-0.289	-0.232
PGRPsc4300	-0.779 **	0.027	-0.185	-0.449 *	-0.208	0.187	-0.408	-0.138
PGRPscNEW	-0.575 *	0.162	0.013	0.261	-0.019	0.194	-0.408	-0.138
PPOact	-0.254	-0.603 **	-0.218	-0.136	-0.240	0.267	-0.227	-0.148
DWV	-0.275	0.300	-0.136	0.149	-0.355	0.006	-0.109	-0.142

Expression and Mite-infestation

Given the influence of host age (HPC) as a factor in worker immune response, all comparisons between cell treatment groups were performed within their respective time past-capping (Table 5). Mean values and graphical displays are included in Appendix A (Table 8) and Appendix B (Figures 3-15), respectively.

Table 5. Overall comparison of treatment groups within each of the sampling time points for all putative targets. F and P values were determined using one-way ANOVA, and letters represent the pairwise differences (α = 0.05) of all post-hoc analyses between the four groups examined (Tukey HSD).

24 HPC — TukeyHSD Pairwise Comparisons

	F value	P-value	NTC	Wound	1 Mite	3+ Mites
RPS5	165.33	<0.0001	A	B	A	A
Alpha Tubulin	17.77	<0.0001	A	B	A	A
Defensin2	5.12	0.0051	A	B	A	A
Abaecin	6.39	0.0016	A	B	A	AB
Hymenoptaecin	8.48	<0.001	A	B	A	A
Apidaecin	36.91	<0.0001	A	B	A	A
Relish	31.88	<0.0001	A	B	A	A
Cactus	11.72	<0.0001	A	B	A	A
PGRPlc710	6.72	0.0012	A	B	A	A
PGRPsc4300	15.10	<0.0001	A	B	A	A
PGRPscNEW	9.91	<0.0001	A	B	A	A
PPOact	0.69	0.57	A	A	A	A
DWV	6.20	0.0018	A	B	AB	B

72 HPC — TukeyHSD Pairwise Comparisons

	F value	P-value	NTC	Wound	1 Mite	3+ Mites
RPS5	2.36	0.087	A	A	A	A
Alpha Tubulin	3.32	0.03	A	AB	AB	B
Defensin2	3.51	0.025	A	AB	AB	B
Abaecin	1.36	0.27	A	A	A	A
Hymenoptaecin	20.50	<0.0001	A	B	B	C
Apidaecin	19.51	<0.0001	A	B	B	B
Relish	6.41	0.0013	A	B	AB	B
Cactus	6.59	0.0011	A	C	AB	BC
PGRPlc710	0.55	0.65	A	A	A	A
PGRPsc4300	3.49	0.025	A	AB	B	AB
PGRPscNEW	4.43	0.0093	A	B	AB	AB
PPOact	3.16	0.036	A	AB	AB	B
DWV	5.44	0.0034	A	A	AB	B

120 HPC — TukeyHSD Pairwise Comparisons

	F value	P-value	NTC	Wound	1 Mite	3+ Mites
RPS5	15.21	<0.0001	A	B	A	A
Alpha Tubulin	21.84	<0.0001	A	B	A	A
Defensin2	1.98	0.13	A	A	A	A
Abaecin	1.65	0.19	A	A	A	A
Hymenoptaecin	4.21	0.012	A	B	AB	B
Apidaecin	94.86	<0.0001	A	B	A	A
Relish	5.46	0.0034	A	B	A	AB
Cactus	24.72	<0.0001	A	C	AC	B
PGRPlc710	3.80	0.018	AB	B	A	A
PGRPsc4300	1.90	0.15	A	A	A	A
PGRPscNEW	9.53	<0.001	A	B	A	A
PPOact	5.12	0.0047	AB	B	A	A
DWV	4.83	0.0063	A	AB	AB	B

192 HPC — TukeyHSD Pairwise Comparisons

	F value	P-value	NTC	Wound	1 Mite	3+ Mites
RPS5	1.15	0.34	A	A	A	A
Alpha Tubulin	0.37	0.77	A	A	A	A
Defensin2	1.69	0.19	A	A	A	A
Abaecin	0.78	0.51	A	A	A	A
Hymenoptaecin	5.67	0.0029	A	A	AB	B
Apidaecin	1.32	0.28	A	A	A	A
Relish	1.44	0.25	A	A	A	A
Cactus	0.33	0.81	A	A	A	A
PGRPlc710	1.29	0.29	A	A	A	A
PGRPsc4300	1.02	0.4	A	A	A	A
PGRPscNEW	1.11	0.36	A	A	A	A
PPOact	0.06	0.98	A	A	A	A
DWV	10.10	<0.0001	A	AB	BC	C

240 HPC — TukeyHSD Pairwise Comparisons

	F value	P-value	NTC	Wound	1 Mite	3+ Mites
RPS5	4.26	0.0094	A	AB	AB	B
Alpha Tubulin	4.28	0.0093	A	A	AB	B
Defensin2	8.61	<0.0001	A	A	A	B
Abaecin	0.09	0.97	A	A	A	A
Hymenoptaecin	1.76	0.17	A	A	A	A
Apidaecin	6.13	0.0013	A	B	AB	AB
Relish	3.19	0.032	A	AB	AB	B
Cactus	1.54	0.22	A	A	A	A
PGRPlc710	13.73	<0.0001	A	B	A	A
PGRPsc4300	3.47	0.023	A	AB	AB	B
PGRPscNEW	15.72	<0.001	A	B	A	A
PPOact	3.97	0.013	A	B	A	AB
DWV	12.52	<0.0001	A	B	B	B

At 24 HPC, the expression of the internal control, RPS5, in the artificially wounded group had a 269-fold decrease in expression relative to the non-treatment control (ANOVA, $p < 0.0001$, TukeyHSD, $p < 0.0001$). Relative to the non-treatment control, the mRNA concentrations (ΔC_T) of the artificially wounded group showed a consistent pattern of upregulation in every immunity-related gene at this time point. Defensin expression was increased in the artificially wounded workers 115-fold relative to the non-treatment control (TukeyHSD, $p = 0.0084$). Abaecin expression was increased in the artificially wounded workers 81-fold relative to the non-treatment control (TukeyHSD, $p = 0.025$). Hymenoptaecin expression was increased in the artificially wounded workers 55-fold relative to the non-treatment control (TukeyHSD, $p = 0.0071$). Apidaecin expression was increased in the artificially wounded workers 271-fold relative to the non-treatment control (TukeyHSD, $p < 0.0001$). Relish expression was increased in the artificially wounded workers 107-fold relative to the non-treatment control (TukeyHSD, $p < 0.0001$). Cactus expression was increased in the artificially wounded workers 608-fold relative to the non-treatment control (TukeyHSD, $p = 0.0011$). PGRPlc710 expression was increased in the artificially wounded workers 77-fold relative to the non-treatment control (TukeyHSD, $p = 0.0035$). PGRPsc4300 expression was increased in the artificially wounded workers 72-fold relative to the non-treatment control (TukeyHSD, $p < 0.0001$). PGRPscNew expression was increased in the artificially wounded workers 28-fold relative to the non-treatment control (TukeyHSD, $p = 0.0013$). PPOact expression was not affected by any treatment (ANOVA, $p = 0.57$). The DWV transcript was significantly increased in the artificially wounded workers (TukeyHSD, $p = 0.0021$) as well as in the highly mite-infested workers (TukeyHSD, $p = 0.0049$).

At 72 HPC, there were no significant changes in scaled RPS5 expression based on treatment (ANOVA, $p = 0.087$). The expression of defensin was increased in the highly mite-infested group relative to the non-treatment control with an approximate 4-fold increase (TukeyHSD, $p = 0.015$). The expression of hymenoptaecin was increased by all treatments. Relative to the negative control, there was a 9-fold increase in the artificially wounded group (TukeyHSD, $p < 0.0001$), a 12-fold increase in the singly mite-infested group (TukeyHSD, $p < 0.0001$), and a 82-fold in the highly mite-infested group (TukeyHSD, $p < 0.0001$). The expression of apidaecin was also increased by all treatments. There was a 68-fold increase in the artificially wounded group (TukeyHSD, $p < 0.0001$), a 30-fold increase in the singly mite-infested group (TukeyHSD, $p = 0.00023$), and a 74-fold in the highly mite-infested group (TukeyHSD, $p < 0.0001$). There was a significant increase in relish expression in the artificially wounded and the highly mite-infested groups. Artificial wounding increased expression 5-fold (TukeyHSD, $p = 0.0038$) while highly infested cells underwent a 9-fold increase in expression (TukeyHSD, $p = 0.0026$). There was a significant increase in cactus expression in the artificially wounded and the highly mite-infested groups. Artificial wounding increased expression 12-fold (TukeyHSD, $p = 0.0013$) while highly infested cells underwent a 10-fold increase in expression (TukeyHSD, $p = 0.021$). PGRPsc4300 expression showed a 67-fold decrease in the singly mite-infested group relative to the non-treatment control (TukeyHSD, $p = 0.014$). PPOact expression showed a 11-fold decrease in the highly mite-infested group relative to the non-treatment control (TukeyHSD, $p = 0.045$). DWV transcripts were increased only in the highly mite-infested cells (TukeyHSD, $p = 0.0030$).

At 120 HPC the expression of RPS5 was again lowered by artificial wounding with a 4-fold decrease relative to the non-treatment control (TukeyHSD, $p < 0.0001$). Hymenoptaecin expression was significantly increased in the artificially wounded and the highly mite-infested groups. Artificial wounding increased expression 5-fold (TukeyHSD, $p = 0.033$) while highly infested cells underwent a 11-fold increase in expression (TukeyHSD, $p = 0.012$). Apidaecin expression was increased in the artificially wounded workers 244-fold relative to the non-treatment control (TukeyHSD, $p < 0.0001$). Relish expression was increased in the artificially wounded workers 5-fold relative to the non-treatment control (TukeyHSD, $p = 0.0058$). Cactus expression was significantly increased in the artificially wounded and the highly mite-infested groups. Artificial wounding increased expression 57-fold (TukeyHSD, $p < 0.0001$) while highly infested cells underwent a 6-fold increase in expression (TukeyHSD, $p = 0.036$). PGRPscNew expression was increased in the artificially wounded workers 11-fold relative to the non-treatment control (TukeyHSD, $p < 0.001$). DWV expression was increased in the highly mite-infested workers 14-fold relative to the non-treatment control (TukeyHSD, $p = 0.0032$).

At 192 HPC, there were no significant changes in RPS5 expression based on treatment (ANOVA, $p = 0.34$). Hymenoptaecin was the only immunity-related gene with a significant difference in expression due to treatment. In the highly mite-infested group expression was increased 6-fold relative to the non-treatment control (TukeyHSD, $p = 0.016$). DWV replication was increased in both mite-infested groups relative to the negative control (TukeyHSD, 1 mite:NTC $p = 0.02$, 3+ mites:NTC $p < 0.0001$).

At 240 HPC, there was a 2-fold decrease in RPS5 expression in the highly mite-infested group (TukeyHSD, $p = 0.0063$). Defensin expression was increased in the

highly mite-infested workers 3-fold relative to the non-treatment control (TukeyHSD, $p < 0.0001$). Apidaecin expression was increased in the artificially wounded workers 22-fold relative to the non-treatment control (TukeyHSD, $p = 0.0012$). Relish expression was increased in the highly mite-infested workers 6-fold relative to the non-treatment control (TukeyHSD, $p = 0.02$). PGRPlc710 expression was increased in the artificially wounded workers 22-fold relative to the non-treatment control (TukeyHSD, $p < 0.0001$). PGRPsc4300 expression was increased in the highly mite-infested workers 5-fold relative to the non-treatment control (TukeyHSD, $p = 0.031$). PGRPscNew expression was increased in the artificially wounded workers 22-fold relative to the non-treatment control (TukeyHSD, $p < 0.001$). PPOact expression was increased in the artificially wounded workers 22-fold relative to the non-treatment control (TukeyHSD, $p = 0.014$). DWV expression was increased in the artificially wounded workers, singly mite-infested group, and highly mite-infested group relative to the non-treatment control (TukeyHSD, $p = 0.012$, < 0.0001, and < 0.0001, respectively).

