# HONEY BEE GENE REGULATION AND THE TRANSCRIPTIONAL EFFECTS OF A PHEROMONE AND A PARASITE

A Thesis

by

LARA ELIZABETH BUTLER

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2008

Major Subject: Genetics

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| Committee Member, | Clare Gill |
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# ABSTRACT

Honey Bee Gene Regulation and the Transcriptional Effects of a Pheromone and a Parasite. (May 2008)

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Co-Chairs of Advisory Committee: Dr. C. Coates
Dr. T. Pankiw

The European honey bee, *Apis mellifera*, is a primarily beneficial insect for mankind. It has been utilized by humans for thousands of years for the products and services it provides. Crop pollination and honey production are two of the most economically beneficial activities of the honey bee. Though they have been important for many centuries and immeasurable amounts of effort have been expended investigating the methods and means to harness their natural abilities, a far lesser amount of attention has been directed towards exploring their molecular makeup. These experiments involve identifying modification of gene transcription as a result of exposure to a pheromone or a parasite. This data will provide information on the general types of transcripts involved in the biochemical response of the honey bee to the two stimuli and will also provide specific candidates for further investigation of their potential role in downstream behavioral events.

# DEDICATION

To those who believe in me without fail. You have helped me become a better person.

This would not be a reality without your support and love.

# ACKNOWLEDGEMENTS

I would like to thank the members of my committee for their support and guidance for my project. Dr. Tanya Pankiw taught me about honey bees and their care and biology. Dr. Craig Coates taught me about molecular biology. Dr. Clare Gill taught me about molecular techniques and gene discovery methods. Thank you all for everything. I would also like to thank all the members of the Coates and Pankiw labs for helping me out whenever I needed it. Brad Metz and Ramesh Sagili offered lots of honey bee assistance. Dr. Christine Gray offered volumes of helpful suggestions for molecular techniques. Paul Barron was an invaluable guide through a process he had already been through. Dr. Ahmed Mohammed offered helpful suggestions, as did the late Dr. Haisheng Tian. Haiwen Li and Dr. Vally KJM were always willing to help if I was too busy and Jennifer Murrell was always there for me with lots of support. I would like to thank Dr. Darren Hagen for not only being my best friend, but for also the years of support, advice, assistance, and kindness he offered from the moment we met upon starting graduate school. Thanks to JKL for the support, both financially and emotionally. Thanks to BJB for being unfailingly encouraging and supportive when I needed it most. Finally, thanks to my parents and my sister. Their support means everything. The people I have encountered in the pursuit of this degree have made a profound impact on me for the better. Thank you all for everything.

# TABLE OF CONTENTS

Page

ABSTRACT ..... iii

DEDICATION ..... iv

ACKNOWLEDGEMENTS ..... v

TABLE OF CONTENTS ..... vi

LIST OF FIGURES ..... vii

LIST OF TABLES ..... viii

INTRODUCTION ..... 1

EFFECT OF A PHEROMONE ON GENE TRANSCRIPTION ..... 5

- Background ..... 5
- Previous Research ..... 7
- Experimental Design and Setup ..... 14
- Results ..... 22
- Conclusion ..... 30

EFFECT OF A PARASITE ON GENE TRANSCRIPTION ..... 33

- Background ..... 33
- Experimental Design and Setup ..... 37
- Results ..... 49
- Conclusion ..... 50

SUMMARY ..... 52

REFERENCES ..... 53

VITA ..... 57

# LIST OF FIGURES

FIGURE Page

1 Illustration of differential expression by rt-PCR in QMP exposed and non-QMP exposed *Apis mellifera* workers ..... 29

2 Illustration of differential expression by rt-PCR in parasitized and non-parasitized *Apis mellifera* drone pupae ..... 49

# LIST OF TABLES

TABLE Page

1 Candidates from Pheromone SSH screen..... 25

2 Candidates from Mite SSH screen ..... 47

# INTRODUCTION

The European honey bee, *Apis mellifera* L., plays an important role in agriculture. Approximately 130 agricultural crops in the US are pollinated by bees, including fruit, nut, vegetable and fiber crops (1). In 2000, the added value of bee pollination to increased yield and crop quality was estimated at approximately $14 billion/year (2).

*Apis mellifera* is a highly social insect living as a colony comprised of one queen, a few drones, and many workers which perform a range of tasks in and out of the hive. These tasks change as the worker ages, a phenomenon called temporal polyethism (3-6). Although there are many possible jobs for the worker to perform within the hive, they do not all perform every job at all possible times (3, 4). Instead, workers tend to perform duties related to colony need for task performance and worker proximity to the task location (7). Tasks are generally divided into three main categories; a) tasks found in the center of the nest such as cell cleaning and capping, brood and queen tending, b) tasks in the periphery of the nest such as comb building, cleaning, and food handling, and c) outside tasks such as ventilating, guarding, and foraging (7). A worker starts out her life performing duties at the center of the hive and moves to the more peripheral tasks as she ages.

Semiochemicals are communication chemicals and are the principal form of communication used by honey bees. Pheromones are chemicals used for communication among members of a species (3). Social insect pheromones are further classified as

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releaser or primer pheromones (8). Honey bee releaser pheromones are generally volatile chemicals, and cause immediate and transient behavioral changes in susceptible individuals (9). Primer pheromones are non-volatile chemicals, altering endocrine, reproductive, and neurosensory systems (9). Changes in behaviors and physiologies in response to primers occur gradually and are permanent (9). Non-volatile pheromone is passed around when bees touch each other in normal contact, and is detected by direct contact with chemoreceptors on the antennae, mouthparts, and feet (10, 11). The pheromone then initiates a molecular signaling cascade in the bee and thus alters the biochemical profile of the bee, resulting in a change in physiology, gene activity, behavior, and/or neurochemistry (5, 12). Pheromones have been shown to have an effect on foraging activity in worker honey bees (13, 14), as has genetic background (15), and social environment (16).

The foraging behavior of honey bees makes them a vital pollinator of agricultural crops, however, *A. mellifera* is vulnerable in that they are preyed upon by a wide host of pests and pathogens. In a natural setting, honey bees are relatively defenseless against invaders that cannot be deterred by stinging. In an apiary, the proximity of hives can facilitate spreading of detrimental hive invaders, though a beekeeper can assist the bees in their fight against some of these illnesses. Common honey bee afflictions are pests like the Small Hive Beetle, the tracheal mite, and the Varroa mite and pathogens such as American and European foulbrood (17).

One of the most devastating problems an apiary can encounter is the Varroa mite, *Varroa destructor* Anderson and Trueman (18). This ectoparasitic mite was first discovered in the U.S. in 1986 (17). Since then, mite infestation has spread throughout *A. mellifera* populations across continental U.S. and most recently in Hawaii (19). Varroa has spread to almost everywhere in the world *Apis mellifera* is reared.

Varroa immunosuppress honey bees, feed on their hemolymph causing reduced adult body weight and protein content, and have been demonstrated to transmit pathogenic viruses causing many bee diseases (20-22). The Varroa mite has the ability to debilitate the colony to the point of elimination if there are no treatments administered. In 2000 it was estimated that there was a shortfall of 200,000 colonies in the U.S. due to Varroa and other associated diseases (23). The worldwide impact of colony losses due to Varroa has not been estimated.

Information about the genes involved in the honey bee's biochemical responses to both a pheromone and to Varroa mite parasitization is appealing because little is known at a molecular level in these two scenarios, though some light has been shed on the effects of a pheromone on gene transcription by another group of researchers. There are many possible techniques that could be employed in this situation, such as differential display, DNA microarray, SAGE, oligo array, or Suppression Subtractive Hybridization (SSH). The two most appealing of these techniques are differential display and SSH because they do not require prior knowledge of mRNA sequence data and are not really prohibitive due to cost. SSH is most appealing though, because it is more sensitive to

lower abundant cDNA transcripts than differential display and has the additional advantage of normalization, or removing common transcripts between the two RNA pools/samples. It is based on a technique called suppressive PCR that combines normalization and subtraction in a single step. This technique is especially desirable for the mite experiment, as sensitive detection methods are needed to specifically isolate the transcriptional differences due to parasitization status between two highly genetically similar honey bee drones. The normalization and suppression components of SSH should eliminate a considerable amount of any background due to the similarity of the bees being compared and thus would isolate a much smaller pool of candidate genes than differential display, which could then be screened for up- and down-regulation of genes due to treatment with the experimental conditions.

# EFFECT OF A PHEROMONE ON GENE TRANSCRIPTION

## Background

The age of foraging onset in worker honey bees is variable, though foraging itself is vital because of the nutrients it provides to all bees existing within the hive. Foragers bring water, nectar, and pollen into the hive from outside sources. These substances provide all necessary nutrients to members of the hive, without which the colony would not be able to sustain itself. Major factors known to alter the probability of foraging onset at a given point in a worker bee's life include genotype (15), social (16), and pheromone environments (13, 14).

There are two main classes of social insect pheromones: releasers and primers. Releaser pheromones cause rapid, transient changes in behavior and are mediated by the nervous system. Some example releaser pheromone responses include alarm, defense, or retinue behaviors. Primer pheromones cause a slower, long-term change that make take hours to days to manifest and occurs through putative response threshold shifts to different stimuli by altering reproductive, endocrine, developmental and neural systems (5, 12). As an example, primer pheromones are responsible for the partial inhibition of worker ovarian development and inhibition of hypopharyngeal gland development (9).

