INFLUENCE OF SOLDIER DERIVED SEMIOCHEMICALS ON *RETICULITERMES FLAVIPES* WORKER CASTE DIFFERENTIATION AND GENE EXPRESSION

By

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To all of those who have helped me along my path

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This dissertation focused on the termite *Reticulitermes flavipes* (Kollar), a serious structural pest in the USA. The goal of this research was to investigate potential impacts that soldier termites have on nestmate caste differentiation. Specifically, studies were conducted to understand the influence of soldier head chemicals on nestmate worker caste differentiation. The central hypothesis was that the chemicals produced by soldiers influence phenotype and gene expression of worker, and responsive genes that show differential expression will play a role in caste differentiation. Results showed that soldier head extracts (SHE), when combined with juvenile hormone (JH), synergistically increased worker-to-presoldier (PS) formation relative to JH alone. Using gas chromatography (GC), mass spectrophotometry (MS), and nuclear magnetic resonance (NMR) analyses, the two major components of SHE were determined to be γ-cadinene and γ-cadinenal.

Through use of quantitative real-time PCR, the expression patterns of 47 genes in response to JH and soldier head chemicals were investigated. The three main groups of genes with significant differential expression were 1) genes encoding enzymes involved in hormone and semiochemical biosynthesis / degradation, 2) hemolymph protein coding genes, and 3) developmental genes. Also, the individual effects of the two major components of SHE (γ-

cadinene and γ-cadinenal) on phenotypic caste differentiation and gene expression of workers was investigated.

Finally, the last phase of this dissertation was to further characterize genes from the chemical biosynthesis / degradation group: two cytochrome P450s and a putative JH esterase (*Cyp15F1*, *Cyp15A2*, and *RfEst1)*. Gene homology analyses, expression profiling, and RNAi studies support the hypothesis that these genes play roles in JH production and degradation.

In summary, this research has led to: 1) a better understanding of the role semiochemicals produced by soldiers play in worker caste differentiation, 2) the impacts that JH, soldier head chemicals, JH+soldier head chemicals, and live soldiers have on nestmate gene expression, and 3) a better understanding of the potential function of three specific genes in caste regulation, or the mediation of worker-to-soldier caste differentiation. As a result of understanding how soldiers are formed, new and novel control methods for this pest can eventually be designed. This dissertation provides a step towards development of more environmentally friendly, next-generation termiticides.

CHAPTER 1
INTRODUCTION

Background

Social insects fall outside the traditional idea of evolution via natural selection in which individuals who are best suited for their environment are able to reproduce and pass their genetic information to the next generation (Thorne, 1997). In social insect colonies, most individuals do not mate; rather, they are altruistic helpers that perform various tasks within the colony. Since members of social insect colonies have high degrees of relatedness, an organism may increase its own fitness by helping its relative's offspring to survive (Dugatkin, 1997). Altruistic behavior, while superficially appearing self-destructive, actually benefits the colony and raises the individual's fitness (Myles and Nutting, 1988).

Organisms that are eusocial share three main characteristics: 1) a reproductive division of labor, 2) overlapping generations, and 3) cooperative care of the young. A number of organisms display a gradient of eusociality, but the two main insect orders that display eusociality are Hymenoptera and Isoptera. Although not all Hymenoptera are eusocial, all Isoptera are eusocial.

Termites are members of the order Isoptera. Termites are small, pale, soft-bodied, hemimetabolous social insects that live in a colony system. Within a colony, termites have a division of labor based on castes that are characterized by both phenotype and behavior.

In contrast to termites, hymenopteran insects (ants, bees, and wasps) have a social structure which is different in the following ways: 1) they are holometabolous, 2) they have a haplodiploid genetic system, and 3) most colony individuals are female. Holometabolous development, also known as complete metamorphosis, is defined as the progression of worm-like larvae through a pupal stage prior to molting into adults.

Because of their haplo-diploid genetic system, hymenopteran workers are more related to one another than they would be to their own progeny (Hamilton, 1964; Thorne, 1997). By providing cooperative care of young, they increase the chance of passing their genetic information to the next generation. The evolution of hymenopteran sociality is discernable because of the wide range of social behaviors observed in hymenoptera, ranging from individuals that work alone (solitary wasps), to the whole colonies that work together for the good of the colony (honey bees). It is difficult to draw a parallel between hymenopteran and isopteran sociality because termites are diploid, hemimetabolous, and all living species are social (Thorne, 1997).

The flexibility to adapt to changing environmental conditions is an important survival strategy for many organisms. Phenotypic plasticity refers to a single genotype that can produce numerous different phenotypes, depending on various conditions encountered through its development (Nijhout, 1999). Phenotypic plasticity can be divided into two major types of responses to signals; reaction norms and polyphenisms. Reaction norms are phenotypically graded responses to environmental factors, while polyphenisms occur as two or more discrete alternative phenotypes, which transpire without intermediate forms (Nijhout, 2003).

Social insects have evolved multiple complex phenotypes. A phenotype is an individual's appearance or behaviors, as produced by the interaction of genes and environment (Nijhout, 1999, 2003; Miura, 2004). Castes are phenotypically and behaviorally discrete individuals that cooperate to perform colony tasks (Miura, 2004). Most termite colonies are made up of three distinct castes that include; workers, soldiers, and reproductives. All termite eggs, except when a rare genetic component might be involved (Hayashi et al., 2007), are totipotent and differentiate into the different castes based on a number of intrinsic and extrinsic factors.