Correlations in Immune-gene Responses

The strongest correlations were found between the three peptidoglycan receptors and nearly every other immune gene measured in this study (Table 6). The target with the least significant correlation to all other transcripts was DWV.

Table 6. Correlation matrix of each cDNA target using Pearson product-moment correlation on raw C_T values. Associated p-values are indicated as follows: p < 0.05*, p < 0.01, p < 0.001***, p < 0.0001****.**

	Def	Aba	Hym	Api	Rel	Cac	710	4300	New	PPO
Defensin2										
Abaecin	0.15 *									
Hymenoptaecin	0.22 **	0.51 ****								
Apidaecin	-0.04	0.4 ****	0.69 ****							
Relish	0.51 ****	0.35 ****	0.47 ****	0.21 **						
Cactus	0.28 ****	0.23 ***	0.43 ****	0.41 ****	0.41 ****					
PGRPlc710	0.41 ****	0.23 ***	0.51 ****	0.31 ****	0.58 ****	0.45 ****				
PGRPsc4300	0.19 **	0.33 ****	0.63 ****	0.55 ****	0.49 ****	0.47 ****	0.61 ****			
PGRPscNEW	0.28 ****	0.22 *	0.41 ****	0.32 ****	0.53 ****	0.44 ****	0.72 ****	0.52 ****		
PPOact	0.16 *	0.16 *	0.44 ****	0.5 ****	0.27 ****	0.44 ****	0.48 ****	0.59 ****	0.34 ****	
DWV	0.27 ***	-0.11	0.19 **	0.02	0.12	0.15 *	0.03	0	-0.05	0.1

CHAPTER IV

DISCUSSION

My study showed that mites do not decrease expression of innate immune-gene responses in their honey bee hosts relative to uninfested cells, which contradicts previous findings (Gregory et al. 2005, Yang and Cox-Foster 2005). Methodologically, I demonstrated that manual introduction of mites can be performed with minimal disturbance in order to mimic natural measures of reproduction and survival. My results support my hypotheses that both mite number and host age affect innate immune responses. While I predicted immunosuppression by mites to be characterized by a pattern of diminishing returns of immune-gene responses with increasing mite number, this was not the case. The data showed a complex, time-dependent interaction with host responses increasing with mite infestation, counter to my initial prediction. In general, the extent of host immune-gene responses to mite infestation increased as the bee developed from its larval to pupal stage. My experiments suggest that the mite-bee interaction involves more variables than previously anticipated and the assertion that mites repress honey bee immunity has to be carefully considered in light of bee development, timing of mite reproductive events, accompanying viral replication, and widespread responses to stressors including physiological wounding created at mite feeding sites.

Mite Introduction and Reproductive Patterns

The reproductive patterns of the experimental mites were comparable to natural patterns (Martin 1994, Ifantidis 1997). Thus, the artificial introduction of mites did not appear to interfere with normal offspring production, as had been described before (Martin 1995). Additionally, the individual, per-mite fitness decreased as initially introduced mite numbers increased. This finding closely matches results of previous studies of multiple mite infestation which show a cost of individual fitness due to intra-species competition (Fuchs and Langenbach 1989, Martin 1995).

Mortality status of offspring was not assessed unless the body of the offspring was visibly dried because the samples had to be processed quickly to prevent sample degradation. Additionally, the presence of nymphal mites undergoing the immobile molting phases of the proto- and deutochrysalis further complicate the ability to confirm offspring successfully surviving to maturity (Ifantidis 1983, Martin 1994). Therefore, I might have counted some dead mite offspring as alive and consequently overestimated the relative success of individual mites. However, the observed timing and overall production of offspring was concordant with the current understanding of the mite reproductive phase as it progresses sequentially after cell-capping (Martin 1994).

The numbers of mite offspring produced in the artificially mite-infested cells were comparable to those previously described under natural conditions (Martin 1994, 1995). The latest time points sampled in this study, at 240 HPC, had a mean of 3.6 offspring produced when a single foundress was present in a cell. Under the assumption that only a single male is produced per cell, the number of female mites present in these cells is

closer to 2.6. Given that the mortality of mite offspring was not measured, it is likely that some immobile nymphs at this final time point might have actually been dead. Based on the assumption that the mortality of nymphal offspring is 48.3% (Martin 1994), the number of surviving female offspring at 240 HPC might have been as low as 1.28 in my study. This result is comparable to the 1.3 surviving female offspring per singly-infested cell at 230 HPC observed in Martin (1995).

It is not uncommon for infertility rates of mites to exceed 20% depending on the subspecies of host as well as mite (Rosenkranz 2010). The 77.5% success rate in this study supports the hypothesis that manual introduction of collected mites into cells does not affect reproductive rates. The number of previous reproductive cycles experienced by a given foundress mite was unknown, which is a difficult factor to determine in studies relating to mite reproduction (Rosenkranz et al. 2010). The random collection of phoretic mites should have overcome the potential issue of including mites of a non-natural reproductive status in the introductions.

Effects of Wounding and Mite-Infestation on Honey Bee Gene Expression

The cuticular wounding of either the positive control or the highly-infested mite groups affected the expression of RPS5, an internal control, at 24 HPC, 120 HPC, and 240 HPC. In order to make meaningful comparisons among and between groups, it is crucial to use an internal control that is not affected by treatment. In my study, RPS5 was selected because it has previously been found to be constitutively expressed in larval and adult honey bees (Evans and Wheeler 2000, Evans 2006). Initial screens of beta actin,

the internal gene used in the mite immunosuppression study by Yang and Cox-Foster (2005), also showed downregulation in the highly mite-infested groups at 240 HPC. Alpha tubulin was also inconsistent as an internal control and exhibited downregulation at 72 HPC where no differences were observed in RPS5. Additionally, RPS5 was more robust and had higher levels of expression overall. Therefore only RPS5 was used as an internal control. While it can be assumed that measuring individual targets relative to an internal control should be informative about expression levels, careful consideration should be given when coming to conclusions about expression levels. The effect of wounding on all housekeeping genes tested in my experiment implies that selection of an internal control for injured worker bees is a sensitive process. When measuring gene expression in the presence of increasing mite load this is also important because the physical injury of multiple feeding sites will potentially lead to a lowered expression of multiple housekeeping genes.

My study showed a complex pattern of immune-gene expression, dependent on mite load and host development time, as well as their interaction. While the time effects in the negative control group revealed the complex natural dynamics of the developing host immune system (Laughton et al. 2011), the interaction effects required a separate comparison among the experimental groups at every investigated time point. At 24 HPC the artificially wounded bees showed a significant increase in the AMP responses (defensin, abaecin, hymenoptaecin, apidaecin), their associated, intermediate transcription factors (relish, cactus), and the three receptor proteins (PGRPlc710, PGRPsc4300, PGRPscNew).

PPOact, a precursor transcript to the melanizing agent ProPO, was not affected by wounding at 24 HPC. It is surprising that PPOact levels did not differ between any

treatments in the presence of natural or artificial wounding, as phenol oxidase is expected to be involved in melanization as well as hemolymph clotting (Chrisophides et al. 2004). It is possible that PPOact was downregulated in response to wounding in a manner similar to RPS5 or that expression of this enzyme occurs at greater levels with increasing bee development time. Laughton et al. (2011) describe an increase in workers' phenol oxidase levels only after adult eclosion. Artificial wounding by capillary needles should not increase bee mortality or microbial infection relative to mite feeding (Herrmann et al. 2005). In spite of this, the introduction of artificial wound sites in my experiment was consistently performed immediately after cell-capping which might predate the initiation of feeding sites by mites. This advanced timing could explain the strong gene expression responses in most immune-genes found in artificially wounded bees at such an early time point.

Mite-infested bees showed low levels of immune-gene expression nearly identical to the negative control cells at 24 HPC. The low expression of immune genes indicates that mite infestation did not elicit an active immune response at this time. This result might be due to immune suppression (Gregory et al. 2005, Yang and Cox-Foster 2005). However, several other explanations exist. First, the manually introduced mites in my study may have not yet initiated feeding at 24 HPC. It is expected that mites will feed as early as 5 HPC (Rosenkranz et al. 2010). The majority of adult mites can't survive more than 36 hours without feeding (Garedew et al. 2004). Additionally, although the positive control treatments were successful in that they did not increase the mortality of developing workers, the depth of the capillary needle was potentially variable and was not controlled for. The artificial wounds were performed on bees at the same time as

mite introductions, and may have initiated a faster response in upregulation in the honey bee larvae than the wounding caused induction of the mite feeding site.

After 72 HPC there was a similar pattern in artificially wounded and highly-infested bees where each group exhibited increased expression of defensin, hymenoptaecin, apidaecin, relish, and cactus. These genes represent both the Toll and Im`d pathway. At this time the singly mite-infested bees did not differ from the non-treated bees in defensin, relish, or cactus expression. The strong immune responses in only those bees receiving artificially-induced wounds and high mite loads supports the alternative hypothesis that cuticle wounding alone, rather than mite feeding, initiates activation of immune-genes in a non-specific manner. The necessity for multiple feeding sites in multiply mite-infested cells increases overall damage to workers, which could explain why bees in this group, rather than the singly mite-infested cells, have higher expression levels of innate responses. 72 HPC is a biologically important time for mite reproduction because it immediately follows the depositing of the first egg by the foundress. The decreased expression of PGRPsc4300 in the singly mite-infested bees at this point could signal the onset of immunosuppression, but this pattern is not seen in the multiply mite-infested group.

There is no evidence of differing mRNA levels in defensin or abaecin expression at the 120 hour time point between any of the groups. The artificial wounding group exhibited expression levels that were similar to the non-treatment control for the remaining immune genes studied. The similarity between positive and negative control groups suggests that the observed upregulation of immune genes is only temporary, a trend that is already indicated at 72 HPC. A true positive control of repeated wounding was not experimentally possible. Therefore, it is difficult to interpret whether the

upregulation of immune genes in the later samples is due to the continued physical injury by mite feeding or other mite-specific factors. Looking at the overall trend of defensin expression, the mite-infested groups appear to initiate increased expression relative to the non-treated bees in nearly every instance except for 120 HPC. The lack of difference in the artificial wound control may be due to error in the determination of ΔC_T values based on the housekeeping gene. As in the 24 HPC samples, the absolute transcription level of RPS5 was significantly lower in the wounding group than in all other groups. The AMPS defensin and apidaecin at 120 HPC showed no difference between the negative control and the mite-infested groups. However, the AMP hymenoptaecin sustained its increased levels with wounding and mite infestation treatment here. This might be due to modulation of the immune system with mite presence. The transcription factors relish and cactus appear to be lower relative to the negative control than previously measured at 72 HPC. These transcription factors are known to regulate AMPs (Christophides et al. 2004).

After 192 hours the lack of differences in expression due to wound or mite treatments for defensin, apidaecin, relish, and cactus could be due to changes in host physiology or modulation by the mites. The increased mRNA concentrations of hymenoptaecin in association with highly mite-infested hosts shows that selected groups of genes do not undergo the same interactions, even under similar circumstances. This might imply that some response peptides are expressed differentially in a time-dependent fashion. It is also around this time point that mRNA concentrations for all genes previously affected by wounding appear to dissipate.