This experiment focuses on primer pheromones, because since more is known about them, the pheromone is commercially available for the experiments. Primer pheromone

causes more permanent changes to the honey bee as well, so the effect can be more carefully quantified because there is not a terribly small window of the pheromone's efficacy. There are only two characterized social insect pheromones; honey bee queen mandibular pheromone (QMP), which communicates the presence of a queen, and brood pheromone (BP), which communicates the presence of larvae (11). QMP works as both a primer and a releaser pheromone in worker honey bees. It consists of five major components, three acids and two aromatics: (E)-9-keto-2-decenoic acid (9-ODA), (R,E)-(-)- and (S,E)-(+)-9-hydroxy-2-decenoic acid (9-HDA), methyl p-hydroxybenzoate (HOB), and 4-hydroxy-3-methoxyphenyl ethanol (HVA) (24), respectively, and is commercially synthesized by PheroTech (Delta, Canada).

Investigations of the regulatory role of QMP on bee colonies have revealed several insights into the many physiological areas that pheromones affect. As a primer pheromone in workers, QMP delays foraging ontogeny (14), suppresses queen rearing behavior (11, 25, 26), affects mandibular gland development (27), variably suppresses ovary development (11, 25, 26, 28), and may affect maturation of adult bee antennal lobes (29). As a releaser pheromone, QMP elicits retinue behavior, which is characterized by a group of workers grooming, licking, and cleaning the queen, thus removing the pheromone from her body and eventually spreading it to other workers throughout the hive (30, 31).

Unfortunately, little is known about the effect of QMP on a transcriptional level. What transcripts are regulated when the pheromone is present? Which transcripts are most

affected? What role do the transcripts play in honey bee biology? This experimental aim was to investigate the molecular impact of a primer pheromone in a social organism.

## Previous Research

A previous study conducted by Grozinger et al. (32), investigated QMP effects on gene expression in the brains of adult honey bee workers. They assessed its effects in both cage and natural settings to test the robustness of the pheromone. Worker bees were produced from queens instrumentally inseminated with the sperm from one drone. In the cage experiment, 35 bees were exposed to 0.1 queen equivalents (QEq) of QMP, which they determined to be sufficient for inhibition of ovary development. The duration of exposure was 1, 2, 3, or 4 days, at which point the heads of the workers were removed and frozen at -80°C until needed.

Field studies involved three groups: a group that retained its original queen, a group that had no queen (0 QEq), and a group that was supplemented with polymer strips infused with ~30 QEq of QMP. After two days, the bees were collected, decapitated, frozen in liquid nitrogen, and stored at -80°C.

To determine pheromone activity in the cage experiment, worker bees were exposed to 0.1 QEq of QMP for 10 days and ovarian development of the workers was assessed according to Veltius. In the field, queen cells were counted to determine if QMP

suppressed queen rearing behavior. In both groups, a difference was seen in either ovarian development or queen rearing behavior.

Pools of 10 worker brains were chosen at random and direct competitive comparisons were performed with matched cDNA samples of QMP+ and QMP- bees from the cage experiments on a brain microarray. For the field experiments, pooled groups of 10 worker brains were applied to the microarray and were directly compared using a loop method for the three groups (original queen, no queen, and pheromone implanted polymer strip).

Grozinger et al. identified 2607 differentially expressed cDNAs in the cage experiment. In the field there were less differentially expressed cDNAs, with 697 between QMP+ and no queen, 1047 between QMP+ and natural queen, and a total of 335 that were co-regulated between the QMP+ and natural queen, relative to the queenless colony (32).

The gene with the biggest difference in mean expression levels in the cage experiments was the Kruppel homolog 1, Kr-h1, a transcription factor which showed a >1.2 fold increase in transcriptional level (32). Of the 129 cDNAs annotated as transcription factors, 39 were significantly regulated by QMP exposure, with 17 being significantly regulated by more than 10%. There were almost twice as many differentially expressed transcription factor cDNAs as the next closest functional category, which was oxidoreductases.

The Grozinger group controlled for age, however they did not incorporate a method for choosing which bee brains were pooled for use in the microarray experiment, thus potentially randomly selecting outliers compared to the average expression patterns. Another problem with their pooling method is that they did not control for the possibility of some brains being over represented in the cDNA mixture applied to the microarray due to differences in brain size or mRNA yield from each individual brain. They looked solely at the effect of the primer pheromone on the brain and failed to investigate its influence within the bodies of the workers. Their chosen method of detection limited them to only expose differences in gene transcripts contained on the microarray, and by their estimates their microarray only represented approximately half of the honey bee genome.

This experiment should yield some of the same transcript expression differences produced by the Grozinger group microarray, especially in up-regulation of transcription factors. However, due to the limitations of the microarray, this experiment should illustrate expression differences not detected by previous research. This should be specifically applicable to differences in the body, as the other group only investigated brain expression differences. By not looking at disparities in the body, they potentially missed differing transcript levels due to pheromonal effects on the reproductive organs.

The technique chosen to investigate transcriptional effects in the honey bee worker body and in the honey bee worker brain due to QMP exposure is Suppression Subtractive Hybridization (SSH), a method that compares transcripts between two samples and does

not utilize a microarray. SSH was chosen for investigating transcript expression differences because it is more economical than a microarray and is not limited to only detecting transcripts present on a microarray. One alternative to SSH for detecting differential expression is differential display, however because of the ability of SSH to identify less abundant transcripts due to its normalization component, it is the best candidate for identifying transcript differences due to QMP exposure.

SSH follows a protocol developed by Diatchenko et al. (33) and is available as a kit from Clontech (Palo Alto, CA) under the name of PCR-Select cDNA Subtraction Kit. The premise of the protocol consists of beginning with two comparable mRNA samples and allows for identifying specific transcripts with differing expression levels of between the two samples. There is a normalization component that results in reducing the contribution of highly abundant transcripts, thus equalizing the relative amounts of each individual transcript. Evans and Wheeler (34) used SSH to isolate a number of differentially expressed transcripts when they compared worker and queen honey bees.

Two subtractions will be performed for this experiment, and are outlined below. Each of those subtractions will be repeated using mRNA from bodies and heads. For each of the subtractions, one of the sources will be designated as the tester mRNA and the other will be identified as the driver mRNA. SSH attempts to accomplish two main goals; a) suppression of the amplification of transcripts common between the two samples resulting in the enrichment of tester specific transcripts and b) normalization of transcript levels by reducing the amplification of abundant tester mRNA transcripts.

a) Driver = Control Bee Heads, Tester = QMP Exposed Bee Heads. This subtraction should identify the transcripts that are up-regulated in the head of the bee by QMP exposure.
b) Driver = Control Bee Bodies, Tester = QMP Exposed Bee Bodies. This subtraction should identify the transcripts that are up-regulated in the body of the bee by QMP exposure.

Since SSH only detects differences based on the tester mRNA sample, the treatments must be switched to detect differences in the other sample.

c) Driver = QMP Exposed Bee Heads, Tester = Control Bee Heads. This subtraction should identify the transcripts that are down regulated in the head of the bee by QMP exposure.
d) Driver = QMP Exposed Bee Bodies, Tester = Control Bee Bodies. This subtraction should identify the transcripts that are down regulated in the body of the bee by QMP exposure.

An assay developed by Pankiw and Page (35), the Proboscis Extension Response-Threshold (PER-RT), will be used to determine the most suitable bees for analysis by identifying representatives with average PER-RT scores. This assay will also directly measure the sensitivity of the worker to sucrose, which has been demonstrated to have a robust association with foraging behavior (35). This is important because QMP is known to affect the age of first foraging (14) and acts as a modulator of sucrose sensitivity, thus a difference is expected in the sucrose response threshold between workers exposed to QMP and workers lacking QMP exposure.

Honey bees respond to sucrose by extending their proboscis when a drop of sucrose solution is touched to their antennae at a concentration that elicits a response. In the PER-RT assay, six concentrations of sucrose are tested; 0.1, 0.3, 1, 3, 10, and 30%. The concentration at which a bee first responds is considered its sucrose threshold and is converted to a score between 1 and 6, where a score of 6 is the most sensitive, corresponding to a response to 0.1% sucrose, and a score of 1 is the least sensitive, corresponding to a response to only 30% sucrose.

Sucrose RT, measured in adults 2-3 weeks before foraging and prior to any feeding or social experience, predicts individual forage choice of nectar, pollen, or water (36-38). Sucrose RT from lowest to highest shows the following individual forager choice behavior respectively; water < pollen < nectar < both (nectar+pollen) < empty. That is, bees with the lowest RTs to sucrose become water foragers, followed by pollen foragers, bees that return the colony carrying both nectar and pollen, nectar foragers, then bees with the highest RTs that return empty. Sucrose RT also organizes nectar choice behavior such that foragers with lower sucrose RTs return with less concentrated nectar than nectar foragers with high RTs (36, 37). Foragers returning empty, carrying nothing, are explained as having such high sucrose RTs that they do not find sufficiently concentrated nectar that elicits proboscis extension, a necessary reflex response for nectar collection (37).

It is unlikely that sucrose RT is causally linked to forage choice decisions of individuals. Not all individuals with measurable sucrose RTs become foragers. Some individuals spend all their lives performing tasks in the nest. It is more probable that the association between sucrose RT and forage choice behavior is indirectly linked through common causal factors acting on sensory perception systems of bees and foraging behavior. Bees with low sucrose RT are more responsive to other stimuli perceived by other sensory modalities. For example, low RT bees are more responsive to pollen in proboscis extension reflex assays, to low intensities of light in phototaxis assays, and are better able to learn patterns and odors in associative learning trials (39).