A recent set of articles discussed genetic caste determination versus environmental caste determination. Past literature has suggested that caste determination in termites is controlled entirely by environment and genetic predisposition does not play a role (Queller and Strassmann, 1998; Lo et al., 2009). However, Hayashi et al. (2007), through an array of complex sexual crosses, suggested that reproductive development (like some ant species) has a genetic component. Matsuura et al. (2009) observed queen reproductive replacement through asexual parthogenesis, and suggested that eusocial insects, because of their unique life histories, can generate unique modes of reproduction. This might possibly explain the unique results observed by Hayashi et al. (2007). Still more work needs to be conducted to define reproductive developmental pathways. There is far more data in existence showing that environment plays a major role in directing termite caste differentiation, especially soldier caste differentiation (Koshikawa, 2005; Scharf et al., 2007; Zhou et al., 2007).

Termite Castes and Caste Differentiation

Termite caste differentiation can proceed along two routes, the imaginal (winged) or the apterous (wingless). All forms are considered immature in lower termites except soldiers, alates and the three reproductive forms. After termite eggs hatch, the first developmental branch is where the larvae differentiate into workers, soldiers or nymphs. Workers can, 1) undergo status quo worker-to-worker molts, 2), differentiate into presoldiers (immediately followed by a molt into a soldier), or 3) differentiate into apterous eyeless third-form reproductives. Nymphs can, 1) regress into worker-like pseudergates, 2) differentiate into fully winged eyed adult alates that disperse, mate and become primary reproductives, or 3) differentiate into second form reproductives that serve as supplemental reproductives (Buchli, 1958; Lainé and Wright, 2003; Scharf et al., 2003a).

Workers constitute the majority of individuals in the colony and perform most of the work. Workers feed other caste members, groom the queen, excavate the nest, make tunnels, tend to larvae, dispose of corpses, perform hygienic behaviors, and forage for food. A true worker is a non-reproductive, non-soldier individual of the third or fourth or a later instar that has diverged early and irreversibly from the imaginal line (Noirot, 1985). Workers are responsible for the majority of damage caused by termites. Pseudergates, sometimes called "false workers," perform the same tasks as workers, but are actually differentiating along the imaginal line and have the ability to eventually differentiate into alates. True workers, pseudergates and nymphs exhibit altruistic helping behaviors that are characteristic of the worker caste.

Within the termite colony the main role of the soldiers is colony defense (Wilson, 1971). Soldiers are morphologically specialized and have large heavily sclerotized heads and well developed mandibles. The sterile soldier caste is at the end of its developmental pathway (it is terminally developed). Soldiers are unable to feed themselves and rely on workers to feed them. Worker termites differentiate into soldiers by first molting into presoldiers, and then into soldiers; a process that takes approximately four weeks. However, the soldier termite's inability to feed itself and its low numbers within the colony has raised many questions of their role within the colony. Recent research suggests soldiers could possible play an influential role in the caste differentiation process. The soldier caste has been theorized to act as a juvenile hormone (JH) "sink" (Henderson, 1998). However there is no evidence that JH can be transferred among nestmates by any other means than cannibalism. In this role, soldiers are hypothesized to either regulate JH titers of nestmates or uninhibit worker maturation by controlling a different primer pheromone (Mao et al., 2005; Park and Raina, 2004, 2005). Thus, soldiers clearly regulate

soldier formation, and may regulate the ability of workers to metamorphose into reproductives, nymphs, or neotenics (Henderson, 1998).

The third caste of termites is the reproductive caste, which is responsible for the production of offspring and passage of genetic information to subsequent generations. There are a number of phenotypic forms of reproductives. Primary and secondary forms develop from the imaginal pathway, while tertiary forms come from the apterous line (Lainé and Wright, 2003; Thorne, 1996; Scharf et al., 2005b). Primary reproductives arise from nymphs that molt into alates which disperse, mate, and form a new colony. Secondary reproductives arise from nymphs that do not disperse and stay in the colony. Tertiary reproductives develop from the apterous line of workers. As discussed above, the specific factors that regulate differentiation of the different reproductives remain unknown. One possibility is that reproductive caste determination could be genetically controlled (Hayashi et al., 2007), but this evidence is not compelling or well-supported.

Socio-Regulatory Factors

As noted above, social insects are unique because they display caste polyphenism and an overlap of generations, resulting in multiple castes of different age classes being present at the same time (Miura, 2004). Also, individuals with the same genetic background express various phenotypes according to intrinsic and extrinsic factors (Koshikawa, 2005; Scharf et al., 2007; Zhou et al., 2007). Intrinsic factors, by definition, "originate or are due to causes within a body, organ, or part." One established intrinsic factor that controls the development of termites is juvenile hormone (JH). Juvenile hormone is a morphogenetic hormone produced by a paired neurosecretory gland (the corpus allatum) that has a broad range of developmental and physiological effects (Nijhout, 1994). In insects, juvenile hormone plays a role in the control of larval development and metamorphosis, but also has been shown to play a role in diapause,

migratory behavior, wing length, seasonal development, and eusocial caste determination (Hartfelder, 2000).

How can this single hormone have such a great diversity of effects? A single conventional JH receptor has yet to be discovered, and an alternative hypothesis is that JH is acting as part of a diverse, multi-receptor lipid signaling system. Wheeler and Nijhout (2003) compared JH action to lipid-soluble signaling molecules found in vertebrates, invertebrates and plants. They suggest that JH may be a lipid signaling molecule that participates in both signal transduction and transcriptional regulation as seen in other organisms. The termite model system may help to address this hypothesis. For example, Zhou et al. (2006a) found a prenylation motif in the termite hexamerin (Hex-1) protein which may covalently bind JH or act as a membrane anchor that mediates signal transduction.