The final time point of the experiment, 240 HPC, shows a significantly higher level of mRNA for defensin, relish, and PGRPsc4300 in the highly mite-infested groups

than in both control groups. Increased defensin transcripts in these bees give evidence of a specific response to an aspect of mite infestation. The associated increase of the transcription relish is likely related to its predicted coregulation of defensin. The increase in expression of defensin to mite presence at this time point is relevant in light of previous findings because it examines a nearly identical time point (Gregory et al. 2005). Furthermore, the effect of this response can't be explained by wounding alone. The effect of artificial wounding reappears at this time point in apidaecin, PGRPls710, PGRPscNew, and PPOact. Thus, in contrast to the results at intermediate time points, the response to wounding seems to be long-lasting.

The patterns of immune-gene responses revealed by my study can be considered in light of the *Varroa* life cycle to gain further understanding of this close bee-mite relationship. Each foundress mite produces a single feeding site which is shared only by her progeny (Donze and Guerin 1994). Cells with multiple foundresses will therefore have multiple feeding sites. As feeding sites are continually visited by mites throughout the course of interaction, the worker hosts will have more time to evoke an immune response. When compared to the artificially wounded hosts, which showed immediate up-regulation of immune-gene responses within the first 24 hours after treatment, the mite-infested hosts displayed a slower reaction that took place over the series of ten days subsequent to cell-capping. While the artificially wounded bees upregulated immune responses faster than mite-infested bees, the location and diameter of the capillary wounds mimics actual mite feeding patterns. AMP expression at 72 HPC indicates that upregulation of host immune-genes matches or even exceeds the artificially wounded bees. Therefore, at 120 HPC, the apparent lack of response in cells containing mites relative to the negative control could potentially be interpreted as immunosuppression

associated with mites. Biologically, this is a meaningful time interval in the mite's life cycle because the first egg has typically been deposited prior to 70 HPC (Rosenkranz et al. 2010, Donze and Guerin 1994, 1997, Martin 1994, Ifantidis 1983). Female mite larvae are then deposited at 92, 118, 144, and 172 HPC (mean values, Martin 1994). It has been hypothesized that immune-gene suppression could aid in maintaining feeding sites; this is potentially a requisite mechanism for offspring to successfully feed (Gregory et al. 2005). If the pattern of individual mite reproduction relates to modulation of host immunity, it would follow that timing immune-gene suppression to correspond with the deposition of mite offspring in order to impart a fitness advantage. If the salivary excretions of the foundress mite suppress normal immune response in the host just prior to the feeding of the first offspring's feeding as a mobile immature at approximately 90 HPC, this timing mechanism could offset a range of normal responses that occur due to piercing the host's cuticle (Ifantidis 1983). The anomalous presence of down-regulation in the singly-infested group just prior to this time point, 72 HPC, strengthens this hypothesis. The host's normal reaction to wounding which involves melanization and a potential range of other effects that might compromise feeding sites makes modulation a potentially crucial strategy to feed the rapidly developing mite offspring. At 120 HPC the expression of defensin in the bees with mites might give evidence to an alternative hypothesis of timed immunosuppression. Defensin does not appear to be up or down-regulated in bees with mites relative to the negative control at 120 HPC. However, defensin expression does continue to respond by increasing in mite-infested bees relative to the controls, as is evident in the time points sampled at 192 and 240 hours.

The findings of Gregory et al. (2005) showed a trend of increasing defensin expression as mite number increased. Because neither time nor presence of offspring was

accurately controlled for in the study, there is a possibility that increasing mite number actually represented both foundresses as well as their nymphal offspring. If offspring were calculated into mite number one would expect the increase in total mite load to increase in a time-dependent manner. Therefore, the pattern of increasing defensin levels in Gregory et al. (2005) might actually capture a similar trend to those evident in my study where older bees nearing emergence exhibit a stronger response of defensin. The importance of time as a determining factor for immune responses in honey bee workers is made evident in my study. Additionally, a recent study reiterates time-dependent immune responses and shows that workers challenged with needles showed increasing expression of AMPs with age (Laughton et al. 2011)

Martin (1994) describes a "dramatic increase in the mortality of the 3rd and 4th offsprings" of *Varroa*, which are deposited around 118 and 144 HPC. This increased mortality of mite offspring laid later in the reproductive cycle might be caused by hardening of the host cuticle (Martin 1994). Alternatively, my results suggest that increased expression of immune-genes in host bees, which might include increased melanization, are affecting the survival of mite offspring. The increased expression of defensin, relish, and hymenoptaecin in the worker hosts at these time points may represent a re-initiation of normal immune-gene responses.

Deformed Wing Virus

DWV is associated with mite infestation and its levels are an important covariate in this study because DWV itself could stimulate particular immune genes and its levels may measure the effectiveness of the bees' immune system. However, no strong

correlations were found between DWV and the immune-genes surveyed in my study. It is relevant to discover increased levels of viral replication in the positive control. This suggests that wounding, irrespective of mites, does increase DWV susceptibility. Analyses of DWV C_T values without RPS5 normalization also show the wound group to be significantly increased in viral level eliminating the possibility that the housekeeping control is creating an artifact in the results. If the effect is real, it is possible that DWV is able to take advantage of a stress response in the workers. This might be due to prioritization of energy expenditure on healing over microbial defense, but the mechanism is not known based on this study.

The data showed a trend of increased DWV replication with increasing mite number. The only group to exceed the positive control in DWV replication was the highly mite-infested group; the singly mite-infested host cells never surpassed the artificially wounded workers. This could be associated with the increased number of feeding sites, thus wound number, in the highly infested bees. Alternatively, multiple foundresses could inoculate the bee host with more virus. Future research could address this open question by investigating my mite samples and relating their virus titers to that of their hosts.

Patterns of Expression and Correlation

Successful mite reproduction was negatively correlated with a few immune genes at different time points. These patterns suggest that suppression of the end product in honey bee immune-genes could potentially be a mechanism to increase fecundity. It could be that either successful mites can actively lower immune function in their hosts.

Alternatively, the natural variability in host immune function allows mite reproduction in some cases but not in other individuals that have naturally higher levels of gene expression. The latter would indicate that selective breeding of mite-resistance could be successful at the physiological level by selecting for high immune-gene expression. Specifically, the negative correlation between the peptidoglycan receptor PGRPsc4300 and mite reproduction could imply a mechanism of suppression induced by *Varroa* that prevents initiation of the Toll pathway at the detection level. Interestingly, a recent study on the effect of the microsporidian *Nosema ceranae* on a panel of 33 *A. mellifera* immune-genes found PGRPsc4300 to be the most responsive gene target to fungal infection (Huang et al. 2012). The recognition targets of this protein might include a wide range of pathogen-associated molecular patterns, and in turn it plays part in regulating the initiation of the Toll cascade (Christophides et al. 2004, Huang et al. 2012). Over all samples, PGRPsc4300 was also found to be correlated with the AMPs hymenoptaecin, abaecin, apidaecin, and PPOact, as expected based on the functional links in the *Drosophila* immune pathways (Schmid-Hempel 2005).

Conclusions

The overall patterns in the data of my study counter previously described patterns of immunosuppression by mites (Gregory et al. 2005, Yang and Cox-Foster 2005). Instead of decreasing the expression of immune-genes in the hosts, mite infestations lead to an increase in the expression of innate immune responses over multiple response pathways. Assessing the dynamics of the host-pathogen interactions proved challenging because developmental processes may cause expression changes in many genes,

irrespective of parasitism. However, my results show how important the time dimension is for understanding host immune responses to parasite attack.

The early, increased expression in nine out of ten immune-genes in response to artificial wounding might indicate a widespread, non-specific response to trauma in the form of cuticle piercing. It is also possible that piercing of the cuticle slows other vital cellular responses in order to divert resources to the immunes system, which would also explain the lowered levels of housekeeping genes in wounded bees. The only screened pathogen in this study, DWV, supports the hypothesis that wounding creates a stress response that ultimately increases immune activity with development due to higher levels of microbial replication.

The coevolutionary origins of the intimate *Varroa*-bee relationship have allowed for great specialization of both host and parasite. While *Varroa* is relatively new to *Apis mellifera*, the differential, host-specific invasion preferences between the drone and worker larvae is evidence of this mite's successful ability to adapt and respond to its environment. Under this reasoning, it would be expected that mechanisms for immune suppression would be tailored to maximize fitness. My study showed an increased response in immunological defenses, rather than a depressed one, in mite-infested bees that were nearing adulthood. There is room in my data for an immunosuppressive effect at time points in the honey bees' development that corresponds to mite reproduction. This follows the biologically meaningful reproductive cycle of mites that follows a strict schedule. Further characterization of honey bee immunity will no doubt play an important role in unraveling the interactions of mites and bees at the molecular level. As it has been demonstrated that mites are harmful in large part due to pathogen

transmission, further understanding of this additional variable is needed to understand host timing of immune-gene responses and potential parasite manipulations. The consequences of selective pressures and evolutionary history should be considered when proceeding with future studies on the *Varroa*-honey bee relationship.

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APPENDIX A.

TABLES

Table 7. Primer sequences of transcriptional targets initially screened, but not investigated, in this study.

	Forward	Reverse
AmEater	CATTTGCCAACCTGTTTGT	ATCCATTGGTGCAATTTGG
Dscam3-7	TTCAGTTCACAGCCGAGATG	ATCAGTGTCCCGCTAACCTG
Domeless	TTGTGCTCCTGAAAATGCTG	AACCTCCAAATCGCTCTGTG
BQCV	TTTAGAGCGAATTCGGAAACA	GGCGTACCGATAAAGATGGA
Defensin1	TGCGCTGCTAACTGTCTCAG	AATGGCACTTAACCGAAACG
IAPVF1aR1	GCGGAGAATATAAGGCTCAG	CTTGCAAGATAAGAAAGGGGG
Basket	AGGAGAACGTGGACATTTGG	AATCCGATGGAAACAGAACG
Bgluc19452	GGACAACCACCTTTTGAACG	AGGAGCTTCCTCTGCACTGA
Dorsal-1	AAATGGTTCGCTCGTAGCAC	TCCATGATATGAGTGATGGAAA
Tab	GCTATCATGCAGCTGTTCCA	ACACTGGGTCAGCCAATTTC
PlS18	TTCACGGCTAACAAAATTAAACA	TTCGCAGAAGTTCCGGTTAC
FungFFR1	GTTAAAAAGCTCGTAGTTG	CTCTCAATCTGTCAATCCTTATT
Bact774_1391	CCATTTGCTTCAGGGAAGAG	CAAGCCAGCGTATGCTGTAA

Table 8. Means of qPCR data across measured treatment groups within each of the sampling time points for all putative targets. Values of housekeeping genes RPS5 and alpha tubulin were scaled to represent original mRNA concentrations (2^(-C_T)). All values for the 11 target transcripts are adjusted using RPS5 as internal control (2^(-ΔC_T)) prior to log-transformation.