In the honey bee, there is a robust association between sucrose response threshold and foraging ontogeny (12, 40, 41). Pankiw (36) measured sucrose response thresholds of newly emerged African and European bees before they were exposed to food or other bees. The race of bee significantly altered sucrose response threshold, such that there was a significantly higher likelihood that Africanized bees would respond to lower sucrose concentrations. A model of sucrose response threshold and onset of foraging, showed that for every unit decrease of sucrose response threshold, probability to forage increased by 14.3% over a 30 day period.

## Experimental Design and Setup

Three hundred worker bees were collected within 0-15 hours of eclosion and the group was divided into two 150 bee units and placed into Plexiglas cages with a feeder allowing them unlimited access to 30% sucrose. A glass microscope slide was inserted into both cages, suspended by wires that were taped to the top of the cage. The slide in the control cage was coated with 5 µL of isopropanol on each side and allowed to dry before placement in the cage and the experimental cage contained a slide treated on each side with one queen equivalent (QEq) of QMP (Pherotech, Inc., Canada) dissolved in 5 µL of isopropanol. The slides were replaced each day to maintain a treatment of 1 QEq of QMP per day. The cages were placed into separate 37°C incubators so no carryover of the pheromone could occur due to loose bees flying between cages. After the groups were exposed to their treatments for 5 days, half of each cage was transferred into new cages containing a glass slide with the opposite treatment for one additional day. The other half of the bees remained in their respective treatment condition.

After 6 days, the bees were individually loaded into an apparatus for performance of the PER-RT assay and given water to repletion before the assay was performed to ensure they were not reacting to thirst. Time was allowed for the bees to calm down because loading into the apparatus excites them, giving them the potential to respond just to the stimulus of touch and not to the sucrose concentration. The sucrose solutions were loaded into syringes dedicated to that concentration and one drop of the solution was touched to the antenna of the honey bee using a 27 gauge needle. Their positive and

negative reactions were noted at each concentration and the first concentration at which the individual bees had positive reactions became their PER-RT score. Upon completion of the assay, the bees were removed from the testing apparatus, heads were removed from bodies, weighed separately, and then placed into individually labeled tubes. They were then flash frozen in liquid nitrogen and stored at -140°C.

To ensure that the bees used were not outliers, the PER-RT assay scores were utilized as a normality gauge. Average scores were calculated for each group. Bees having an average score were used in the SSH procedure, while the remaining bees were placed into storage for future comparisons. Total RNA was extracted from bees with the mean PER score using the column based RNeasy Mini Kit (Qiagen, Valencia, CA). Poly A+ mRNA was isolated from total RNA with the Oligotex direct mRNA Mini Kit utilizing a bead-based recovery system (Qiagen Valencia, CA).

Messenger RNA was used because it consists of a transcriptional profile of an individual, instead of using DNA or RNA which do not necessarily reflect genes or transcripts actively being utilized by an individual. It is important to isolate mRNA for these experiments, rather than using total RNA, because total RNA contains ribosomal RNA which swamps out the mRNA transcripts, making detection of actual differential expression above background near impossible using the employed technique. Even though an oligo dT primer is used to synthesize the cDNA, and would thus specifically target the A tail of mRNA for transcription into cDNA, mRNA only comprises around 1-3% of the total RNA of a cell. By specifically isolating mRNA, this increases the

probability that reverse transcriptase will hybridize with mRNA instead of ribosomal RNA, which can comprise more than 80% of a cell's total RNA. This also ensures that the lower abundant mRNA transcripts are represented because if total RNA were used in the reverse transcription reaction, the probability of lower abundant mRNA being transcribed into cDNA would be very unlikely.

To ensure there was an equal mRNA contribution from each bee, total RNA concentration was used for a rough input estimate based on spectrophotometric data, because mRNA is difficult to quantify. Double stranded cDNA was synthesized using the pooled mRNA as a template, as mRNA is not a template for PCR. The resulting cDNA became the starting material for the SSH, which was performed according to a protocol outlined in the PCR-Select cDNA Subtraction Kit (Clontech Mountain View, CA). The cDNA was digested with a restriction enzyme (*Rsa* I) and two specific adapters included in the kit were ligated onto the ends of only the tester cDNA in two separate reactions. The actual SSH procedure entailed two rounds of hybridizations followed by two PCR amplifications outlined in the kit's protocol. In brief, two reactions were set up for each driver cDNA sample. In each reaction, driver cDNA was combined with one of the two adapter ligated tester cDNA samples and 4X Hybridization buffer. The samples were denatured at 98°C for 90 seconds and then were left for 8 hours at 68°C. In the second round of hybridization, the two samples from the first hybridization were mixed together. Fresh denatured driver was added to the first hybridization mixture, along with fresh 4X hybridization buffer, to further enrich for differentially expressed sequences and was incubated at 68°C overnight in a second

round of hybridization. After the hybridizations, an aliquot of subtracted cDNA (the sample had been hybridized twice) and a corresponding aliquot of unsubtracted tester cDNA (the sample had not been subjected to hybridization) were subjected to two rounds of PCR. The primers were specific to the adapters ligated onto the ends of the tester cDNA molecules. The first PCR program started with 75°C for 5 minutes to extend the adaptors. The samples then immediately went thru 27 cycles of 94°C for 30 seconds, 66°C for 30 seconds, and 72°C for 1.5 minutes. A 1:10 dilution was taken from the first PCR and subjected to a secondary PCR using nested primers which were also specific to the adapters. The secondary PCR program consisted of 14 cycles of 94°C for 30 seconds, 68°C for 30 seconds, and 72°C for 1.5 minutes. An aliquot of the results were analyzed on a 2.0% agarose/Ethidium Bromide (EtBr) gel run in 1X TAE buffer. The SSH process allowed for identification of the expression differences between average representatives of the control group and the QMP group. Bodies and heads were subjected to the SSH procedure independently to enable a comparison to the results generated by the Grozinger group.

The products from the SSH procedure were cloned into the TOPO 2.1 vector from Invitrogen (Carlsbad, CA) and transformed into TOP10 electrocompetent cells (Invitrogen, CA). The cells were placed into a shaking incubator at 200 rpm and 37°C for an hour and were then plated on LB/agarose plates containing Kanamycin. Large 250 mL plates (Genetix Boston, MA) were used for plating and the colonies were picked robotically by a Q-bot (Genetix Boston, MA) and placed into 384 well plates (Genetix Boston, MA) containing liquid freezer media. Of the culture that was plated, the robot

searched for colonies that met certain criteria for colony size, shape, and diameter. The robot picked 1145 colonies from the body library of bees exposed to QMP (QMP+), 995 colonies from the QMP+ head library, 1876 colonies from the QMP- (non-QMP exposed) body library, and 1869 colonies from the QMP- head library. After allowing the plates to grow in an incubator at 37°C for 24 hours, a manual replicator was used to spot replicate colonies onto nylon membranes resting on Kanamycin50/LB plates. The membranes were allowed to grow for 12 hours at 37°C in an incubator and then were pulled off the plates. The colonies on the membranes were chemically denatured by placing on Whatman paper saturated with 0.5M NaOH, and 1.5M NaCl for 4 minutes and neutralized by placing on Whatman paper saturated with 0.5M Tris-HCl, pH 7.4, and 1.5M NaCl for 4 minutes. The colonies were then fixed by crosslinking the DNA to the membranes using an auto-crosslinker (Stratagene UV Stratalinker-2400 La Jolla, CA, on the 'auto crosslink' setting).

Radioactive probes were created using unsubtracted and subtracted products from the SSH procedures. Each SSH experiment produced a collection of cDNAs, an unsubtracted set and a subtracted set. The unsubtracted set is one that had not gone through the SSH procedure, thus it represents the cDNA profile before any portion of the subtraction procedure was performed. The subtracted cDNA set was the result of the SSH procedure, so it was expected to have differing levels of cDNA copy numbers than the original stock due to suppression of common transcripts within the sample and subtraction of common transcripts between the two samples being compared. Radioactive probes were synthesized using these cDNAs as templates. To create the

probes, 40-100 ng of cDNA from the secondary PCR was combined with dNTPs (lacking dATP), a random primer mix, α -$P^{32}$ ATP, and 2-5 units of Klenow. This mixture was incubated at 37°C for 30 minutes. EDTA was added to terminate the reaction and then the reaction was cleaned up using a column based cleanup procedure to remove free nucleotides. The PCR Select Differential Screening Kit from Clontech (Mountain View, CA) was used to screen the clones, as it contains all materials needed to synthesize the probes used in screening the membranes (except for the radioactive nucleotide). For the QMP+ head library, there was an unsubtracted QMP+ head probe that consisted of cDNA that did not go thru the SSH procedure and a subtracted QMP+ head probe that was the end result of the SSH procedure. There were also probes for the QMP- head library, the QMP+ body library, and the QMP- body library, making a total of eight α- $P^{32}$ radiolabelled probes for this experiment.