In termites, JH shows characteristics of primer pheromones. Primer pheromones are chemical messengers that are passed among individuals and trigger physiological responses in recipients (Wilson and Bossert, 1963). At high JH titers a worker termite differentiates into a presoldier, which is directly followed by a molt into a soldier. The role of JH in soldier development is apparently the opposite of the "normal" role of JH among insects, which is apparently to maintain immature features (Truman and Riddiford, 1999). Previous studies have shown that exposure of worker termites to various JH homologues, including JH III, induces soldier differentiation (Howard and Haverty, 1979; Scharf et al., 2003b). Morphogenic hormones, such as JH, thus appear to be directly responsible for at least soldier caste differentiation. The trigger for JH production by the corpora allata however may be extrinsic, as described below.

Factors that affect individuals from outside the body (extrinsic factors) also clearly play a role in termite differentiation and development. Extrinsic factors, by definition, "originate from or on the outside, originating outside a part and acting upon the part as a whole." Examples of extrinsic factors in termite caste differentiation are colony caste composition, reproductive type and number, seasonality (temperature and rainfall), and nutrition (food quality, presence or absence of food). Two specific extrinsic factors that play a role in termite development are environmental factors such as seasonality and food quality. Cabrera and Kamble (2001) showed *R. flavipes* that were pre-exposed to a reduced thermo-photoperiod had an increased survival rate at low temperatures. Liu et al. (2005) monitored JH levels in field collected termites and demonstrated that they fluctuate throughout the year. Specifically, JH titers peaked with rising temperatures in early spring in correspondence with increased alate and soldier formation, but decreased thereafter. Scharf et al. (2007) demonstrated a correlation between temperature, caste differentiation, hexamerin protein levels, and JH sequestration. They suggested that hexamerin proteins are part of an environmentally and nutritionally responsive switching mechanism that helps regulate caste composition.

The influence of colony nestmates also plays a role in the regulation of caste differentiation. The termite colony produces individual castes based on the necessities of the colony. If there are low numbers of soldiers in the colony, some evidence suggests that workers will change into soldiers; while if a colony has too many soldiers then none will be formed (Park and Raina, 2003; Mao et al., 2005). Soldiers within the colony appear to play a key role in caste regulation (Park and Raina, 2004, 2005; Mao et al., 2005; Henderson, 1998). It has also been hypothesized that presoldiers and soldiers can act as JH "sponges" in the colony by absorbing JH from the colony, thus inhibiting soldier formation and stimulating the worker-to-alate

transformation (Henderson, 1998). Mao and Henderson (2006) suggest the enlarged functional labrum and broad soft mandibles of the presoldiers could be absorbing exogenous JH. However, there is no evidence that JH can be transferred among individuals. Okot-Kotber et al. (1991) and Korb (2003) showed live soldiers and soldier head extracts (SHE), in combination with synthetic JH analogs, could block soldier differentiation. Dong et al. (2009) suggested that physical contact between workers and soldiers is also important in soldier caste regulation.

Termite Biology and Control

Although termites represent model social organism for studying phenotypic plasticity, they remain one of the most highly destructive insect pests. An estimated 20 billion US dollars is spent globally on termite damage and control each year (Su, 2002). Termites cause structural damage to buildings by eating cellulose-based building materials or chewing through non-cellulose material. Throughout the history of termite control, a number of methods have been developed and employed. Chemical treatments for subterranean termite control include liquid soil treatments (repellent and non-repellent), baits, and wood treatments. Non-chemical control methods include barriers, such as aggregates and stainless steel meshes. Also, removal of high moisture conditions and vegetation in contact with the structure helps prevent termite infestations (Bennett et al., 2003). A drawback of chemical control methods is that they can be highly toxic, non-selective, require large amounts of chemical, and may not provide complete coverage (Forschler, 1993). Non-chemical treatments can be ineffective if termites are already established in a structure (Culliney and Grace, 2000). Understanding the unique social structure of termites, as well as its regulation, could lead to more efficient and safer termite control methods.

Termite Socio-Genomics Research

Previous research has identified differentially expressed genes across termite castes, and has led to the identification of a number of candidate genes that might play a role in caste

differentiation. Miura et al. (1999) used mRNA differential-display and found one gene, *SOL1*, that was expressed specifically in mature soldiers. Hojo et al. (2005) identified another soldier - specific protein (Ntsp1) in the frontal gland of a nasute termite. The protein has homology with known insect secretory carrier proteins, which they suggest could be a carrier of JH or related defensive terpenes. Koshikawa et al. (2005), using fluorescent differential display, identified 12 upregulated genes expressed in developing soldier mandibles. These genes included cuticle proteins, nucleic acid binding proteins, ribosomal proteins, and actin-binding proteins, which Koshikawa et al., inferred to be involved in caste-specific morphogenesis (2005).

Wu-Scharf et al. (2003) executed a pilot study in *R. flavipes* using expressed sequence tags (ESTs), or partial cDNA sequences, to identify 88 high quality ESTs. Next, Scharf et al., (2003b) used cDNA macroarrays to compare gene expression between polyphenic castes. This experiment was the first that provided a summary of more than 20 caste-associated genes in termites. They found cellulase genes expressed in only workers and nymphs, genes relating to transcriptional and translational regulation and signal transduction in soldiers, genes associated with musculature and cytoskeletal architecture in soldiers, genes encoding vitellogenin in presoldiers, and several unidentified genes present in some castes but not others. Scharf et al., (2005a) used the same approach to identify 34 nymph-biased genes. These genes had associations with vitellogenesis, nutrient storage, juvenile hormone sequestration, ribosomal translational and filtering mechanisms, fatty acid biosynthesis, apoptosis inhibition, and both endogenous and symbiont cellulases. Scharf et al. (2005b) used model bioassays to identify specific genes and hemolymph proteins that change expression during the worker-to-presoldier transition. This study also validated the usefulness of JH model assays for inducing synchronized molecular changes in worker to presoldier differentiation. Having a JH model assay system

enables controlled experiments to be conducted, which allows the direct comparison between treatments and controls. This bioassay system is considered to have an advantage over other experimental approaches that are based on behavioral observation alone (e.g., Whitman and Forschler, 2007) or physiological comparisons of caste phenotypes after differentiation alone (e.g., Korb et al., 2009a).