24 HPC — Mean Expression Values

	NTC	Wound	1 Mite	3+ Mites
RPS5	1E-07	4E-10	1E-07	1E-07
Alpha Tubulin	1E-09	4E-11	8E-10	2E-09
Defensin2	8E-04	0.094	0.001	0.026
Abaecin	6.888	559.8	5.353	8.717
Hymenoptaecin	1.398	77.45	0.799	1.15
Apidaecin	0.729	197.7	0.251	0.32
Relish	0.003	0.374	0.004	0.003
Cactus	0.002	0.987	0.002	8E-04
PGRPlc710	0.039	2.637	0.059	0.044
PGRPsc4300	0.039	2.824	0.04	0.061
PGRPscNEW	0.028	0.787	0.021	0.04
PPOact	6E-04	0.043	6E-04	8E-04
DWV	4E-06	0.663	12.04	0.143

72 HPC — Mean Expression Values

	NTC	Wound	1 Mite	3+ Mites
RPS5	1E-08	9E-09	3E-08	1E-08
Alpha Tubulin	4E-09	1E-09	1E-09	6E-10
Defensin2	0.034	0.037	0.023	0.127
Abaecin	1.986	8.093	3.91	42.73
Hymenoptaecin	0.153	1.396	1.903	12.54
Apidaecin	0.062	4.176	1.835	4.569
Relish	0.007	0.033	0.013	0.065
Cactus	0.008	0.09	0.016	0.078
PGRPlc710	2.419	0.14	0.136	0.216
PGRPsc4300	3.369	0.102	0.051	0.171
PGRPscNEW	0.14	0.117	0.022	0.112
PPOact	0.384	0.056	0.05	0.036
DWV	1E+07	294.7	916.3	9423

120 HPC — Mean Expression Values

	NTC	Wound	1 Mite	3+ Mites
RPS5	2E-07	4E-08	1E-07	1E-07
Alpha Tubulin	2E-09	3E-10	4E-09	2E-09
Defensin2	0.001	0.003	0.002	0.002
Abaecin	0.223	0.27	1.248	0.673
Hymenoptaecin	0.039	0.184	0.215	0.423
Apidaecin	0.003	0.651	0.004	0.004
Relish	0.003	0.013	0.004	0.004
Cactus	4E-04	0.021	8E-04	0.002
PGRPlc710	0.038	0.205	0.037	0.044
PGRPsc4300	0.006	0.037	0.008	0.012
PGRPscNEW	0.002	0.021	0.002	0.003
PPOact	6179	0.057	7E-05	3E-04
DWV	0.867	76.15	6.48	11.86

192 HPC — Mean Expression Values

	NTC	Wound	1 Mite	3+ Mites
RPS5	3E-08	5E-08	5E-08	5E-08
Alpha Tubulin	2E-10	3E-10	5E-10	4E-10
Defensin2	0.047	0.009	1.258	0.038
Abaecin	0.533	1.866	118.5	1.196
Hymenoptaecin	0.125	0.053	1.339	0.796
Apidaecin	0.43	0.326	1.099	0.067
Relish	0.008	0.009	0.155	0.012
Cactus	0.071	0.024	0.124	0.01
PGRPlc710	0.349	0.24	0.27	0.618
PGRPsc4300	0.291	0.021	0.027	0.037
PGRPscNEW	0.322	0.093	0.187	0.273
PPOact	0.017	0.026	0.003	0.005
DWV	8417	31.32	97.58	265

240 HPC — Mean Expression Values

	NTC	Wound	1 Mite	3+ Mites
RPS5	3E-08	8E-09	4E-08	2E-08
Alpha Tubulin	2E-10	1E-10	2E-10	3E-11
Defensin2	0.005	0.014	20.19	1.524
Abaecin	0.258	0.961	3.904	15.21
Hymenoptaecin	0.016	0.137	0.117	0.123
Apidaecin	0.004	0.082	0.07	0.085
Relish	0.01	0.057	0.372	0.548
Cactus	0.005	0.014	0.205	0.141
PGRPlc710	0.049	1.11	0.154	0.102
PGRPsc4300	0.004	0.02	0.128	0.116
PGRPscNEW	0.057	0.394	0.036	0.042
PPOact	8E-05	0.003	4E-04	7E-04
DWV	4E-04	0.018	271.1	194.1

APPENDIX B.

FIGURES

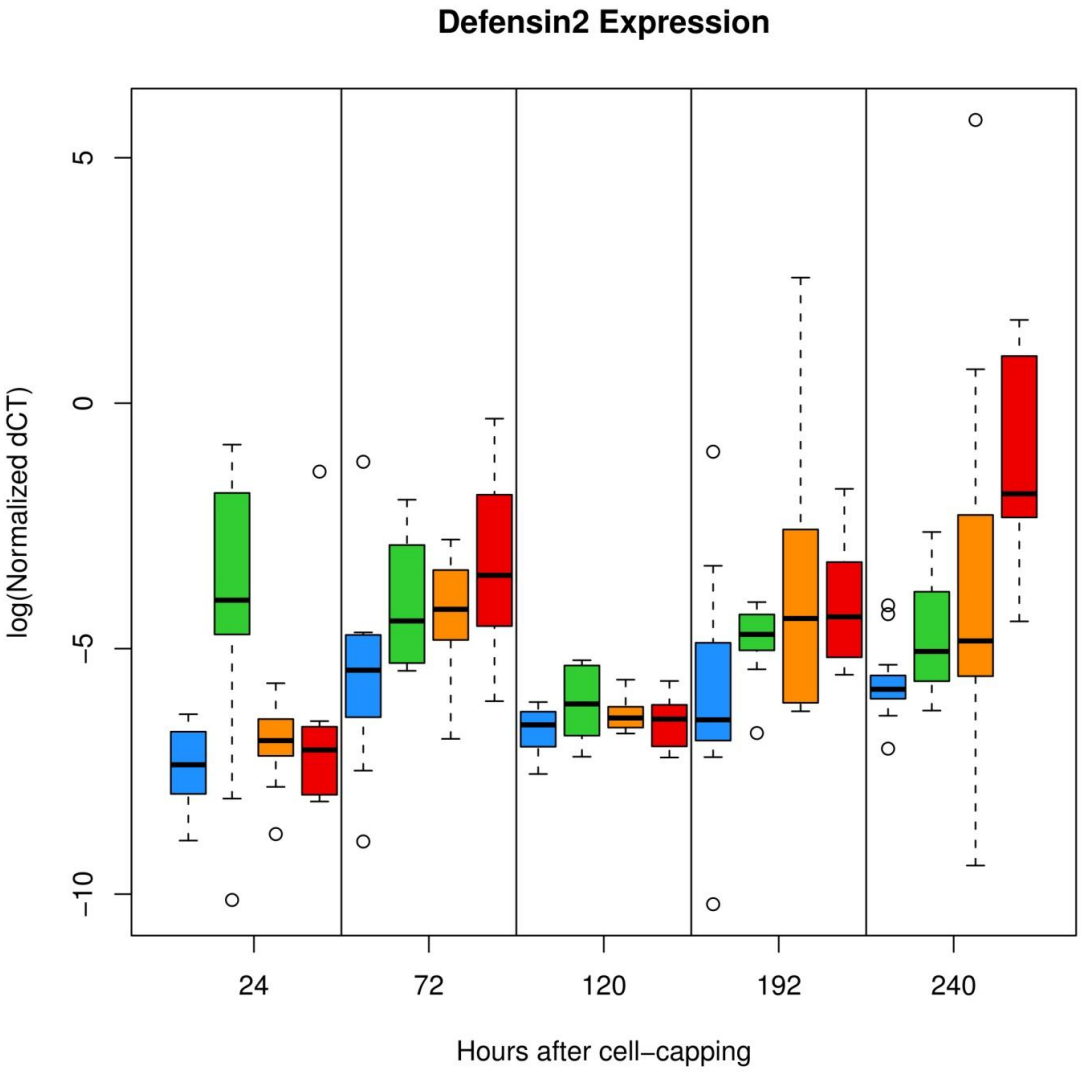

Figure 3. Boxplot showing expression levels of the antimicrobial peptide defensin with increasing host development time in hours post-capping. The non-treatment control (blue) was compared to the artificially wounded positive control (green), singly mite-infested (orange), and highly mite-infested (red) cells. The black bar indicates the mean for each group with the box and whisker representing the inner-quartile range and overall range, respectively. Points considered as potential outliers are represented by open circles; however, all data were included in statistical analyses.

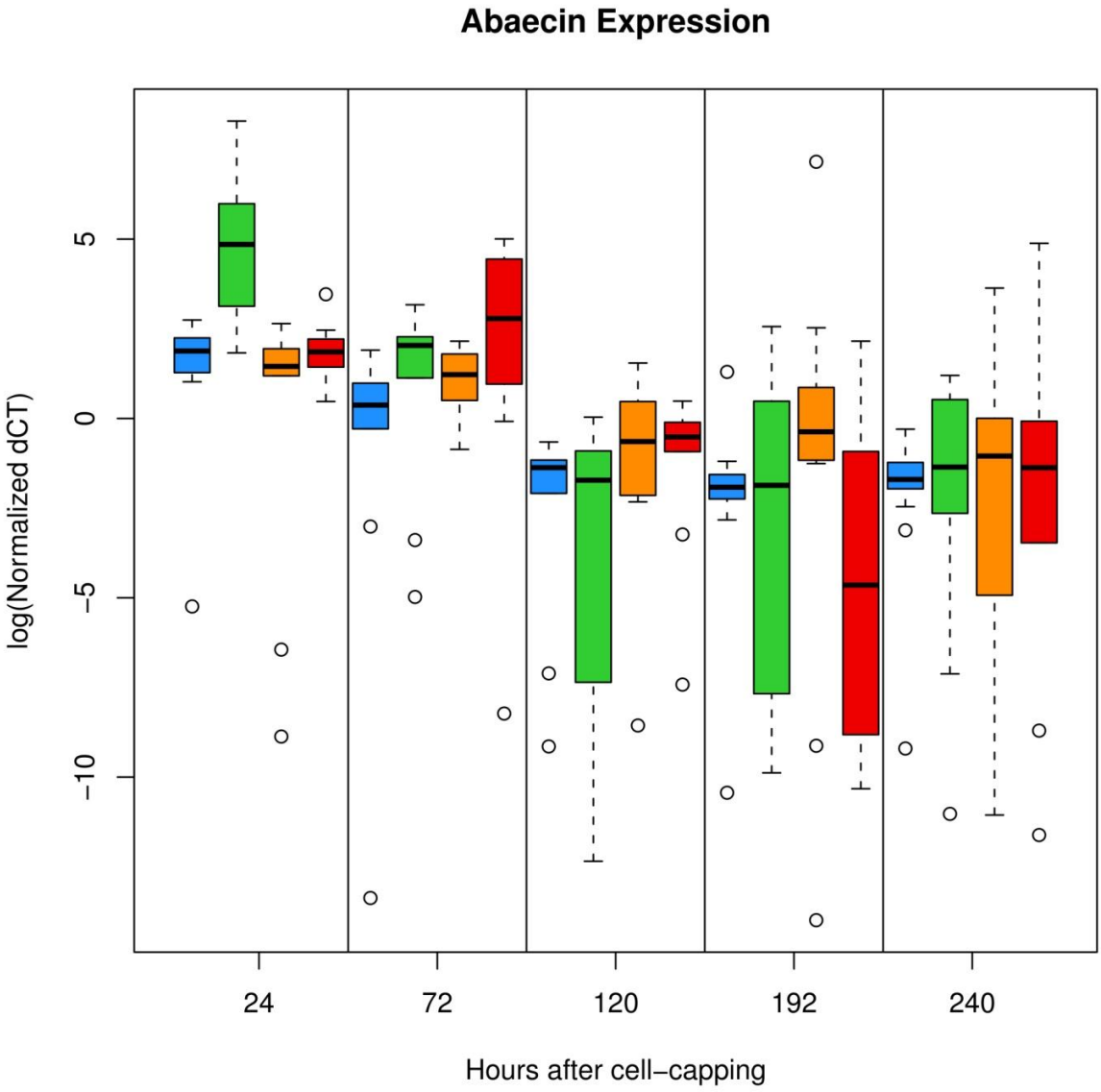

Figure 4. Boxplot showing expression levels of the antimicrobial peptide abaecin with increasing host development time in hours post-capping. The non-treatment control (blue) was compared to the artificially wounded positive control (green), singly mite-infested (orange), and highly mite-infested (red) cells. The black bar indicates the mean for each group with the box and whisker representing the inner-quartile range and overall range, respectively. Points considered as potential outliers are represented by open circles; however, all data were included in statistical analyses.