The membranes were pre-hybridized with a 1:1 solution of 20X SSC and a blocking solution made by Clontech (Mountain View, CA) from their PCR-Select Differential Screening Kit, specifically designed to work by blocking the adapters used in the SSH procedure. This prevented hybridization of the probe with the sequences specific to the adapters, ensuring that the probe bound only to a complementary sequence in the cDNA library. The membranes were hybridized with an individual probe for 16 hours and then washed twice with a low stringency buffer (2X SSC, 0.5% SDS) and then twice with a high stringency buffer (0.2X SSC, 0.5% SDS) for 20 minutes per wash with agitation at 68°C to reduce background hybridization. The membranes were then heat-sealed in plastic film and placed in film cassettes with Kodak Bio-Max film to visualize

hybridizing signals. Membranes from each original 384-well plate were eventually exposed to all four of the probes corresponding to that library (if the plate was from the QMP+ head library, it would be allowed to hybridize with unsubtracted QMP+ head, subtracted QMP+ head, unsubtracted QMP- head, and subtracted QMP- head probes with stripping of each probe in between each exposure). The autoradiographs from the four hybridizations were compared and differences were noted. The best candidates for further differential expression analyses were those that were very visible in the library they were originally from (if it was from the QMP+ library, it was desirable to see a very visible signal on the films from the QMP+ probes) and little to no signal when hybridized with probes from the reverse subtraction or non-subtracted material (QMP- in this example).

Signal intensities were initially estimated manually comparing autoradiograms from hybridizations with different radioactive probes. Comparisons focused on the probes created from the forward and reverse subtracted cDNAs. Clones showing signal intensity differences between the forward and reverse probes were the primary focus of this investigation. Candidates were identified that showed a signal when the membrane was exposed to the probe from one library but not when exposed to the probe from the corresponding library. Five candidates from each of three groupings (high intensity, medium intensity, and low intensity) were isolated and sequenced for further analysis from all four libraries (QMP+ head, QMP- head, QMP+ body, QMP- body), for a total of 60 initial sequences. The three intensity groups represented transcripts that appeared to have high, moderate, and low differential expression levels. Since the candidates

were cloned into TOPO 2.1, M13 reverse primer was used for PCR sequencing, using the following parameters: 30 seconds at 94°C, 15 sec at 55°C, and 1 minute at 72°C for 40 cycles.

Using the Vector NTI 8.0 suite of programs (InforMax, MD), flanking vector sequence was removed from the sequence data obtained from the 60 candidate clones. EMBL EBI-Heidleberg (European Molecular Biology Laboratory), which was the depository for the initial honey bee genome sequence data (before the data from the sequencing effort was fully assembled in the honey bee genome project), was queried for longer sequence data using personal sequence information from the initial 60 candidates sequenced. The sequence data obtained from this experiment was compared to the sequencing database utilizing BLAST, which is a sequence alignment algorithm. If the query produced a significant match to something in the database (an e value of $10^{-4}$ or less), then the full length data was used to provide the deduced amino acid sequence to be used for continued analyses. If there were no significant matches, then the initial sequence data was used. NCBI (42) was then utilized to attempt to infer a potential function for the differential expression candidates by using BLAST to find homologous sequences with known functions in other species. Primers were then developed for candidate genes which appeared multiple times in the sequence data and reverse transcriptase Polymerase Chain Reaction (rt-PCR) was performed using experimental bees to illustrate differential expression. The bees used in the rt-PCR had the same PER score as the bees used in the SSH procedure. rt-PCR is a technique used to investigate the presence of a desired sequence by taking the mRNA from a sample, transcribing it

into cDNA, and then attempting to amplify a sequence using specific primers. The results are then run on a 2.0% agarose/EtBr gel in 1X TAE buffer to visualize.

## Results

QMP is known to affect the age of foraging onset, thus differences between the PER-RT score of workers reared with or without QMP were expected. Indeed, a mean PER score difference of almost 1.5 was detected between the two groups. The control group had a mean PER score of 3.96 and the QMP exposed group had an average of 2.49. Utilizing the average scores to select the bees to be included in the experimental procedures prevented use of outliers that were either very sensitive or insensitive to QMP (which would skew the SSH results because the bees would produce different amounts of mRNAs based on the altered sensitivity, thus it would affect gene transcription levels). Bees having a score of 4 in the control group and 2 or 3 in the QMP rearing environment group were chosen for use in the SSH comparison.

Table 1 lists the differential expression candidates isolated through this experiment, though only a few were selected for further study. Of the QMP+ libraries, two transcripts from the body were chosen to investigate, both of which appeared 2 out of 15 times in the sequenced candidates: cytochrome oxidase (Cypm3r9), which is involved in metabolism, and myosin 3 light chain (mlc1), which is the regulatory light chain of myosin. Only one was chosen from the QMP+ head library: Rab9A, which is involved in protein transport and appeared twice.

In the QMP- libraries (both head and body), only one of the two libraries provided any pursuable transcripts to explore. The QMP- body library provided two candidates to investigate further: troponin c (TpnC), observed in 3/15 of the candidate sequences, which is involved in cell signaling, and imaginal disc growth factor 2 (Idgf2), which is involved in embryonic development and is also expressed in the ovary. Unfortunately a problem arose with the QMP- head library and the sequences from 14/15 of the transcript candidates most closely resembled viruses transmitted by mites. Mite viruses were also seen in 8/15 of the QMP- body sequences. The viruses identified were deformed wing virus, Kakugo virus, and Varroa destructor virus 1.

Primers were designed for the 5 candidates utilizing the published sequences on NCBI. Vector NTI 8.0 analyzed the sequences and identified primers which would yield a 400-500 base pair PCR product. The primer sequences for cytochrome oxidase were CYPm3r9rev-CGAGGTACAGCCTTGGCAT and CYPm3r9fwd-CGAGCCAATGGTTGGTTT, myosin 3 light chain were Mlc3rev-GGGCAGGTACTGAAGGCAGCGATT and Mlc3fwd-TAGATGCATGCTCGAGCGGC, Rab 9A were Rab9Arev-GCCGAGGTACAATTCGCAATATAGTA and Rab9Afwd-GCTGATGTTCAAGAAGGATCAACA, Troponin C were TpnCrev-TCGCGGCCGAGGTACAAGAT and TpnCfwd-CGTGGAAAACGCGCGATT, and imaginal disc growth factor primer sequences were Idgf2rev-

AGCACGGGACACCGGGTTCCAAGAT and Idgf2fwd-TACTACTTGTCGAGCGCGAGGCGTC.

The reactions were subjected to the following parameters: an initial denaturing step of 94°C for 5 minutes, followed by 25 cycles of 94°C for 30 seconds, the primer specific annealing temp for 30 seconds, and 72°C for 1 minute. After the 15 cycles, an aliquot was removed from the tube for analysis and the remaining sample was subjected to 5 more cycles of amplification. This continued until there were 6 aliquots from 15, 20, 25, 30, 35, and 40 cycles. The results were analyzed on a 2.0% agarose/EtBr gel in 1X TAE buffer.

The rt-PCR reaction results supported the findings illustrated in the SSH screening procedure (Fig. 1). Cytochrome oxidase, myosin 3 light chain, and Rab9A transcripts were expressed at higher levels in QMP reared bees, though Rab9A expression was only slightly higher than in non-QMP reared bees. Both Troponin c and Idgf transcript levels were also confirmed to be expressed at higher levels in bees without exposure to QMP.

Table 1: Candidates from Pheromone SSH screen. Sequences were queried against NCBI database in an attempt to infer function. Candidates are grouped according to library they were a part of. Also included in the table is the corresponding score and E-value of the blast search of each candidate, along with the number used for identification and how the candidate was scored in the screening process.

| **Library** | **Candidates showed most similarity to…** | **In organism…** | **blast Score** | **blast E-value** | **Number of candidates matching blast search** | **Intensity difference in screen (high, medium, or low)** |
|---|---|---|---|---|---|---|
| **QMP+ Body** | | | | | | |
| | putative cytochrome bc-1 | Haematobia irritans irritans | 228 | 2e-68 | 2 (1AF2, 1AF19) | High |
| | diacylglycerol O-acyltransferase 1 (Dgat1) | Rattus norvegicus | 322 | 1e-85 | 1 (1AK21) | High |
| | Oat mRNA | Drosophila ananassae | 489 | e-136 | 1 (1AL21) | High |
| | putative MLC3 protein | Lonomia oblique | 223, 223 | 7e-57, 7e-57 | 2 (1AN2, 1AN1) | High, Medium |
| | CG7430-PA | Drosophila melanogaster | 644 | 0.0 | 1 (1AD24) | Medium |
| | putative MLC1 protein | Apis mellifera | 246 | 3e-64 | 1 (1AM1) | Medium |
| | myofibril-associated Zeelin1 protein | Apis mellifera | 248 | 7e-65 | 1 (1AP3) | Medium |
| | testis enhanced gene transcript-like protein | Paralichthys olivaceus | 155 | 2e-36 | 1 (1AD1) | Low |
| | large ribosome mitochondrial | Apis mellifera | 179 | 5e-43 | 1 (1CA8) | Low |
| | cytochrome oxidase subunit 1 | Apis mellifera | 108 | 1e-22 | 1 (1CE16) | Low |