In focusing on two candidate caste regulatory genes, Zhou et al. (2006a) identified two hexamerin genes that help regulate caste differentiation by binding JH. Using RNAi, they showed that by silencing the *Hex-1* and *Hex-2* genes, protein expression was lowered. The hexamerin silencing apparently limited JH sequestration, resulting in greater pre-soldier differentiation. Zhou et al. (2006b, 2007) provide additional evidence that the Hex-1 and Hex-2 proteins participate in the regulation of caste-differentiation by modulating JH availability. They demonstrated that *Hex-1* and *Hex-2* have elevated expressions in caste phenotypes that differentiate in response to rising JH titers, and that *Hex-1* and *Hex-2* have distinct protein structures. Also results from *Hex* silencing has similar effects on gene expression as JH treatment. These findings were the first demonstration of a status quo regulatory mechanism for worker caste retention, providing the first example of a physiological caste regulatory mechanism in a social insect.

Hypotheses, Rationale and Objectives

Although the work reviewed above provides evidence that soldiers influence nestmate caste differentiation, and that socio-genomic factors contribute to nestmate caste differentiation, what remains unknown is how these two mechanisms work together. To resolve this problem, the following research sought to investigate the relevant interactions between termite soldiers (and the chemicals they produce) and their nestmates. To understand this relationship, I tested the central hypothesis that the chemicals produced by *R. flavipes* soldiers influence phenotype

and gene expression of workers. To reach the overall objective, the following specific aims were pursued: 1) characterize and identify the effects soldier head chemicals (SHE) have on worker caste differentiation, 2) identify genes that respond to multiple socio-environmental and semio-chemical factors including SHE, and 3) further characterize three genes (*Cyp15F1*, *Cyp15A1*, and *RFest1*) that potentially play roles in worker termite caste differentiation.

CHAPTER 2
EFFECTS OF SOLDIER-DERIVED TERPENES ON SOLDIER CASTE DIFFERENTIATION IN THE TERMITE *RETICULITERMES FLAVIPES*

Introduction

Social insect castes are groups of phenotypically, morphologically and behaviorally distinct individuals that cooperate to perform colony tasks (Wilson, 1971; Miura, 2004). Caste differentiation plays an important and necessary role in creating an effective division of labor. It is imperative that colonies find ways to regulate caste differentiation within this system. Improper regulation could result in the over-abundance or absence of specific castes, making colony tasks such as food acquisition, grooming, defense, and reproduction inefficient or even impossible.

Polyphenisms are alternative morphological phenotypes that differentiate in response to environmental conditions (Nijhout, 2003). Termites use polyphenism to produce different castes that perform complementary roles within the colony (Miura, 2004). Termite colonies are made up of three distinct castes that include workers/pseudergates, soldiers, and reproductives. Only soldiers and reproductives are considered adults in lower termites, while all castes can be adults in higher termites. Termite caste differentiation can proceed along two routes; the imaginal (winged) or the apterous (wingless) route. The first developmental branch point occurs when larvae differentiate into either workers or nymphs after the second instar (Buchli, 1958; Lainé and Wright, 2003). Workers can: (1) undergo status quo worker-to-worker molts, (2) differentiate into presoldiers (immediately followed by soldier differentiation) or (3) differentiate into apterous and eyeless third-form reproductives, or "ergatoid neotenics." Nymphs can either; (1) regress into worker-like pseudergates, (2) differentiate into fully winged and eyed adult alates that disperse, mate, and become primary reproductives, or (3) differentiate into wingless and

eyed non-dispersive second form reproductives, or “brachypterous neotenics” that serve as supplemental reproductives (Buchli, 1958; Lainé and Wright, 2003).

Caste polyphenism in social insects is distinct from solitary insects because multiple castes that perform non-overlapping tasks are present in colonies at the same time (Miura, 2004). Individuals in termite colonies with the same genetic background can differentiate into alternate phenotypes depending on a number of intrinsic and extrinsic factors (Lenz, 1976; Greenberg and Tobe, 1984; Koshikawa et al., 2005; Scharf et al., 2007). One intrinsic factor is juvenile hormone (JH) (Scharf et al., 2003b; Park and Raina, 2004, 2005; Mao et al., 2005). Juvenile hormone is a morphogenetic hormone produced by a neurosecretory gland (the corpus allatum) that has a broad range of developmental and physiological effects (Wigglesworth, 1935; Schal et al., 1997; Truman and Riddiford, 1999; Gilbert et al., 2000; Truman et al., 2006). For example, in insects juvenile hormone plays a role in the control of larval / nymphal development and metamorphosis, diapause, migratory behavior, wing length, seasonal development, reproduction, and caste determination (Hartfelder, 2000).