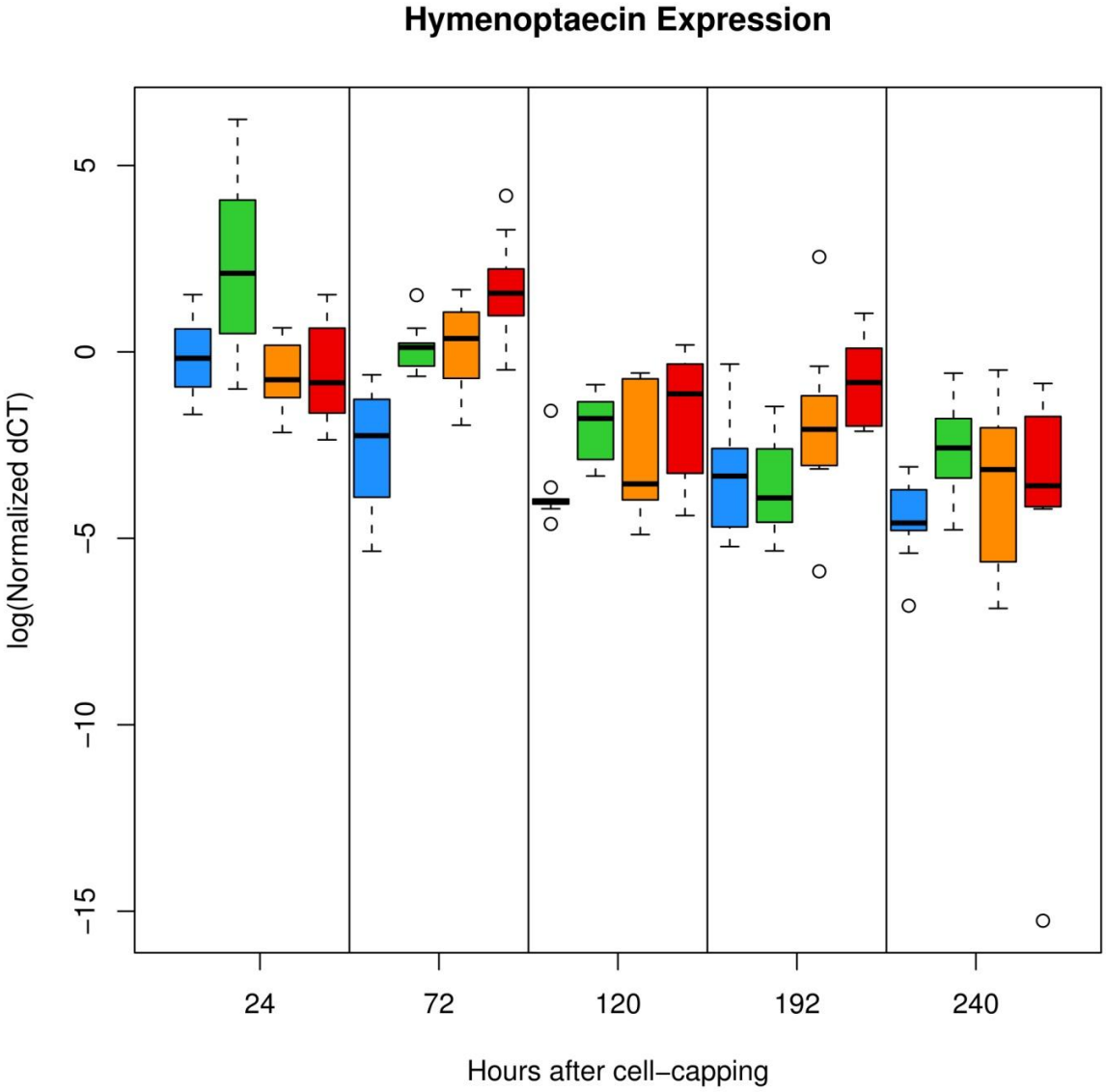

Figure 5. Boxplot showing expression levels of the antimicrobial peptide hymenoptaecin with increasing host development time in hours post-capping. The non-treatment control (blue) was compared to the artificially wounded positive control (green), singly mite-infested (orange), and highly mite-infested (red) cells. The black bar indicates the mean for each group with the box and whisker representing the inner-quartile range and overall range, respectively. Points considered as potential outliers are represented by open circles; however, all data were included in statistical analyses.

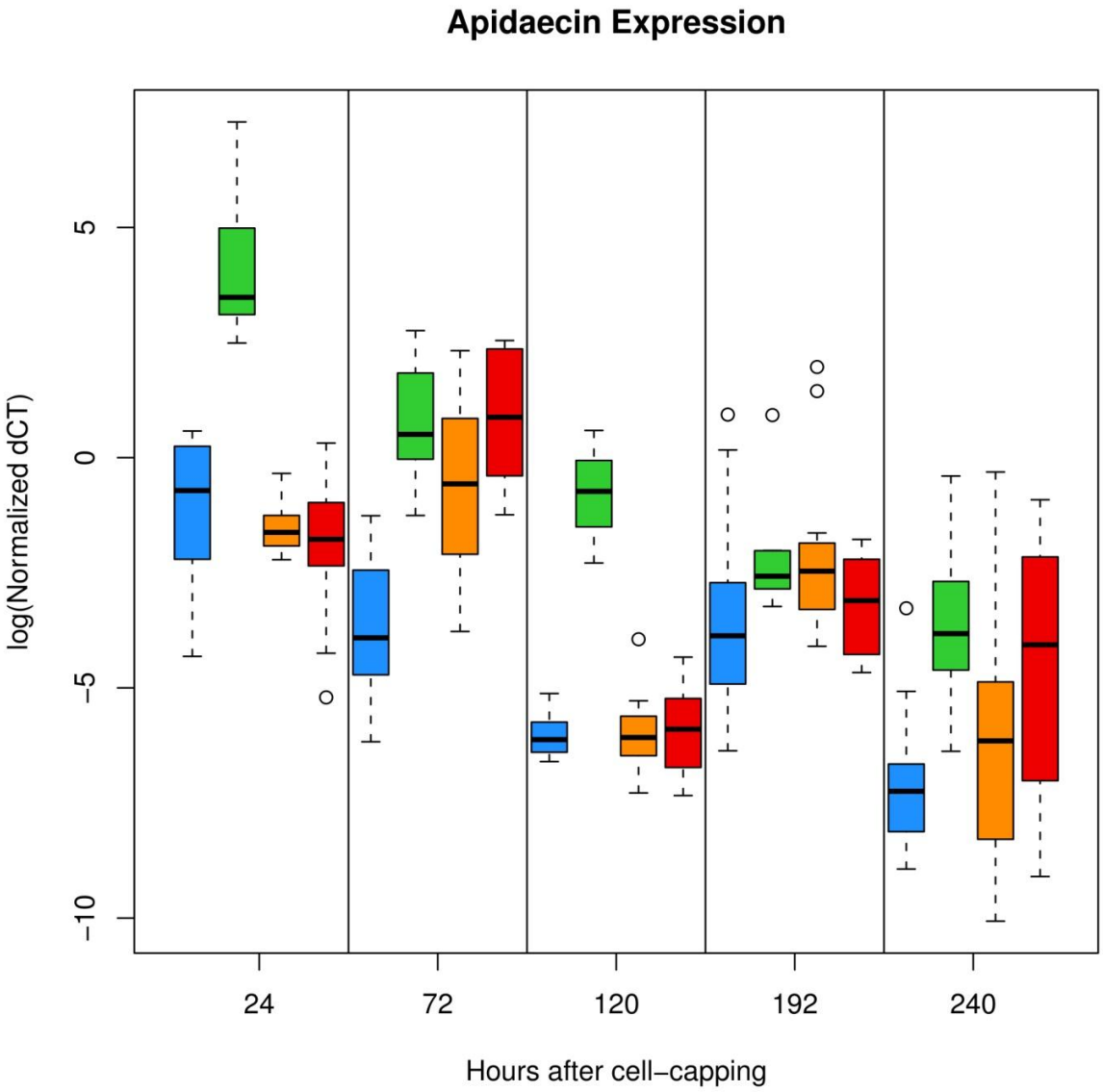

Figure 6. Boxplot showing expression levels of the antimicrobial peptide apidaecin with increasing host development time in hours post-capping. The non-treatment control (blue) was compared to the artificially wounded positive control (green), singly mite-infested (orange), and highly mite-infested (red) cells. The black bar indicates the mean for each group with the box and whisker representing the inner-quartile range and overall range, respectively. Points considered as potential outliers are represented by open circles; however, all data were included in statistical analyses.

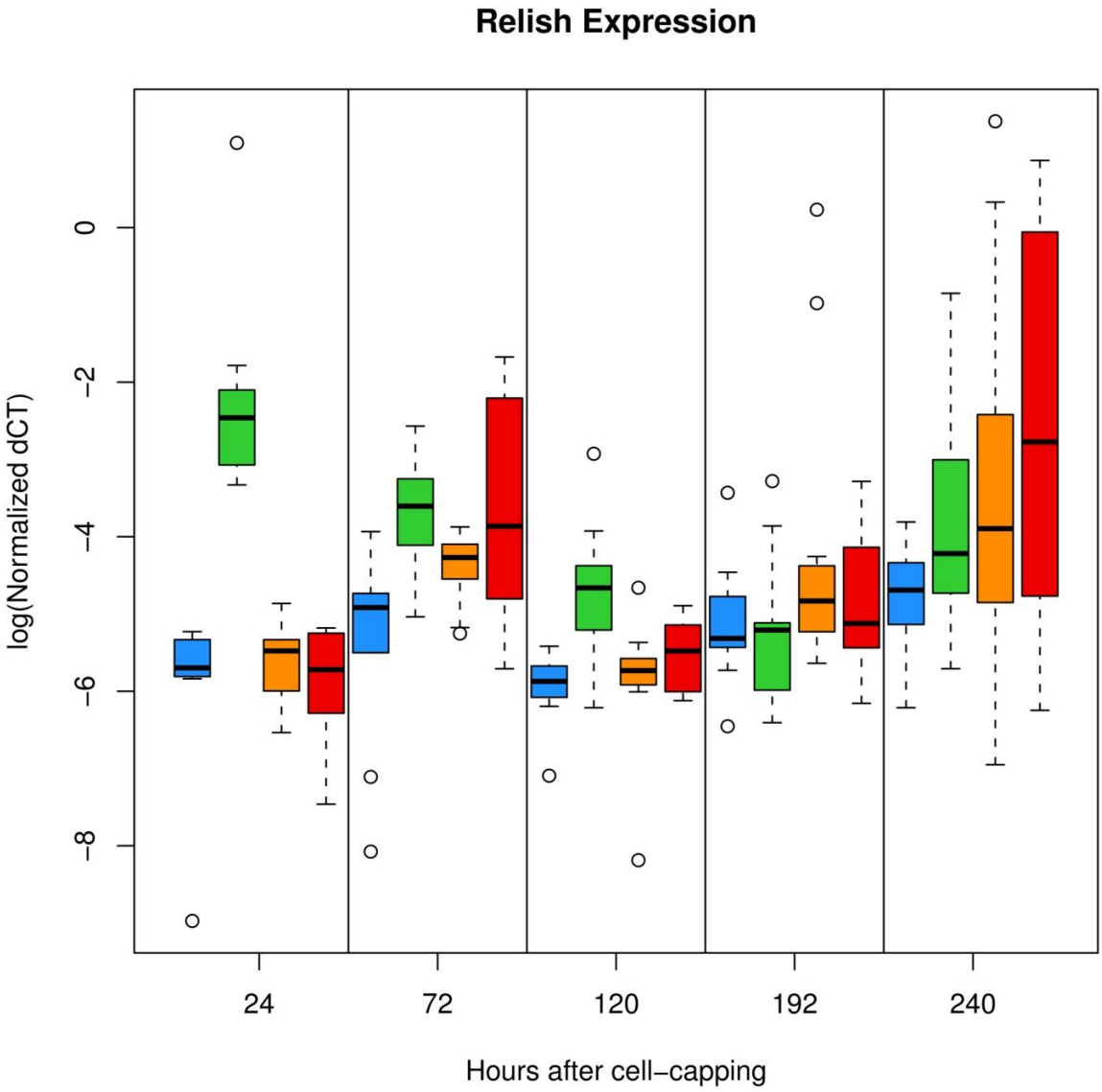

Figure 7. Boxplot showing expression levels of the transcription factor relish with increasing host development time in hours post-capping. The non-treatment control (blue) was compared to the artificially wounded positive control (green), singly mite-infested (orange), and highly mite-infested (red) cells. The black bar indicates the mean for each group with the box and whisker representing the inner-quartile range and overall range, respectively. Points considered as potential outliers are represented by open circles; however, all data were included in statistical analyses.