Table 1 Continued

| Library | Candidates showed most similarity to… | In organism… | blast Score | blast E-value | Number of candidates matching blast search | Intensity difference in screen (high, medium, or low) |
|---|---|---|---|---|---|---|
| **QMP+ Head** | | | | | | |
| | CG10737-PB | Apis mellifera | 1346 | 0.0 | 1 (2AA2) | High |
| | phosphotidylinositol 4-phosphate 5-kinase (CG3682) | | | | 1 (2AB4) | High |
| | Ras-related protein Rab-9A | Apis mellifera | 389 | e-107 | 1 (2AD22, 2BJ22) | High, High |
| | CG4710-PA | Apis mellifera | 60 | 3e-08 | 1 (2AA1) | Medium |
| | myosin 1 light chain | | | | 1 (2AA4) | Medium |
| | WD repeat domain 37 | Xenopus laevis | 214 | 4e-54 | 1 (2AA6) | Medium |
| | CG15279-PA | Apis mellifera | 711 | 0.0 | 1 (2AB2) | Medium |
| | CG7867-PC, isoform C nuclear fallout (nuf) | | | | 1 (2BC22) | Medium |
| | K channel tetramerisation domain containing 9 | Apis mellifera | 252 | 1e-65 | 1 (2CA20) | Medium |
| | Partial 16 S rRNA Gene | Uncultured bacterium | 96 | 5e-18 | 1 (2AB20) | Low |
| | glucose oxidase (dehydrogenase) | | | | 1 (2AP21) | Low |
| | RE32966p | | | | 1 (2BP18) | Low |
| | putative fructose 1,6-bisphosphate aldolase | | | | | Low |
| | CG8110-PA, isoform A Sunday driver (syd) | Apis mellifera | 2021 | 0.0 | 1 (2CO9) | Low |

Table 1 Continued

| Library | Candidates showed most similarity to… | In organism… | blast Score | blast E-value | Number of candidates matching blast search | Intensity difference in screen (high, medium, or low) |
|---|---|---|---|---|---|---|
| **QMP-Body** | | | | | | |
| | Deformed wing virus polyprotein | | 444, 417, 235, 424 | e-122, 3e-59, e-114, e-116 | 4 (3AB6, 3BA16, 3AD1, 3DB24) | High, High, Medium, Low |
| | Deformed wing virus isolate PA | | 301, 232, 249, 282 | 1e-81, e-117, e-125, 6e-74 | 4 (3BO23, 3AO8, 3CA6, 3CF1) | High, Medium, Low, Low |
| | Troponin C, type IIIB and IIIA | Apis mellifera | 150, 234, 238 | e-102, e-100, e-106 | 3 (3BE18, 3BE24, 3AO9) | High, High, Medium |
| | Imaginal disc growth factor (Idgf) | Pieris rapae | 400 | e-109 | 1 (3BB18) | Medium |
| | Partial 16 S rRNA Gene | Uncultured bacterium | 101 | 2e-19. | 1 (3BB19) | Medium |
| | ENSANGP00000011882 | Anopheles gambiae | 204 | 3e-51 | 1 (3CA7) | Low |

Table 1 Continued

| Library | Candidates showed most similarity to… | In organism… | blast Score | blast E-value | Number of candidates matching blast search | Intensity difference in screen (high, medium, or low) |
|---|---|---|---|---|---|---|
| **QMP-Head** | | | | | | |
| | Deformed wing virus isolate PA | | 230, 180, 194, 305, 188, 231, 389 | 2e-78, 1e-43, e-107, 3e-83, 8e-46, 2e-76, e-106 | 7 (4AF23, 4AN5, 4CL23, 4AA10, 4AC10, 4CB24, 4AI1) | High, High, High, Medium, Medium, Medium, Low |
| | Deformed wing virus polyprotein | | 215, **, 456, 232, 239, 320 | 6e-54, **, e-126, 5e-59, 3e-61, 3e-85, | 6 (4CF2, 4CC24, 4BP23, 4BB6, 4BB8, 4CA4) | High, Medium, Medium, Low, Low, Low |
| | virus 1 | Varroa destructor | 292 | 6e-77 | 1 (4BI22) | High |
| | tbetaRI gene for TGF-beta Type I receptor, exons 1-10 | Crassostrea gigas | 91 | 3e-16 | 1 (4BI9) | Low |

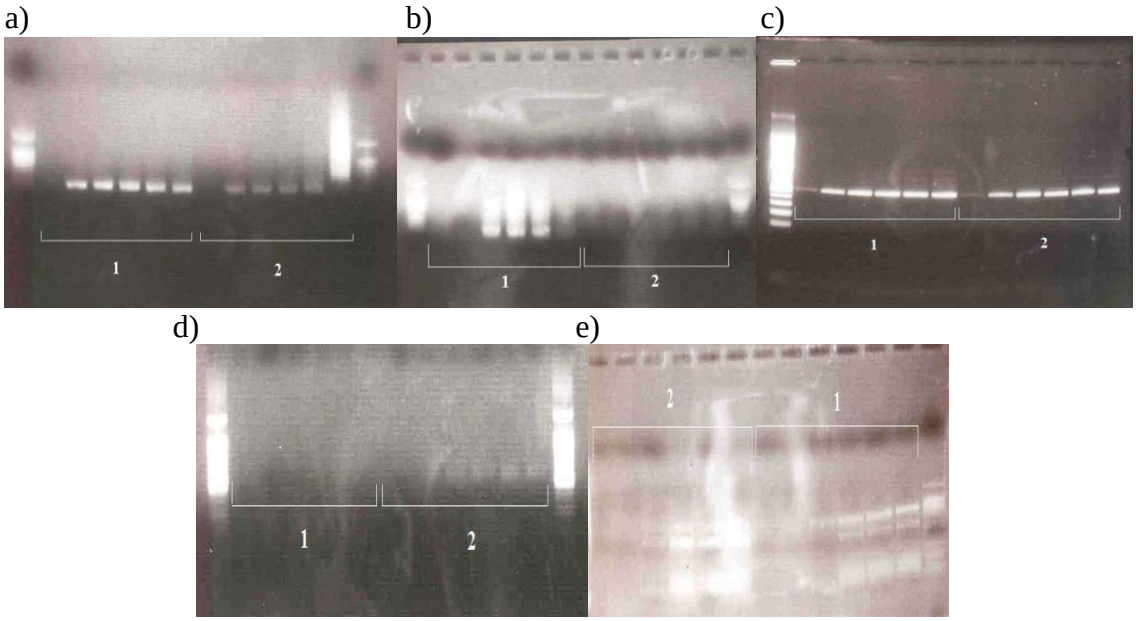


Fig. 1. Illustration of differential expression by rt-PCR in QMP exposed and non-QMP exposed *Apis mellifera* workers. Transcripts represented are Cytochrome oxidase (a), Myosin (b), Rab9A (c), Troponin C (d), and Idgf2 (e). The image contains the visualization of amplification using specific primers on both a QMP exposed worker (1) and a non-QMP exposed worker (2). There are six lanes per individual for 15-40 cycles in increasing 5 cycle increments.

# Conclusion

Cytochrome oxidase is involved in metabolism. An increase in transcript levels was observed in the bodies of QMP reared bees. This was expected because the pheromone presence would hypothetically delay development of foraging behavior retaining bees in the nest to perform in-hive tasks associated with colony growth and maintenance (14). For example honey bees working in the hive show increased hypopharyngeal and mandibular gland activity and amount of extractable protein related to larval food production compared to foragers (43-48).

Myosin transcripts were expressed at higher levels among workers reared with QMP. This appears counter-intuitive because bees reared with QMP are expected to show delayed foraging ontogeny and consequentially delayed age of first flight. Alternatively, delayed foraging suggests extended brood rearing. An important component of brood rearing is maintaining a brood nest temperature from 30°C to 35°C (3). Workers maintain brood nest temperature by increasing their own body temperature by contracting their thoracic muscles (3). QMP reared bees may be expressing myosin transcripts as a consequence of thoracic muscle development for brood nest temperature maintenance and potentially a greater sensitivity to brood rearing environment temperature.

Rab9A was shown to have higher expression levels in QMP reared worker heads. Rab9A is involved in protein transport. The increased Rab9A transcription could be due

to the pheromonal signal's impact on the brain. With the presence of the signal, perhaps the brain orchestrates the body development at a given rate with many specific proteins, and without the QMP signal the protein transmission rates are slower and much more erratic and disorganized.

Troponin c transcripts were seen at higher levels in the bodies of workers not reared with QMP. This transcript encodes a product with calcium binding capabilities and is putatively involved in calcium-mediated signaling. Troponin c is also often involved in muscular contraction. Though a decrease in myosin was seen in bees not reared with QMP, troponin c transcripts may be expressed at higher levels because of an increased need in the body for calcium mediated signaling. Foraging ontogeny proceeds at a faster rate in colonies that are not supplemented with QMP (14). In this context, troponin c may reflect flight muscle development associated with accelerated foraging ontogeny.

Imaginal disc growth factor 2 (Idgf2) transcripts were expressed at higher levels in non-QMP reared bees. Idgf2 is normally expressed in many developing tissues in the embryo and larvae, and is also present in both nurse cells and oocytes of adult ovarian tissue, which is particularly interesting in this scenario. Many workers reared in colonies without a queen and/or diploid larvae have well developed ovaries and are able to lay unfertilized eggs (49). An increase in Idgf2 transcription could potentially demonstrate the ovaries are at least partially functional in these early adult workers. This would make sense because when a queen is present ovarian development is suppressed by QMP. When it is not present, the ovaries of workers partially regain functionality.

Future work with these candidates would include investigating the expression levels in workers with PER-RT scores deviating from the average to see if the expression levels change based on the bee's sensitivity to sucrose (and theoretically QMP). Expression patterns could also be investigated in bees from the groups with the 24 hour treatment shifts at the 5 day time point to determine which transcripts are the earliest influenced by the addition or removal of QMP.