Primer pheromones are chemical messengers that are passed among individuals and trigger physiological responses in recipients (Wilson and Bossert, 1963). Primer pheromones are distinct from “releaser” pheromones, which elicit rapid behavioral responses in recipients (Vander Meer et al., 1998). Two examples of releaser pheromones in termites are the trail pheromone (Z,Z,E)-3,6,8-dodecatrien-1-ol (Matsumura, 1968) and the phagostimulatory pheromone hydroquinone (Reinhard et al., 2002). Three examples of primer pheromones from the honey bee are worker behavioral maturation inhibitory pheromone (ethyl oleate; Leoncini et al., 2004), brood pheromone (fatty acid esters; LeConte et al., 2006), and queen mandibular pheromone (5 carboxylate and aromatic components; Grozinger et al., 2007). Although no

primer pheromones have been identified in termites, JH has been proposed as a possible termite primer pheromone (Henderson, 1998). Previous studies have shown that ectopic exposure of worker termites to JH III readily induces soldier caste differentiation (Howard and Haverty, 1979; Scharf et al., 2003b, 2005, 2007; Zhou et al. 2006a,b, 2007), indicating that JH can act via exogenous exposure. Under natural conditions, high endogenous JH titers in worker termites cause differentiation into presoldiers, and then into soldiers (Park and Raina, 2004; Mao et al., 2005). Regardless of whether JH acts exogenously as a primer pheromone, or as an endogenous hormone, or both, the role of JH in soldier development is unique and contrasts the immature "status quo" role of JH among insects (Henderson, 1998).

It has been hypothesized that termite soldiers may play a role in regulating worker differentiation to other caste phenotypes (Henderson, 1998). For example, JH titers in workers rise upon removal from the colony (Okot-Kotber et al., 1993; Mao et al., 2005), which can result in presoldier / soldier formation (Mao et al., 2005). However, if workers are held with soldiers, worker JH titers remain below threshold levels and presoldier formation is attenuated (Mao et al., 2005; Park and Raina, 2005). It has been theorized that soldiers can down-regulate worker JH titers by acting as a JH "sink" (Henderson, 1998; Mao et al., 2005) or by lifting some other primer pheromone's inhibition on worker differentiation (Park and Raina, 2004, 2005; Mao et al., 2005).

Previously, Lefeuve and Bordereau (1984) investigated live soldiers and the effects of methylene chloride (dichloromethane; DCM) soldier head extracts (SHE) on caste differentiation in the higher termite *Nasutitermes lujae*; they found that SHE inhibited worker-to-soldier differentiation. They further suggested that soldier termites may secrete an inhibitory pheromone that contributes to worker-soldier homeostasis in termite societies. Korb et al. (2003) also

reported that DCM SHE inhibited soldier formation in the lower termite *Cryptotermes secundus*. Additionally, Okot-Kotber et al. (1991) also showed that soldier formation in *R. flavipes* was reduced by DCM SHE when co-applied in combination with synthetic JH analogs. While these studies have verified primer pheromone-like effects for SHE, no bona-fide termite primer pheromones have yet been chemically identified. Thus, two important outstanding questions in termite research relate to whether or not caste-regulatory primer pheromones exist, and if so, what are their chemical structures and modes of action?

Reticuliterme. flavipes and its European synonym *R. santonensis* are common and economically destructive termites in the U.S. and Europe; thus, there is a need to define their chemical ecology with respect to caste regulation. The central objective of this study was to investigate chemical constituents of *R. flavipes* SHE as possible primer pheromones. To meet this objective, we conducted studies to (1) investigate SHE effects on JH-dependent soldier caste differentiation, (2) identify SHE constituents, and (3) compare constituent activity with previously identified soldier head chemicals. Through these studies, we provide evidence supporting the idea that soldier-derived terpenes play roles as caste-regulatory primer pheromones in termites.

Materials and Methods

Termites

Reticulitermes flavipes colonies were collected from various locations on the University of Florida campus. Termites were brought back to the laboratory and held for at least 2 months before use. Laboratory colonies were maintained in darkness within sealed plastic boxes, at 22 ^{o}C. A total of 9 termite colonies were tested, all of which contained male and female neotenic reproductives. Termite workers were considered workers if they did not possess any sign of wing buds or distended abdomens. Termites were identified as *R. flavipes* from sequence of the *16S*

mitochondrial-ribosomal RNA gene, (Szalanski et al., 2003), gut fauna (Lewis and Forschler, 2004), and soldier morphology (Nutting, 1990).

Dish Assays

Dish assays were conducted at 27 ℃ as described previously (Scharf et al., 2003b). Paired paper towel sandwiches (Georgia Pacific) were treated with respective control, JH III, and SHE treatments delivered in solvent (acetone). JH III (75% purity; Sigma; St. Louis, MO) was provided at 112.5 µg per dish in a volume of 200 µl acetone. This JH quantity was chosen based on its maximal efficacy and minimal mortality observed in previous concentration range studies (Scharf et al., 2003b). SHE was tested at several different quantities (see next section). After solvent evaporation, paper towel sandwiches were placed in 5 cm plastic Petri dishes and then received 150 µl of reverse osmosis water. Fifteen worker termites were placed in each dish. Every five days termites were counted, presoldier formation was noted, and deionized water was added if needed.

Solider Head Extracts

Soldier head extract (SHE) was prepared by collecting soldiers from laboratory colonies, removing their heads, and then by homogenizing the heads (~80-150 total, depending on the experiment) in acetone with a Tenbroeck glass homogenizer. SHE was fractionated by passing it through a glass Pasteur pipette filled with approximately 250 mg of silica gel (60-200 mesh) on top of a glass wool plug. The eluting solvent in fractions consisted of 10 column volumes of the extraction solvent (acetone). The fractionated SHE was then brought to 50 ml in a volumetric flask.

SHE Concentration Response and Investigation of Colony Variation

SHE prepared in acetone was tested at multiple concentrations on three *R. flavipes* colonies (Colonies 7, 8 and 9). Seven different treatments were tested: control (300 µl acetone), JH III

(200 µl acetone containing 112.5 µg JH III), SHE alone (4 head equivalents), and JH III plus a range of soldier head extract equivalents (0.5, 1, 2 and 4). Each treatment was replicated six times.