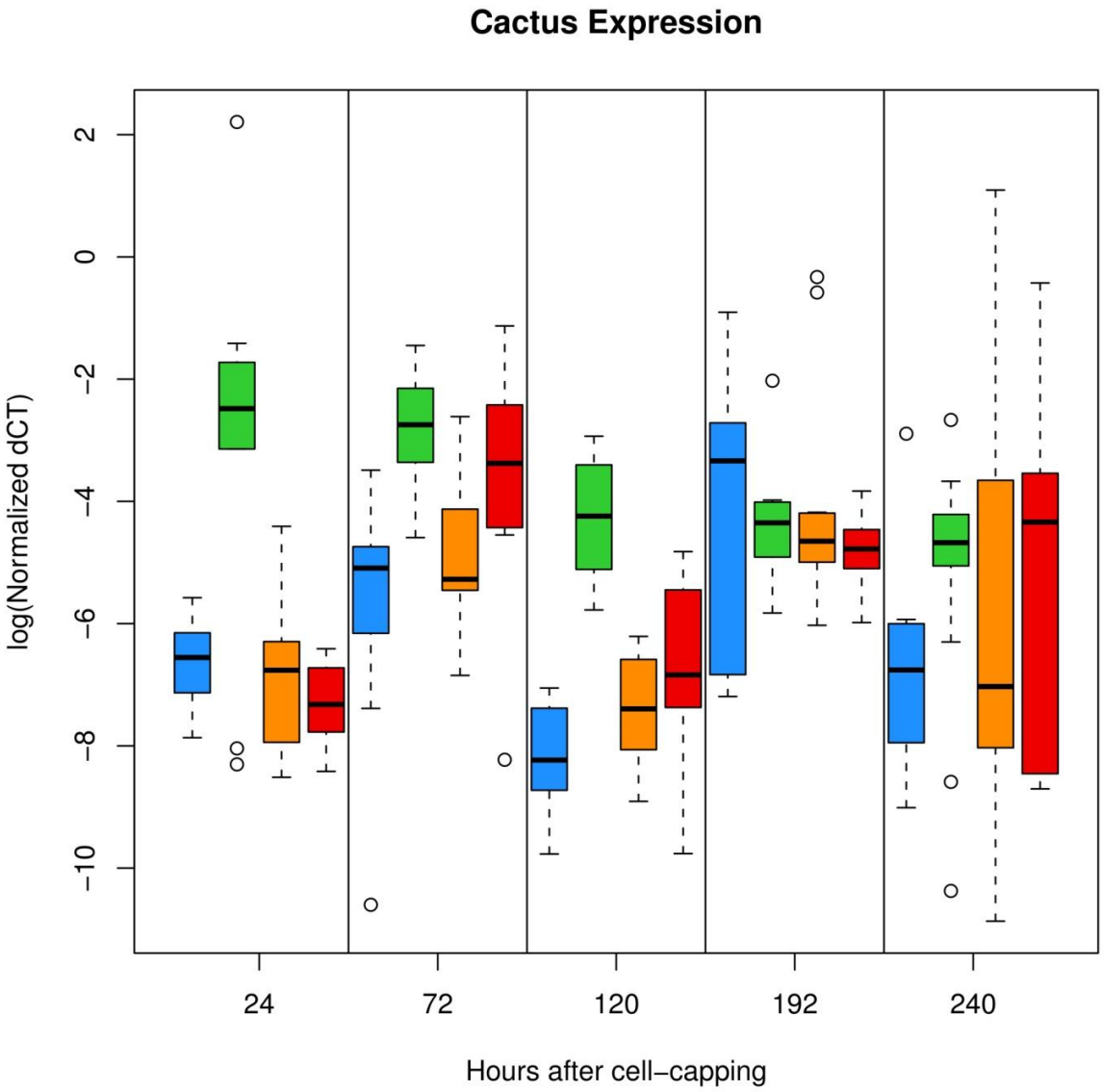

Figure 8. Boxplot showing expression levels of the transcription factor cactus with increasing host development time in hours post-capping. The non-treatment control (blue) was compared to the artificially wounded positive control (green), singly mite-infested (orange), and highly mite-infested (red) cells. The black bar indicates the mean for each group with the box and whisker representing the inner-quartile range and overall range, respectively. Points considered as potential outliers are represented by open circles; however, all data were included in statistical analyses.

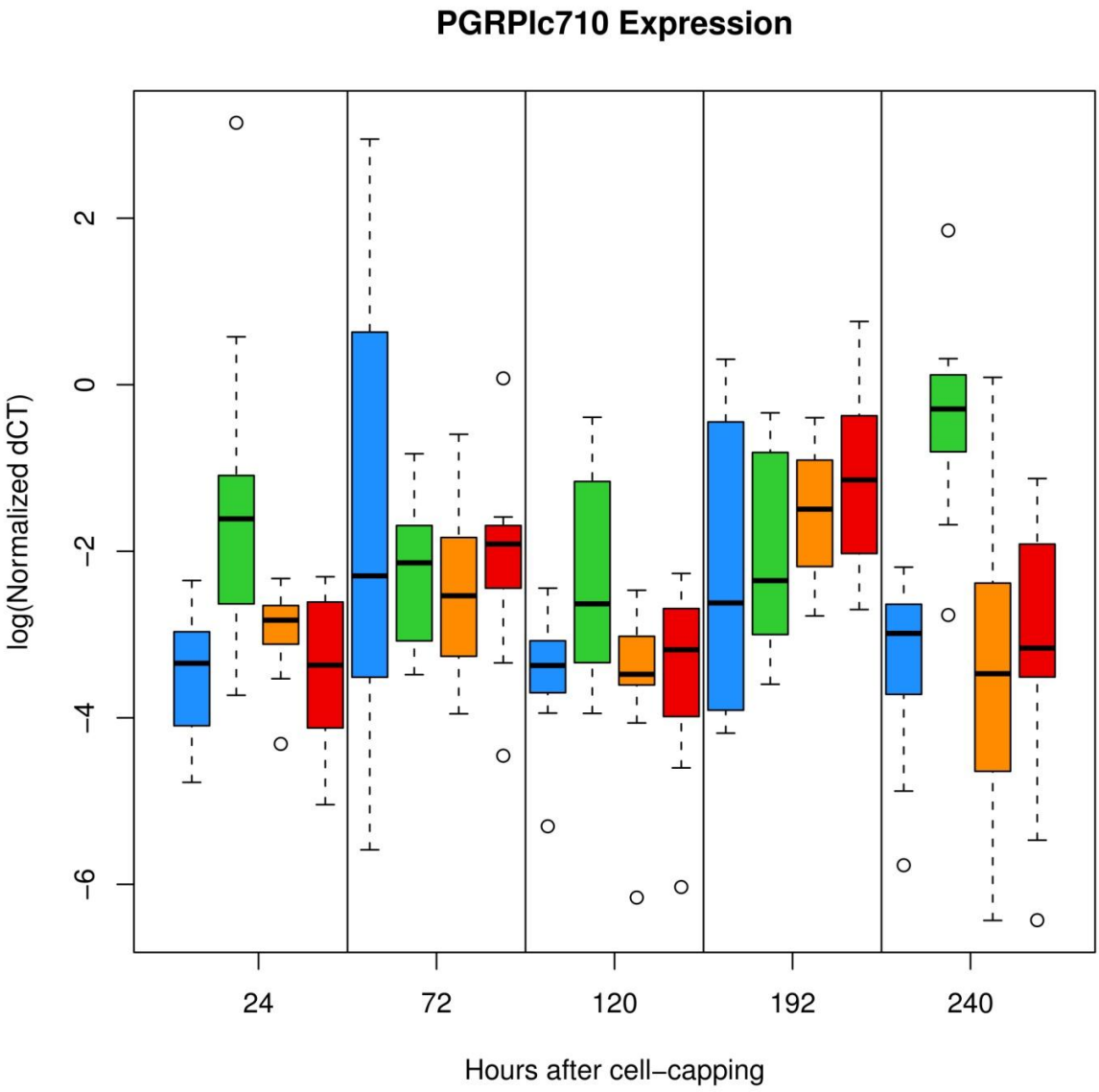

Figure 9. Boxplot showing expression levels of the pathogen recognition protein PGRPlc710 with increasing host development time in hours post-capping. The non-treatment control (blue) was compared to the artificially wounded positive control (green), singly mite-infested (orange), and highly mite-infested (red) cells. The black bar indicates the mean for each group with the box and whisker representing the inner-quartile range and overall range, respectively. Points considered as potential outliers are represented by open circles; however, all data were included in statistical analyses.