Another course of research would be to investigate why the mite virus transcripts were so prevalent in bees reared without QMP. Perhaps the lack of QMP stresses the bees and causes their immunity to drop enough for viruses to take hold. Or perhaps the lack of QMP in the hive encourages the Varroa mite to capitalize on the weakened colony and their numbers skyrocket, as does the level of mite viruses among the honey bees. This viral transcript was quite prevalent in bees reared without QMP, and was not identified in any of the sequences recovered from the bees reared with QMP.

# EFFECT OF A PARASITE ON GENE TRANSCRIPTION

## Background

Despite being an important pollination source, honey bees also have value for the products they make, such as honey and wax. Honey production is a trait unique to honey bees, and they are responsible for over $100 million worth of honey production per year. According to the National Honey Board, in 2001 the US per capita honey consumption was 2.1 pounds. To make one pound of honey, honey bees typically visit around 200 million flowers. The wax produced by honey bee is used in countless products such as candles, cosmetics, crayons, lotions, soaps, and creams. Thus, anything that negatively affects the honey bee potentially has an unfavorable influence on a number of industries and products.

The most detrimental organism a colony of European honey bees can encounter is the Varroa mite (*Varroa destructor*), particularly as the bees have not developed a defense mechanism against the mite. The recent introduction of the pest to the European honey bee has not allowed enough time for selective pressure to develop an innate protective mechanism. This particular host-parasite relationship has not yet reached a state of homeostasis. Instead the mite population continues growing even after it reaches the maximum load a colony can handle without suffering as a whole unit from the feeding burden the mites create. The mites increase in number until the colony dies, either because it has insufficient numbers of workers tending to activities within the hive, lacks

the foraging resources to gather enough materials to live through the winter, or has such a lowered immunity that other afflictions, such as Acute Bee Paralysis Virus or American or European Foulbrood, are able to replicate and kill the colony.

The Asian honey bee, *Apis cerana*, is the natural host of the Varroa mite. It is not known how the Varroa was introduced to *Apis mellifera*. The shift to a new host was fortunate for the mite because the Asian honey bee adapted a grooming behavior, which allows them to remove the mite from their body and kill it. It was quite unfortunate for the European honey bee because its grooming behavior is not sufficient to remove mites, and thus it has no natural defense against this mite. The exportation of hives of bees from infected areas to non-infected areas helped spread the mite quite rapidly. When infested bees were transported into new areas by beekeepers, sometimes the mite would hitch a ride on foraging adult workers and on occasion the worker would mistakenly enter the wrong hive, sometimes an uninfected hive, allowing the mite to move off its carrier and infect a new colony. This enabled the infestation to spread across large geographic distances. To date there are few regions with European honey bee colonies that are not battling against the invasion and establishment of the Varroa mite.

The Varroa mite preferentially feeds on hemolymph of drone pupae. If there are no drone pupae in the hive due to the seasonality of drone presence, the mites feed on worker pupae and even worker adults. The life cycle of the mite is much shorter than the honey bee, allowing them to develop from egg to reproductive adult before a bee emerges as an adult from its cell. Briefly, a mated female mite enters the cell of the bee

just before it is sealed with wax by the workers. The mite is thus enclosed with the late instar bee larva. The female mite feeds on the larva until it reaches the pre-pupal stage, at which point she will lay 2-6 eggs. The bee then turns into a pupa. A lone male usually hatches first, followed by females. It takes 6 days for male mite eggs to hatch and 10 days for female mite eggs to hatch. The mites emerge from their eggs and feed from the wound their mother made on the bee pupae. The females then mate with the male and he usually dies shortly after. The bee emerges from the capped cell as an adult, with the mated females riding on its back. The females will continue to feed on the hemolymph of adult bees for a few days to a few weeks and then choose another brood cell to enter and finish their life cycle.

The most effective current treatment consists of a miticide strip containing either tau-fluvilinate, a pyrethroid insecticide, or coumaphos, an organophosphate insecticide, (50), which is administered to the hive 2-3 times per year. There are three main problems associated with this treatment method. First, the strips can only be used at certain times of the year because the chemicals are absorbed by honey and can become concentrated in wax (51-53), so it is not suggested for use at times when bee products will be used commercially, so as not to contaminate consumer products with the chemicals. Second, the Varroa mite has begun developing resistance to both of the miticide strips (54-56), such that this form of control will not be effective indefinitely. The last problem associated with miticide treatments is the cost. Since the treatment must be administered multiple times throughout the year, beekeepers must recoup their costs in the sale of

honey and wax (57). This in turn increases the cost for consumers who buy goods manufactured with bee products.

Because mite generation time is shorter than bee generation time, the mite has more opportunities to evolve and drive selection for traits that will enable them to thrive. This includes resistances to chemicals and deterrent methods used in apiaries. Unfortunately for beekeepers, natural selection within the mite population for the resistance traits will mean that methods developed to combat the mites will generally only work for short periods of time, until the mite population develops high levels of resistance to that treatment. In the short term, treatments are absolutely vital for combating the effects of the mites on bee colonies. In the long term, alternative methods need to be developed to facilitate an innate defense mechanism for the bee in its battle with the mite so that it will not need chemical assistance.

The primary aim of this experiment is to identify gene transcripts that are differentially expressed due to parasitization by the Varroa mite. If more were understood about the biochemical effects on the bee due to mite feeding, perhaps a strain of bee could be developed expressing a protein that gives it an advantage due to mite invasion. The experimental goal is to identify transcripts turned on or off when the mites feed. Identifying those transcripts affected by mite parasitization will provide some insight into the mechanisms used by the honey bee to combat the influence of Varroa mite feeding. This information could be used to potentially constituitively express a protein

to deter the interest of the mite or even select for bees in an apiary with higher basal levels of specific Varroa mite combative proteins.

## Experimental Design and Setup

A virgin queen obtained from Wooten Golden Queens (Palo Cedro, CA) was introduced into a hive of packaged bees obtained from Weaver Apiaries (Navasota, TX). After successful introduction, the queen was caged against a single frame for 24 hours for egg laying. This insured the eggs were all within 24 hours of each other in developmental stage. The queen was then excluded from the frame using a box constructed out of queen excluders. The box was left in the hive to facilitate nurturing by the nurse bees. The eggs were left in the hive until they developed into pre-pupae. After this stage they no longer needed to rely on the worker bees within the hive for nourishment. The frame was removed from the hive and taken into an incubator. The larvae were removed from the frame using a pair of blunt ended featherweight forceps (BioQuip Products, Inc., Rancho Dominguez, CA) to prevent wounding the larvae. The larvae were then placed into size 00 gelatin capsules obtained from Henry Schein, Inc (Melville, NY). Three holes were poked into the ends of the capsules using a number 2 insect pin before the larvae were placed into them to facilitate air circulation. Approximately half of the capsules also had a mite added to them. The mite used for placement in the capsule was removed from a capped larval cell, because that indicated that the mite was searching for a blood meal to develop her offspring. The mite was placed into the gelatin capsule to allow her an opportunity to feed on the immature bee. Only bees that had a feeding

wound were counted as being parasitized. This was evident by melanization around the feeding site. Bees placed into the capsules with a mite, but showing no evidence of being fed upon, were discarded since no definite conclusion could be determined regarding their parasitization status.

Capsules were placed in a labeled 24-well plate lid and positioned into another container containing wet paper towels to provide moisture. The containers were put into a walk-in incubator set at 37°C and the bee larvae were allowed to develop. When the bees pupated and developed purple eyes, they were removed from the capsules. The mites were separated from the pupae and placed in individual tubes for identification. The pupae were placed into individual tubes, weighed, flash frozen, and stored at -140°C until they were processed.

SSH was used to investigate differential gene expression due to parasitization in wild type bees. This method was chosen over differential display because it reduces the amount of background one would expect to see when comparing highly similar subjects. In this case, it was expected to see a high amount of similarities between parasitized and non-parasitized drones because of the common maternal contribution. SSH allowed the commonalities to be removed from the screening pool, leaving virtually only the differential expression candidates. By controlling the situation with a single queen laying the eggs used, there were fewer genetic differences between the samples. Utilizing drones enabled the exploitation of two factors; the mite's natural preference for drones and the lack of a paternal contribution since drone bees are haploid.

Pupae were taken and ground up using Qiagen's RNEasy Kit (Valencia, CA) and blue pestles. One male offspring from a single queen was used for each of the two treatment conditions in the SSH procedure. Poly A+ mRNA was isolated from the total RNA using the Oligotex direct mRNA Mini Kit which is a bead-based recovery system (Qiagen Valencia, CA).

Messenger RNA was used because it consists of a transcriptional profile of an individual, instead of using DNA or RNA which do not necessarily reflect genes or transcripts actively being utilized by an individual. It is important to isolate mRNA for these experiments, rather than using total RNA, because total RNA contains ribosomal RNA which swamps out the mRNA transcripts, making detection of actual differential expression above background near impossible using the employed technique. Even though an oligo dT primer is used to synthesize the cDNA, and would thus specifically target the A tail of mRNA for transcription into cDNA, mRNA only comprises around 1-3% of the total RNA of a cell. By specifically isolating mRNA, this increases the probability that reverse transcriptase will hybridize with mRNA instead of ribosomal RNA, which can comprise more than 80% of a cell's total RNA. This also ensures that the lower abundant mRNA transcripts are represented because if total RNA were used in the reverse transcription reaction, the probability of lower abundant mRNA being transcribed into cDNA would be very unlikely.