Gas Chromatography (GC) and Mass Spectrometry (MS)

Thirty soldier and worker heads from two different colonies (colonies 5 and 7) were extracted as described above (acetone) in a volume of 2 ml and evaporated under N_2 to 400 µl. Samples were first analyzed by GC/MS (electron ionization, 70eV) to confirm the presence of the previously published predominant terpenoids, namely γ-cadinene and γ-cadinenal (Nelson et al., 2001), and then subsequently quantified using a 6890 gas chromatograph (Agilent; Santa Clara, CA) coupled to a flame ionization detector as described in full by Schmelz et al. (2001). We also examined pine wood extracts, prepared from the same "shim" wood used to provision lab colonies (seasoned and kiln-baked), to specifically test the hypothesis that SHE chemicals are produced in termites *de novo*. Fresh pine wood sawdust (1.26 g) was extracted and analyzed as described above for head extracts (acetone).

To quantify semiochemical levels found in individual soldier heads, five individual soldier heads were extracted in a similar manner as above. Individual extracts were in a final volume of 400 µl; an internal standard of 400 ng of nonyl acetate was added to each sample. Samples were then separated by GC. Peaks were analyzed and quantified by comparing to the nonyl acetate standard.

Nuclear Magnetic Resonance (NMR) Analysis

NMR analyses were performed to accurately identify the cadinene chemicals from the soldier heads. The two main peaks of the SHE were separated using preparative GC and analyzed by NMR. Initial sample preparation of soldier head solvent extracts utilized vapor

phase extraction at 80 ºC on polymeric adsorbent traps, followed by dichloromethane elution to remove less volatile contaminants (Schmelz et al., 2004). Micropreparative gas chromatography (GC) was accomplished using an Agilent (Santa Clara, CA) 6890 gas chromatograph (He carrier gas; 5.7 ml min-1; cool on-column injector set to track oven) with an DB-1 column (30 m long, 530 µm i.d., 0.50 µm film thickness) with the temperature programmed from 35 °C (2 min hold) at 10 °C min-1 to 260 °C (hold for 5.5 min). Recovery of separated GC fractions followed from Heath and Dueben (1998) with slight modification. Specifically, a glass press-fit splitter was used at the end of the DB-1 column, coupling a 0.5 m (150 µm i.d. fused silica) capillary to the flame ionization detector (FID) and a second 0.5 m (350 µm i.d. fused silica) capillary directed to the heated transfer line and chilled glass capillary for sample collection. Under these conditions, the two predominant soldier head sesquiterpenes eluted at 16.1 and 18.9 min. Authentic standards of γ-cadinene were similarly chromatographed, eluted at 16.1 min and recollected for NMR.

One-dimensional (1-D) and two-dimensional (2-D) NMR spectra were acquired at 20 ºC with standard techniques using TopSpin® (version 2.1) software on a Bruker Avance-II-600 spectrometer equipped with a 1 mm high-temperature superconducting (HTS) CryoProbe (Brey et al. 2006). Solutions of the SHE γ-cadinene, ~ 10 µg/15 µl, the authentic γ-cadinene, ~ 25 µg/17 µl and of the SHE γ-cadinene aldehyde, ~ 50 µg/10 µl, were prepared in $CDCl_3$ (99.96 atom % D). These solutions were added via a 110 mm-needled 10 µl syringe to 1 mm O.D. x 0.73 mm I.D. x 100 mm long capillary NMR tubes (Norell, Inc.). The capillaries were then attached to an appropriate Bruker MATCHTM apparatus before being lowered into the NMR magnet for analysis. Proton spectra were acquired at 600.23 MHz using 45º pulses, 32768 complex points over an 11 ppm spectral width (SW) – corresponding to a 2.48 second

acquisition time (AT), and a 3 second relaxation delay (RD). The ^{1}H data was processed by zero filling the FID's to 32768 real points before application of line broadening (LB) and Fourier transformation. An exponential LB value of 0.4 Hz was used for integrated spectra, and a negative LB value of (–) 0.2 Hz was used for "peak picking." The ^{1}H chemical shift axis was referenced to $CHCl_3$, assigned to 7.26 ppm (Gottlieb et al., 1997). Abbreviations in ^{1}H spectra: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, J = apparent coupling constants in Hertz. Two-Dimensional $^{1}H/^{1}H$-COSY data sets (SW = 8 ppm, AT = 0.21 seconds, RD = 2 seconds, 2-8 transients) were acquired with Bruker's "cosygpqf" pulse sequence as 2048 complex points in the directly detected dimension (DD) and 512 increments in the indirect dimension (ID), and they were processed with sine-function apodization into 1024 x 1024 point spectra. Carbon-13 spectra were acquired at 150.93 MHz using 45º pulses, 65536 complex points over a 220 ppm SW – corresponding to a 0.98 second AT, and a 3 second RD. The 13C FID's were Fourier transformed after zero filling to 65536 real points and applying an exponential LB value of 2 Hz. The ^{13}C chemical shift axis was referenced to $CDCl_3$, assigned to 77.16 ppm (Gottlieb et al., 1997). Multiplicity-edited 2-dimensional $^{1}H/^{13}C$-HSQC data sets (1H SW = 8 ppm, ^{13}C SW = 170 ppm, AT = 0.14 seconds, RD = 2 seconds, 48-96 transients) were acquired with Bruker's "hsqcedetgpsisp2.2" pulse sequence as 1348 complex points in the DD dimension and 256 increments in the ID dimension, and they were anodized with cosine squared-functions into 2048 x 512 point spectra.