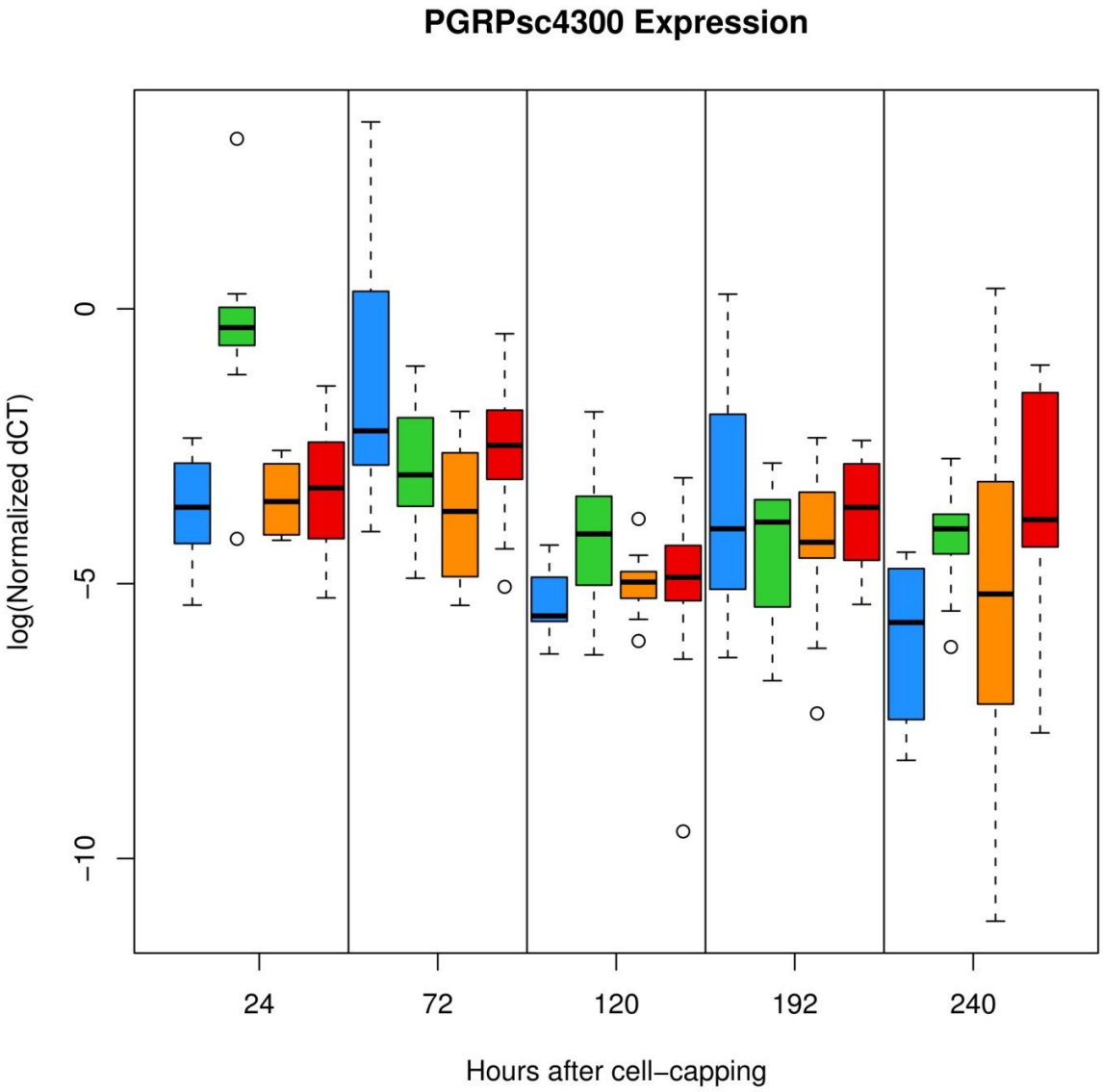

Figure 10. Boxplot showing expression levels of the pathogen recognition protein PGRPsc4300 with increasing host development time in hours post-capping. The non-treatment control (blue) was compared to the artificially wounded positive control (green), singly mite-infested (orange), and highly mite-infested (red) cells. The black bar indicates the mean for each group with the box and whisker representing the inner-quartile range and overall range, respectively. Points considered as potential outliers are represented by open circles; however, all data were included in statistical analyses.

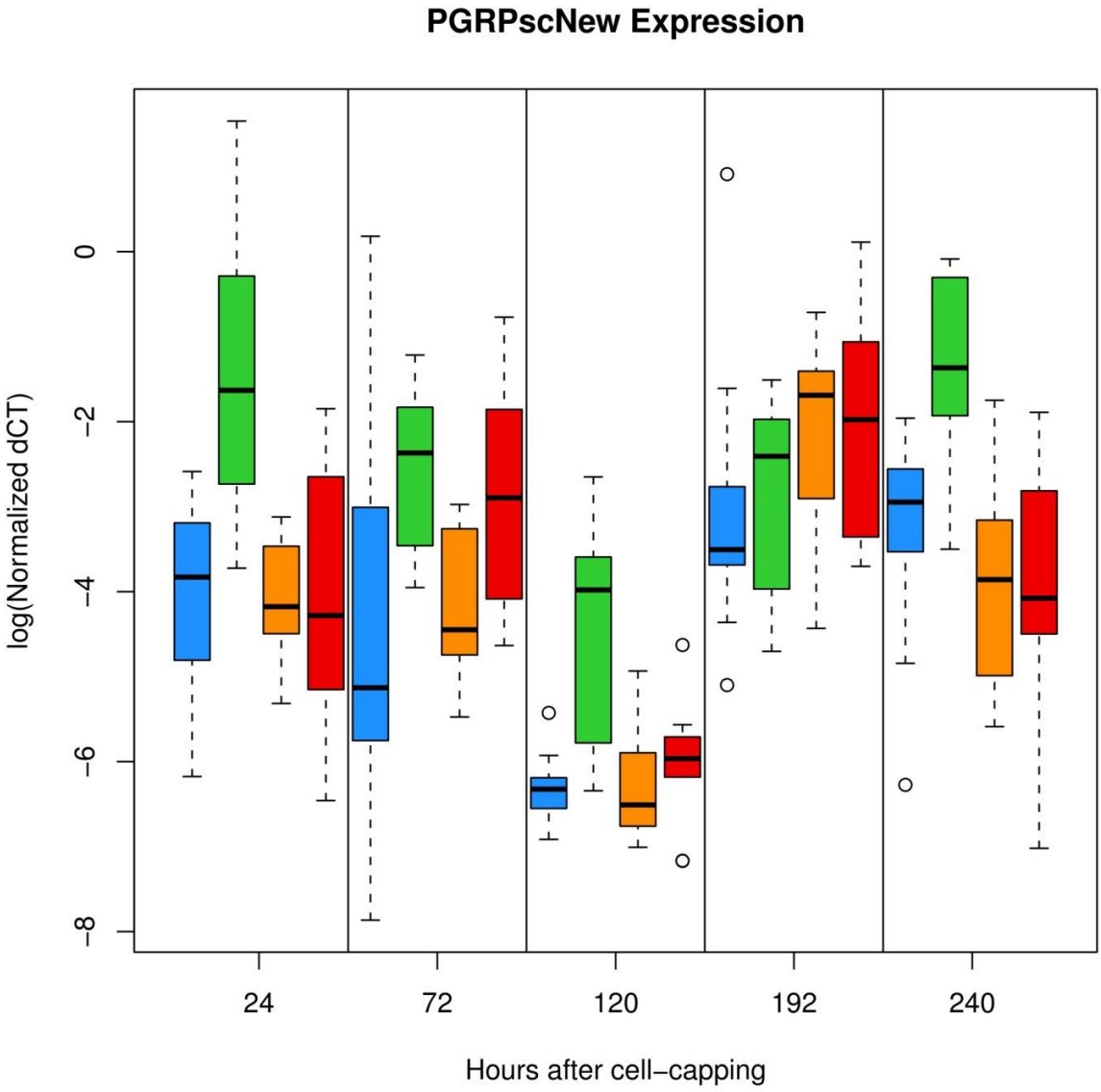

Figure 11. Boxplot showing expression levels of the pathogen recognition protein PGRPscNew with increasing host development time in hours post-capping. The non-treatment control (blue) was compared to the artificially wounded positive control (green), singly mite-infested (orange), and highly mite-infested (red) cells. The black bar indicates the mean for each group with the box and whisker representing the inner-quartile range and overall range, respectively. Points considered as potential outliers are represented by open circles; however, all data were included in statistical analyses.

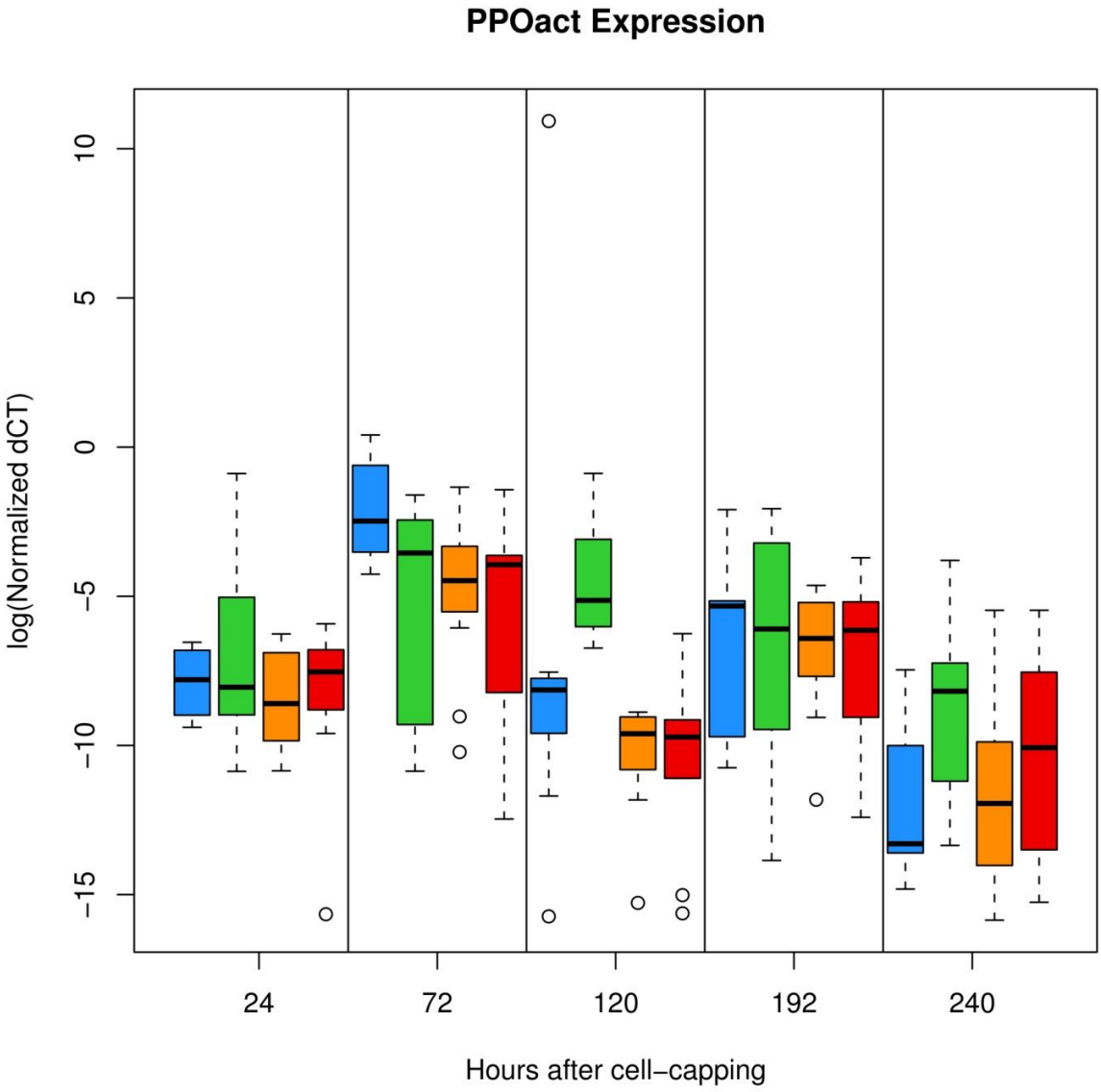

Figure 12. Boxplot showing expression levels of the phenol oxidase precursor PPOact with increasing host development time in hours post-capping. The non-treatment control (blue) was compared to the artificially wounded positive control (green), singly mite-infested (orange), and highly mite-infested (red) cells. The black bar indicates the mean for each group with the box and whisker representing the inner-quartile range and overall range, respectively. Points considered as potential outliers are represented by open circles; however, all data were included in statistical analyses.