To ensure there was an equal mRNA contribution from each bee, total RNA

concentration was used for a rough input estimate based on spectrophotometric data, because mRNA is difficult to quantify. Double stranded cDNA was synthesized using the pooled mRNA as a template, as mRNA is not a template for PCR. The resulting cDNA became the starting material for the SSH, which was performed according to a protocol outlined in the PCR-Select cDNA Subtraction Kit (Clontech Mountain View, CA). The cDNA was digested with a restriction enzyme (*Rsa* I) and two specific adapters included in the kit were ligated onto the ends of only the tester cDNA in two separate reactions. The actual SSH procedure entailed two rounds of hybridizations followed by two PCR amplifications outlined in the kit's protocol. In brief, two reactions were set up for each driver cDNA sample. In each reaction, driver cDNA was combined with one of the two adapter ligated tester cDNA samples and 4X Hybridization buffer. The samples were denatured at 98°C for 90 seconds and then were left for 8 hours at 68°C. In the second round of hybridization, the two samples from the first hybridization were mixed together. Fresh denatured driver was added to the first hybridization mixture, along with fresh 4X hybridization buffer, to further enrich for differentially expressed sequences and was incubated at 68°C overnight in a second round of hybridization. After the hybridizations, an aliquot of subtracted cDNA (the sample had been hybridized twice) and a corresponding aliquot of unsubtracted tester cDNA (the sample had not been subjected to hybridization) were subjected to two rounds of PCR. The primers were specific to the adapters ligated onto the ends of the tester cDNA molecules. The first PCR program started with 75°C for 5 minutes to extend the adaptors. The samples then immediately went thru 27 cycles of 94°C for 30 seconds, 66°C for 30 seconds, and 72°C for 1.5 minutes. A 1:10 dilution was taken from

the first PCR and subjected to a secondary PCR, using nested primers which were also specific to the adapters. The secondary PCR program consisted of 14 cycles of 94°C for 30 seconds, 68°C for 30 seconds, and 72°C for 1.5 minutes. An aliquot of the results were analyzed on a 2.0% agarose/ EtBr gel run in 1X TAE buffer. The SSH process allowed for identification of the expression differences between parasitized and non-parasitized individuals.

The products from the SSH procedure were cloned into the TOPO 2.1 vector from Invitrogen (Carlsbad, CA) and transformed into TOP10 electrocompetent cells (Invitrogen, CA). The cells were placed into a shaking incubator at 200 rpm and 37°C for an hour and were then plated on LB/agarose plates containing Kanamycin. Large 250 mL plates (Genetix Boston, MA) were used for plating and the colonies were picked robotically by a Q-bot (Genetix Boston, MA) and placed into 384 well plates (Genetix Boston, MA) containing liquid freezer media. Of the culture that was plated, the robot searched for colonies that met certain criteria for colony size, shape, and diameter. For the Mite- library, the robot selected 757 candidates based on the default criteria and 2341 Mite+ candidates. After allowing the plates to grow in an incubator at 37°C for 24 hours, a manual replicator was used to spot replicate colonies onto nylon membranes resting on Kanamycin50/LB plates. The membranes were allowed to grow for 12 hours at 37°C in an incubator and then were pulled off the plates. The colonies on the membranes were chemically denatured by placing on Whatman paper saturated with 0.5M NaOH, and 1.5M NaCl for 4 minutes and neutralized by placing on Whatman paper saturated with 0.5M Tris-HCl, pH 7.4, and 1.5M NaCl for 4 minutes. The

colonies were then fixed by crosslinking the DNA to the membranes using an auto-crosslinker (Stratagene UV Stratalinker-2400 La Jolla, CA, on the 'auto crosslink' setting).

Radioactive probes were created using unsubtracted and subtracted products from the SSH procedures. Each SSH experiment produced a collection of cDNAs, an unsubtracted set and a subtracted set. The unsubtracted set is one that had not gone through the SSH procedure, thus it represents the cDNA profile before any portion of the subtraction procedure was performed. The subtracted cDNA set was the result of the SSH procedure, so it was expected to have differing levels of cDNA copy numbers than the original stock due to suppression of common transcripts within the sample and subtraction of common transcripts between the two samples being compared. Radioactive probes were synthesized using these cDNAs as templates. To create the probes, 40-100 ng of cDNA from the secondary PCR was combined with dNTPs (lacking dATP), a random primer mix, $\alpha$-$P^{32}$ ATP, and 2-5 units of Klenow. This mixture was incubated at 37°C for 30 minutes. EDTA was added to terminate the reaction and then the reaction was cleaned up using a column based cleanup procedure to remove free nucleotides. The PCR Select Differential Screening Kit from Clontech (Mountain View, CA) was used to screen the clones, as it contains all materials needed to synthesize the probes used in screening the membranes (except for the radioactive nucleotide). For the Mite+ library, there was an unsubtracted Mite+ probe that consisted of cDNA that did not go thru the SSH procedure and a subtracted Mite+ probe that was the end result of the SSH procedure. There were also probes for the Mite- library,

making a total of four $\alpha$-$P^{32}$ radiolabelled probes for this experiment.

The membranes were pre-hybridized with a 1:1 solution of 20X SSC and a blocking solution made by Clontech (Mountain View, CA) from their PCR-Select Differential Screening Kit, specifically designed to work by blocking the adapters used in the SSH procedure. This prevented hybridization of the probe with the sequences specific to the adapters, ensuring that the probe bound only to a complementary sequence in the cDNA library. The membranes were hybridized with an individual probe for 16 hours and then washed twice with a low stringency buffer (2X SSC, 0.5% SDS) and then twice with a high stringency buffer (0.2X SSC, 0.5% SDS) for 20 minutes per wash with agitation at 68°C to reduce background hybridization. The membranes were then heat-sealed in plastic film and placed in film cassettes with Kodak Bio-Max film to visualize hybridizing signals. Membranes from each original 384-well plate were eventually exposed to all four of the probes corresponding to that library (if the plate was from the Mite+ library, it would be allowed to hybridize with unsubtracted Mite+, subtracted Mite+, unsubtracted Mite-, and subtracted Mite- probes with stripping of each probe in between each exposure). The autoradiographs from the four hybridizations were compared and differences were noted. The best candidates for further differential expression analyses were those that were very visible in the library they were originally from (if it was from the Mite+ library, it was desirable to see a very visible signal on the films from the Mite+ probes) and little to no signal when hybridized with probes from the reverse subtraction or non-subtracted material (Mite- in this example).

Signal intensities were initially estimated manually comparing autoradiograms from hybridizations with different radioactive probes. Comparisons focused on the probes created from the forward and reverse subtracted cDNAs. Clones showing signal intensity differences between the forward and reverse probes were the primary focus of this investigation. Candidates were identified that showed a signal when the membrane was exposed to the probe from one library but not when exposed to the probe from the corresponding library. Five candidates from each of three groupings (high intensity, medium intensity, and low intensity) were isolated and sequenced for further analysis from both libraries (Mite+ and Mite-), for a total of 30 initial sequences. The three intensity groups represented transcripts that, based on the intensity of the hybridization signal, appeared to have high, moderate, or low differential expression levels. Since the candidates were cloned into TOPO 2.1, M13 reverse primer was used for PCR sequencing, using the following parameters: 30 seconds at 94°C, 15 sec at 55°C, and 1 minute at 72°C for 40 cycles.

Using the Vector NTI 8.0 suite of programs (InforMax, MD), flanking vector sequence was removed from the sequence data obtained from the 30 candidate clones. EMBL EBI-Heidleberg (European Molecular Biology Laboratory), which was the depository for the initial honey bee genome sequence data (before the data from the sequencing effort was fully assembled in the honey bee genome project), was queried for longer sequence data using personal sequence information from the initial 30 candidates sequenced. The sequence data obtained from this experiment was compared to the sequencing database utilizing BLAST, which is a sequence alignment algorithm. If the

query produced a significant match (an e value of $10^{-4}$ or less) to something in the database, then the full length data was used to provide the deduced amino acid sequence to be used for continued analyses. If there were no significant matches, then the initial sequence data was used. NCBI (42) was then utilized to attempt to infer a potential function for the differential expression candidates by using BLAST to find homologous sequences with known functions in other species. Primers were then developed for candidate genes which appeared multiple times in the sequence data and rt-PCR was performed using experimental bees to illustrate differential expression. The bees used in the rt-PCR were subjected to the same treatment as the bees used in the SSH procedure. rt-PCR is a technique used to investigate the presence of a desired sequence by taking the mRNA from a sample, transcribing it into cDNA, and then attempting to amplify a sequence using specific primers. The results are then run on a 2.0% agarose/EtBr gel in 1X TAE buffer to visualize.

The entire list of sequenced candidates can be found in Table 2 on P. 49, though only four were chosen for further analysis in these two libraries. From the parasitized library, heat shock protein 70 (hsp70), which is stress-related, was present 6 times in the 30 candidates sequenced. Socius, which may play a role in the reorganization of the actin cytoskeleton, was present twice in the parasitized library. From the library containing transcripts more prevalent in the non-parasitized drones, two hexamerin transcripts, which are larval storage proteins, were chosen to investigate further; hexamerin 70b (hex70b) which was identified in 3 of the 30 sequences and hexamerin 2 (hex2) which was present twice.

Vector NTI 8.0 was used to analyze the sequences to determine a possible set of primer sequences. The primer sequences for hsp70 were Hsp70rev GCCGAGGTACACCTCTAGGT and Hsp70fwd-GCAGAATTCGCCCTTAGC, socius were Sociusrev-TCGCGGCCGAGGTACCTTTTA and Sociusfwd-TAGATGCATGCTCGAGCGGC, hex70b were Hex70brev-TCGCGGCCGAGGTACTGATT and Hex70bfwd-TAGATGCATGCTCGAGCGGC, and hex2 primer sequences were Hex2rev-TCGCGGCCGAGGTACAATCT and Hex2fwd-TAGATGCATGCTCGAGCGGC.