Previously Identified Chemicals

Past research (Zalkow et al., 1981; Bagnères et al., 1990; Nelson et al., 2001; Quintana et al., 2003) and my own GC-MS efforts (current chapter) have identified a number of chemicals from termite soldier heads. Chemicals (or close structural analogs) were tested individually in

dish assays on a single *R. flavipes* colony (Colony 5). All treatments were applied at 50 µg/dish, with and without JH III (300 µl acetone containing 112.5 µg JH III). Individual chemical treatments were provided at a quantity equivalent to approximately one half of the JH III dose in order to test synergistic effects on JH III-induced presoldier differentiation. This amount [50 µg/dish] approximates endogenous cadinene levels found in 25 soldier head equivalents, based on GC-MS analysis. Treatments were as follows: controls (300 µl acetone), SHE alone (4 head equivalents in acetone), α-humulene (CAS: 6753-98-6, Fluka, Sigma Aldrich, St. Louis, MO), β -farnesene (CAS:18794-84-8, Bedoukian, Danbury, CT), cadinene (CAS: 29350-73-0, Vigon International, East Stroudsburg, PA), geranyl linalool (CAS: 1113-21-9, Acros, New Jersey, NJ), linalool (CAS: 78-70-6, Aldrich), farnesol (CAS: 4602-84-0, Bedoukian), (+)β-pinene oxide (CAS: 6931-54-0, Acros), limonene (CAS: 5989-27-5, Aldrich), nootkatone (CAS: 4674-50-4, Bedoukian), nerolidol (CAS: 7212-44-4, Bedoukian), α-pinene (CAS: 80-56-8, Acros), and geranylgeraniol (CAS: 24034-73-9, Fluka, Sigma Aldrich). Control treatments included acetone, JH III alone, SHE alone, JH III+SHE. All SHE was prepared in acetone. Each treatment was replicated three times.

Statistical Analyses

In all experiments the number and caste of each termite in each dish was counted every five days. The percentage of presoldiers formed out of the total number of workers put into each assay was used in statistical analyses (Scharf et al., 2003b, 2005; Zhou et al., 2006a,b). Data were first analyzed for normality using the Levene test. If the data were not normal, the data were transformed to ranked averages and means separated using the Tukey-Kramer test ($p<0.05$). For bioassays with previously identified soldier chemicals, ranked averages were separated using a LSD Student t-test ($p<0.05$).

Results

SHE Concentration Response

Three colonies were examined in SHE dose-response bioassays using SHE prepared in acetone (Figure 2-1). Two of the three colonies responded similarly, but one colony (colony-9) responded slightly differently, which led to a significant colony effect in the ANOVA ($df=2,117$, $F=4.788$, $p=0.01$). Nonetheless, a pooled dose-response analysis of the three colonies was conducted. Presoldier induction significantly increased when termite workers were co-exposed to SHE and JH III, as compared to treatments of JH III alone ($p<0.05$). Controls treated with either acetone or SHE alone resulted in no presoldier formation. Presoldiers first appeared between days 10 and 15, and reached maximum levels by day 25 in all SHE + JH III and JH III-alone treatments. This analysis verifies that SHE does indeed cause a significant increase in presoldier formation when combined with JH III, however, this effect is not significantly dose-dependent in the range of 0.5 - 4 head equivalents ($df= 6,117$, $F= 32.32$, $p<0.0001$).

GC-MS and NMR Analysis

GC-FID analyses of soldier head extract identified two major sets of peaks (Figure 2-2). Retention times, peak size, and GC-MS spectra of the two sets of peaks have similar profiles as Zalkow et al. (1981) and Nelson et al. (2001), who identified γ-cadinene and γ-cadinenal as major whole-head extract components (Figure 2-3). The first peak, γ-cadinene, was identified by comparing its spectra with those in the literature, as well as by a gas-chromatographic comparison with the same sample. Additionally, comparison of the SHE γ -cadinene and of an authentic sample of γ-cadinene (kindly provided by Dr. Bartelt, USDA-ARS-NCAUR; Peoria, IL) by GC-MS analysis (EI, 70 eV) gave the same EI mass spectra and identical GC retention times. The mass spectrum and the 1H (600 MHz) NMR spectrum of the SHE γ -cadinene were the same as those described for γ-cadinene by Quintana et al. (2003). My analyses further

confirmed that the SHE γ-cadinene and the authentic sample of γ-cadinene produced the same NMR spectra. That is, except for trace impurities in the natural sample, they gave identical 1-dimensional (^{1}H) and 2-dimensional (^{1}H/^{1}H-COSY and ^{1}H/^{13}C-HSQC) NMR spectra.

The corresponding γ-cadinene aldehyde (γ-cadinenal), assumed to arise from allylic oxidation of the olefinic methyl group of γ-cadinene, was identified by comparison of its ^{1}H NMR spectrum (see data below) and EI-mass spectrum to those reported by Kaiser and Lamparsky (1983). Since we observed some small differences between their 400 MHz ^{1}H spectrum and ours at 600 MHz, we also report the details of the ^{1}H NMR spectrum here, along with the fifteen chemical shifts for the ^{13}C NMR resonances of the SHE γ -cadinene aldehyde.