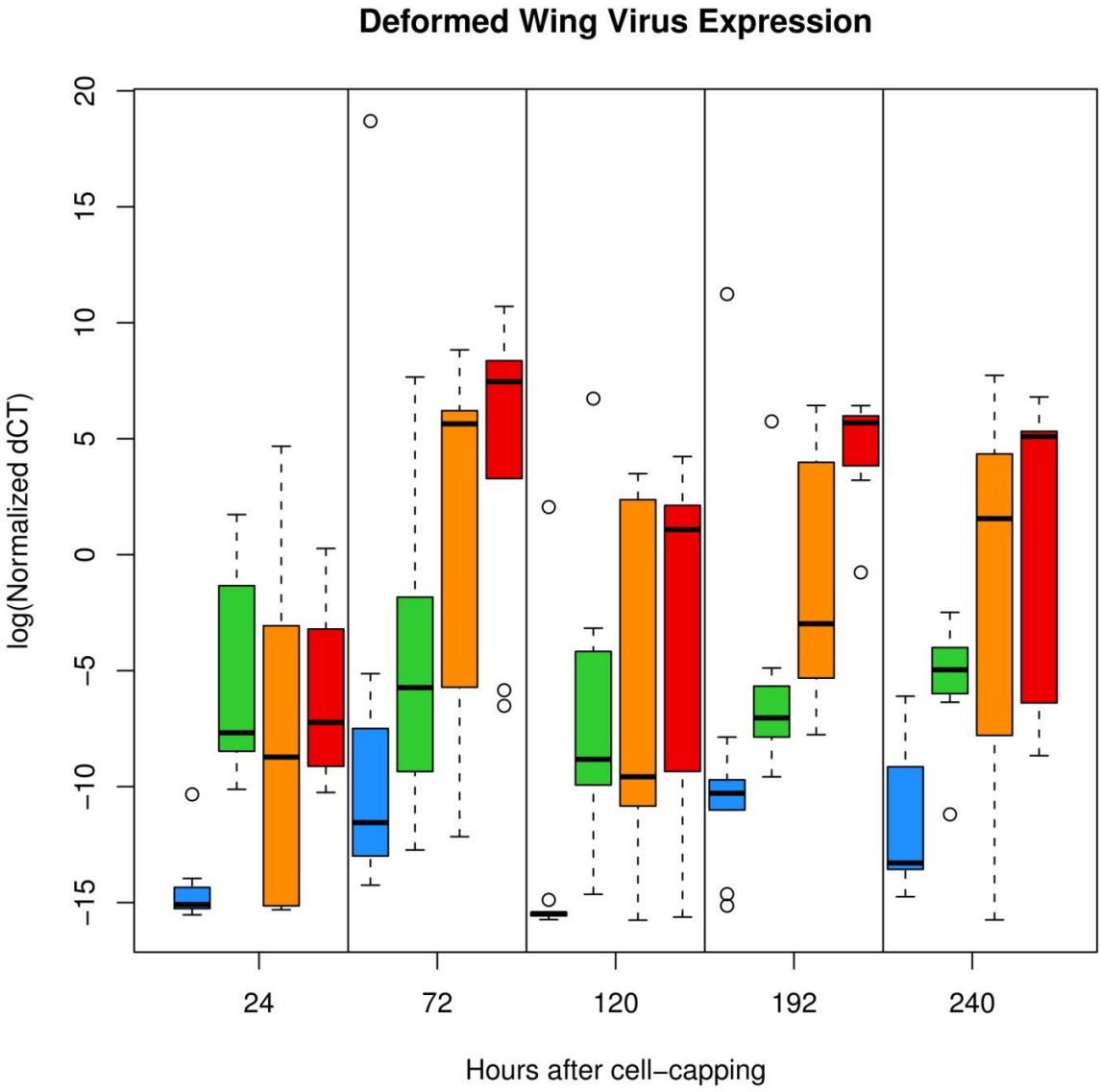

Figure 13. Boxplot showing expression levels of deformed wing virus with increasing host development time in hours post-capping. The non-treatment control (blue) was compared to the artificially wounded positive control (green), singly mite-infested (orange), and highly mite-infested (red) cells. The black bar indicates the mean for each group with the box and whisker representing the inner-quartile range and overall range, respectively. Points considered as potential outliers are represented by open circles; however, all data were included in statistical analyses.

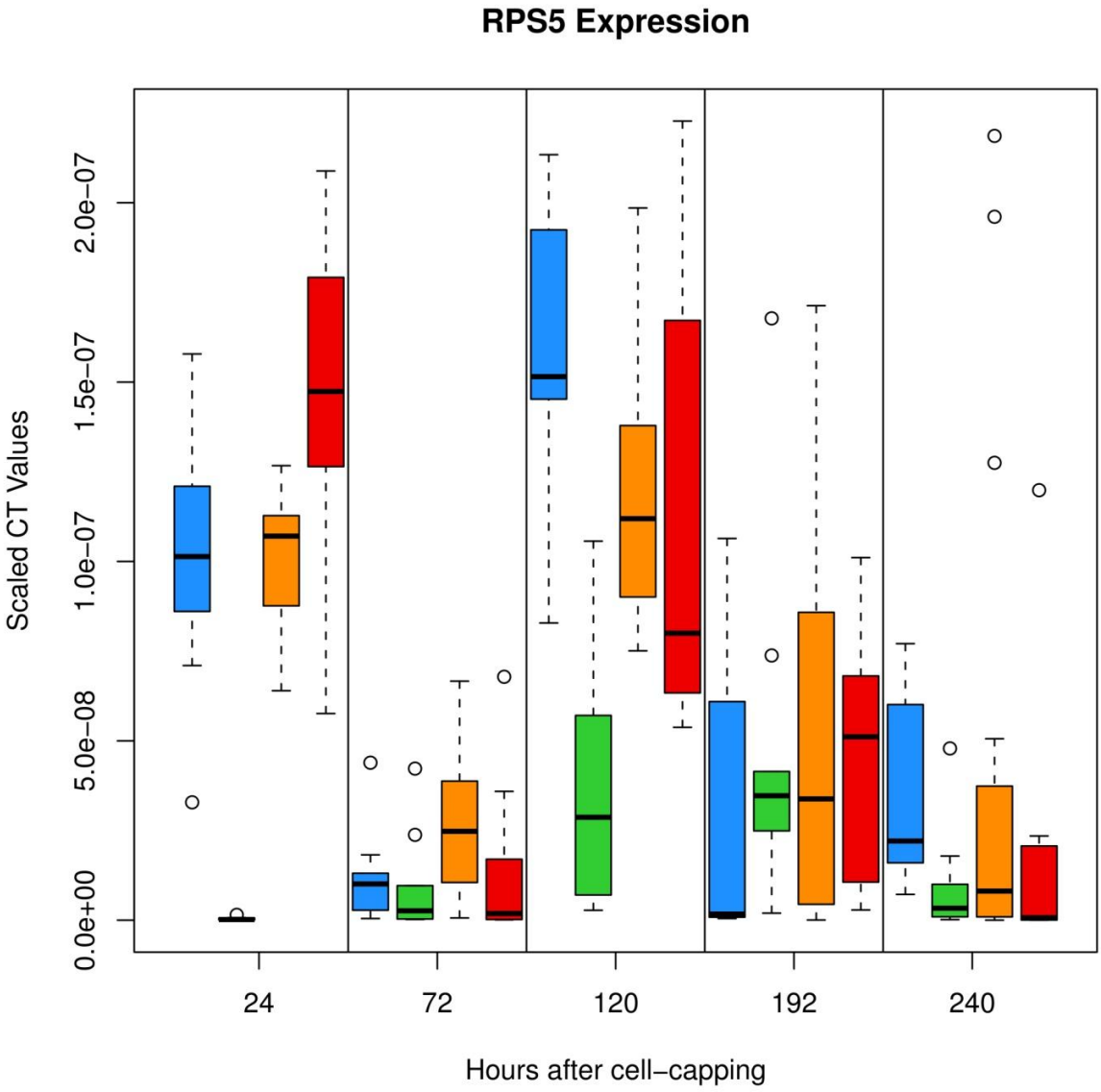

Figure 14. Boxplot showing expression levels of the housekeeping gene RPS5 with increasing host development time in hours post-capping. The non-treatment control (blue) was compared to the artificially wounded positive control (green), singly mite-infested (orange), and highly mite-infested (red) cells. The black bar indicates the mean for each group with the box and whisker representing the inner-quartile range and overall range, respectively. Points considered as potential outliers are represented by open circles; however, all data were included in statistical analyses.

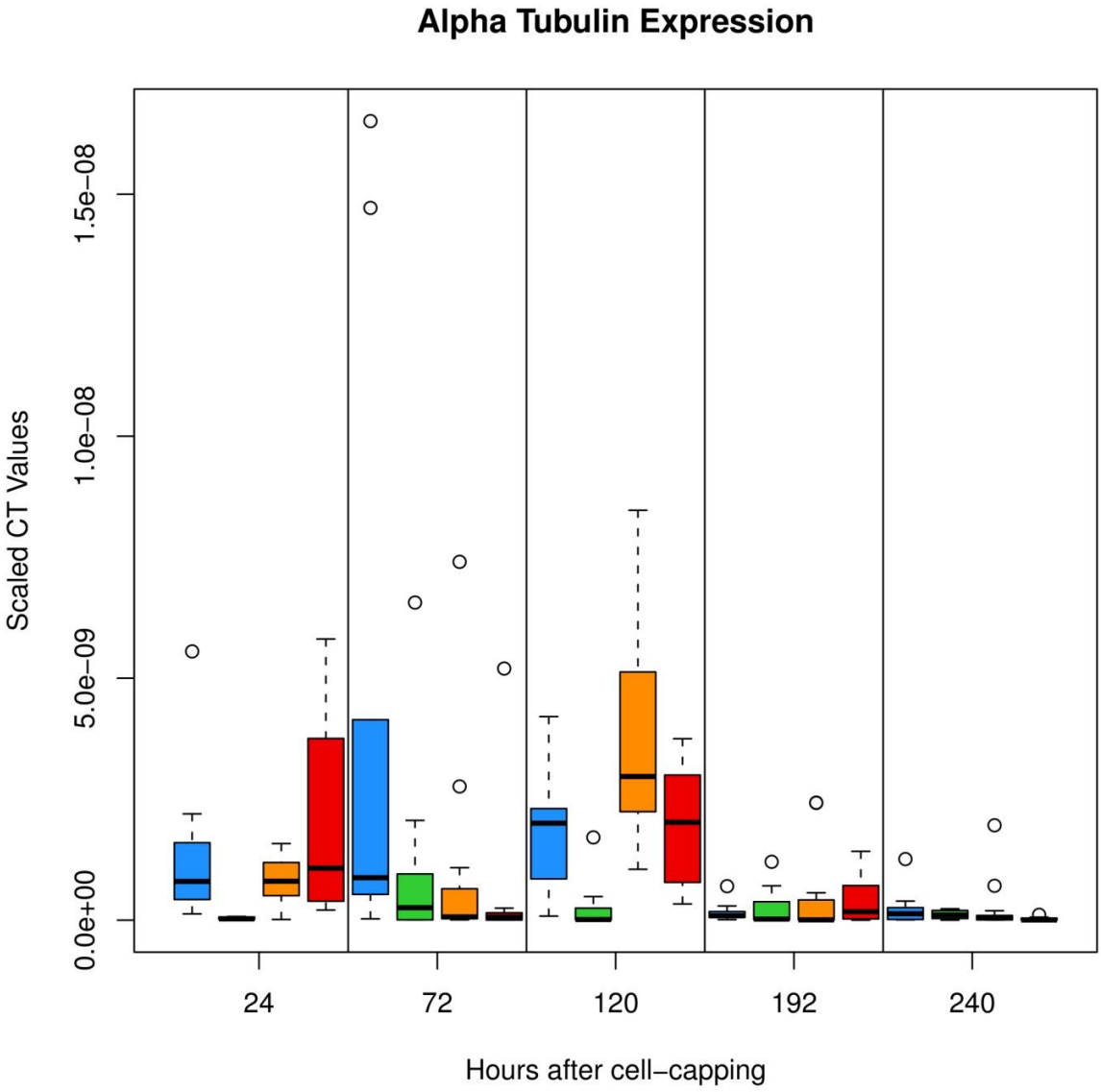

Figure 15. Boxplot showing expression levels of the housekeeping gene alpha tubulin with increasing host development time in hours post-capping. The non-treatment control (blue) was compared to the artificially wounded positive control (green), singly mite-infested (orange), and highly mite-infested (red) cells. The black bar indicates the mean for each group with the box and whisker representing the inner-quartile range and overall range, respectively. Points considered as potential outliers are represented by open circles; however, all data were included in statistical analyses.