The reactions were subjected to the following parameters: an initial denaturing step of 94C for 5 minutes, followed by 25 cycles of 94C for 30 seconds, the primer specific annealing temp for 30 seconds, and 72C for 1 minute. After the 15 cycles, an aliquot was removed from the tube for analysis and then remaining sample was subjected to 5 more cycles of amplification. This continued until there were 6 aliquots from 15, 20, 25, 30, 35, and 40 cycles. The results were analyzed on a 2.0% agarose/EtBr gel in 1X TAE buffer.

Table 2: Candidates from Mite SSH screen. Sequences were queried against NCBI database in an attempt to infer function. Candidates are grouped according to library they were a part of. Also included in the chart is the corresponding Score and E-value of the blast search of the candidates, along with the number used for identification and how the candidate appeared in the screening process.

| **Library** | **Candidates showed most similarity to…** | **In organism…** | **blast Score** | **blast E-value** | **Number of candidates matching blast search** | **Intensity difference in screen (high, medium, or low)** |
|---|---|---|---|---|---|---|
| **Mite+** | | | | | | |
| | Zgc:92707 | | | | 1 (2MCL5) | High |
| | CG7152-PB | Apis mellifera | 556, 556 | e-157, e-157 | 2 (2MDA15, 2MED11) | High, High |
| | heat shock cognate 70 protein mRNA | Manduca sexta | 942 | 0.0 | 5 (2MAK14, 2MAK24, 2MAL8, 2MBB20 2MBF18) | High, High, High, High, Medium |
| | Socius | | | | 2 (2MAM8, 2MCM12) | High, Medium |
| | G-protein coupled receptor 172A | | | | 1 (2MAM4) | Medium |
| | 16S Ribosomal subunit | Uncultured bacterium | | | 1 (2MAM5) | Medium |
| | Sequence from patent | | | | 1 (2MBD14) | Medium |
| | blackjack | Apis mellifera | 1259 | 0.0 | 1 (2MCP14) | Medium |
| | Mitogen activated protein kinase | Apis mellifera | 1216 | 0.0 | 1 (2MDJ8) | Medium |
| | Sequence 4 from patent | | 105, 108 | 2e-20, 2e-21 | 2 (2MCC10, 2MDL23) | Medium, Low |
| | Pacrg (Parkin coregulated gene protein) | Danio rerio | 265 | 2e-69 | 1 (2MBC18) | Low |
| | Glutathione S transferase | | | | 1 (2MBP24) | Low |
| | Stam (signal transduction adapter molecule) | Drosophila melanogaster | 446 | e-123 | 1 (2MEM17) | Low |

Table 2 Continued

| Library | Candidates showed most similarity to… | In organism… | blast Score | blast E-value | Number of candidates matching blast search | Intensity difference in screen (high, medium, or low) |
|---|---|---|---|---|---|---|
| **Mite-** | | | | | | |
| | Nucleoplasmin | | | | 1 (1MAA11) | High |
| | Chaperonin containing TCP1, subunit 5 (epsilon) | Xenopus laevis | 337 | 1e-90 | 1 (1MAK2) | High |
| | CG12214-PA | | | | 1 (1MAM1) | High |
| | RE72245p | | | | 1 (1MBA13) | High |
| | Sequence from patent | | 100 | 5e-19 | 1 (!MBD23) | High |
| | Hexamerin 2 | Apis mellifera | 714 | 0.0 | 1 (1MBE23) | High |
| | Hexamerin 70b | Apis mellifera | 1368, 1368, 1368 | 0.0, 0.0, 0.0 | 3 (1MAD24, 1MAP4, 1MBM15) | High, High, Low |
| | Zfr protein | | | | 1 (1MAC24) | Medium |
| | NADH dehydrogenase | | | | 1 (1MAF8) | Medium |
| | Psmc2 protein | | | | 1 (1MAH17) | Medium |
| | MGC81123 protein | Xenopus laevis | 172 | 7e-42 | 1 (1MAK24) | Medium |
| | Tdp1 | Drosophila melanogaster | 419 | e-115 | 1 (1MAN22) | Medium |
| | Jitterbug | Apis mellifera | 3067 | 0.0 | 1 (1MAO5) | Medium |
| | Pgant7 | Drosophila melanogaster | 830 | 0.0 | 1 (1MAP7) | Medium |
| | CG18335-PA | Drosophila melanogaster | | | 1 (1MAI21) | Low |
| | Clone NAP-d-08 | Anopheles gambiae | 198 | 3e-49 | 1 (1MAA22) | Low |
| | Rpt4 | Drosophila melanogaster | 560 | e-158 | 1 (1MBM17) | Low |

# Results

After rt-PCR analysis, the Hsp70 transcript was determined to be up-regulated in parasitized pupae compared to non-parasitized pupae, as was expected based on the initial SSH screening procedure. Socius transcript levels in parasitized and non-parasitized pupae were not easily discernable utilizing rt-PCR detection methods. Hex2 transcript levels were higher in parasitized pupae than in non-parasitized pupae, unlike what was expected based on the SSH screen. Hex70b transcript levels were as expected, and were at higher levels in non-parasitized pupae than in parasitized pupae.

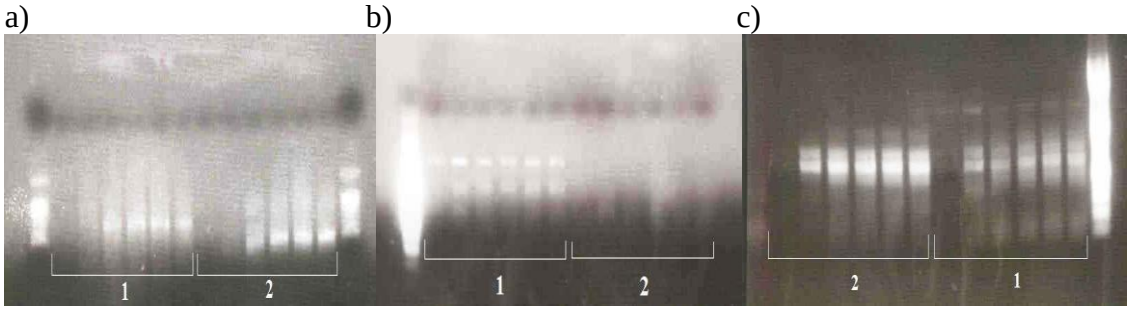


Fig. 2. Illustration of differential expression by rt-PCR in parasitized and non-parasitized *Apis mellifera* drone pupae. Transcripts represented are Hsp70 (a), Hex2 (b), and Hex70b (c). The image contains visualization of amplification utilizing specific primers on both a parasitized drone pupae (1) and a non-parasitized drone pupae (2). There are six lanes per individual for 15-40 cycles in increasing 5 cycle increments.

# Conclusion

The four genes investigated with rt-PCR were good candidates because of their potential involvement in or response to ecto-parasitization and were the most commonly detected transcripts were investigated.

Heat shock protein 70 (hsp70) is a stress related protein. It was seen more prevalently in parasitized drones. Perhaps the increased hsp70 transcript expression was due to the drone being stressed from the mite consuming its hemolymph.

Unfortunately not much was discernable for the socius transcript levels. In the initial SSH screening procedure, it was seen at higher levels in parasitized pupae. Unfortunately it appears that the transcript was not abundant enough to be detected using rt-PCR as detectable levels of socius were not seen in either parasitized or non-parasitized drones.

Hexamerin 70b (hex70b) transcripts were detected at higher levels in mite free pupae. Hexamerins are larval amino acid storage proteins, and since the drones analyzed were pupae, it would be logical for there to still potentially be larval storage protein transcripts needed in the drone. Perhaps the reason hex70b was more prevalent in the mite free drone was because with a parasitization event the pupae may put more effort into producing transcripts necessary for an immune response.

Hexamerin 2 (hex2) transcripts were detected at higher levels in parasitized pupae, unlike that seen in the initial SSH screen. The two hexamerin sequences were quite similar on one end, so it was possible to use one common primer (the forward primer for both). Perhaps the unique primer sequence for hex2 closely resembled that of another transcript present in the parasitized pupae and the primers also annealed to that sequence, thus producing non-specific amplification. Or perhaps the individual used for the rt-PCR amplification did indeed have higher hex2 transcript levels, but not higher hex70b levels.

Future work would include further investigation into the transcripts not highlighted in this thesis work, as there were many more candidates. Further investigation into possible reasons for the hexamerin2 differential results between the SSH screen and the rt-PCR would also be helpful. Another possible course of investigation would be to artificially wound a drone with an insect pin to see if it has a similar reaction to mite feeding, or up-regulate other unique transcripts (which would potentially indicate something in the saliva of the mite induces a different reaction in the drone). These results could also be compared to the response of drones to other pests and pathogens in the hive to determine if these transcripts are common in all instances or are specific only to the Varroa mite.

# SUMMARY

SSH was a useful tool to facilitate the identification of differentially expressed transcripts in both experiments. It was possible to determine some of the genes involved directly or indirectly with a response in the worker due to QMP and the drone due to Varroa parasitization. The genes analyzed were only a small fraction of what was actually detected by screening the SSH products.

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