NMR results are as follows; additional data and structural information can be provided upon request. ^{1}H NMR (600 MHz, $CHCl_3$ = 7.26 ppm (Gottlieb et al., 1997)) 9.47 (s, 1 H), 6.91 s, 1 H), 4.74 ("d", J = 1.5, 1 H), 4.62 ("d", J = 1.3, 1 H), 2.52-2.46 (m, 1 H), 2.44 (ddd, J = 2.9, 4.0, 13.0, 1 H), 2.26 (d septets, J = 3.3, 6.9, 1 H), 2.15-2.07 (multiplets, 2 H), 2.06 (broad dt, J = 4.5, 13.1, 1 H), 1.97-1.91 (t of "five-line patterns", 1 H), 1.90-1.84 (multiplets, 2 H), 1.50-1.41 (multiplet, 1 H), 1.42 (tt, J = 3.2, 11.6, 1 H), 1.21 (dq, J = 4.2, 12.8, 1 H), 0.99 (d, J = 6.9, 3 H), 0.82 (d, J = 6.9, 3 H).

^{13}C NMR (151 MHz, $CDCl_3$ = 77.16 ppm (Gottlieb et al. 1997)) - 194.76, 151.78, 151.75, 141.77, 104.65, 46.37, 46.05, 44.07, 36.05, 26.69, 26.65, 24.45, 21.87, 21.57, 15.34.

The average amount of γ-cadinene and γ-cadinenal from soldiers was 1.44 ± 0.29 and 9.42 ± 1.75 µg, respectively. The amount of γ-cadinenal was significantly higher than the amount of γ-cadinene (df=1,8, F=20.2864, p=0.0020). Although weakly abundant in the worker extracts, the γ-cadinene and γ-cadinenal were substantially more prevalent in the soldier heads (Figure 2-2). Pine wood extracts prepared using an identical extraction method did not indicate any

similarity to chemicals found in SHE (Figure 2-2), supporting that cadinene and cadinenal are produced *de novo*.

Cadinene and Previously Described Soldier Chemicals Enhance JH-Induced Presoldier Differentiation

Twelve previously identified soldier-derived chemicals, including cadinene, were tested for their ability to induce presoldier formation in dish assays. All of these previously described chemicals (except nootkatone and nerolidol), when tested in combination with JH III, caused significant increases in presoldier differentiation relative to JH III alone. When tested without JH III, the soldier chemicals caused no presoldier differentiation ($df=26,63$, $F=14.4633$, $p<0.0001$) (Figure 2-4). Similar to all previous assays, no presoldiers were observed in acetone controls, while high presoldier induction levels (~80%) were observed in SHE + JH III treatments. Treatments of JH III alone induced significantly lower presoldier levels (~20%), which are comparable to results of preceding experiments as presented above and Appendix A.

Discussion

In previous research, termite soldier-produced chemicals have mostly been investigated as a taxonomic tool for species identification (Zalkow et al., 1981; Prestwich, 1983; Bagneres et al., 1990; Nelson et al., 2001; Quintana et al., 2003, Nelson et al., 2008). Such research has identified a number of chemicals in soldier secretions, but little consideration has been given to roles of these chemicals in caste differentiation. The study presented here confirms the effects of *R. flavipes* SHE on JH-induced presoldier differentiation. Results from multiple bioassays on different colonies at different times of the year indicated that SHE synergistically increases worker to soldier morphogenesis when applied in combination with JH III. These findings support the idea that the soldier caste, in addition to playing a defensive role, also plays a part in caste regulation within termite society (Henderson, 1998).

This study also supports previous research showing ectopic JH III treatments cause some workers to molt into presoldiers (and onto soldiers) (Scharf et al., 2003b, 2005, 2007; Zhou et al., 2006a,b). The JH III mediated worker-to-soldier molt is an atypical example of a JH III response when compared to other insect groups. In most insects, JH causes insects to remain as immature forms during a molt, while the absence of JH causes the insect to molt into an adult form. Thus, termites have apparently co-opted JH for a different function than other insect groups.

The combination of SHE with ectopic JH III treatments synergistically enhanced presoldier development relative to JH III alone, while SHE by itself caused no presoldier induction. This suggests that SHE probably does not contain significant quantities of JH. Preliminary thin layer chromatography separations of JH III and SHE showed no common bands (MRT unpublished) and GC-MS of SHE identified no JH III, supporting the absence of JH III in SHE. Therefore, in these assays, I conclude that chemicals from soldier heads modulate the termite response to ectopically applied JH III, thereby enhancing JH III activity. It is hypothesized that, endogenously, the synergistic effect of these SHE terpenes is manifest only in individuals with elevated JH titers.

The results from this study are in contrast with past reports concluding that soldiers and extracts from soldiers inhibit presoldier formation (Lefeuve and Bordereau, 1984; Okot-Kotber et al., 1991; Korb et al., 2003). There are several differences between the current and past research that could at least partially explain these discrepancies. First, Lefeuve and Bordereau (1984) exposed groups of 200 workers of the higher termite *Nasutitermes lujae* to one of three treatments that included nothing, live soldiers, or SHE extracted in dichloromethane (DCM). Differences between this and the current study include extraction solvent, termite species, and group size. Korb et al. (2003) tested the effect of precocene II, an allatectomizing agent, and

SHE extracted in DCM on whole colonies of the lower termite, *Cryptotermes secundus*. Differences between this study and that of Korb et al. (2003) include solvent, termite species, and treatment size. Also, Korb et al. did not test precocene in combination with natural JH or SHE. Okot-Kotber et al. (1991) tested combinations of methoprene and SHE extracted in DCM on *R. flavipes* in a dish assay, similar to the experiment described here, and found that the combination resulted in less presoldier formation than treatments of methoprene alone. We found no difference between SHE extracted in DCM or acetone (Appendix A), eliminating the effect of solvent. However, we used JH III in this study while Okot-Kotber et al. (1991) used the JH analog methoprene. Other factors that may explain some of the differences between this study and preceding studies may be colony conditions at the time of testing and the time of year at which testing was performed; e.g., responses to SHE and JH may vary among termite colonies, as well as within a colony over a year according to season. An additional potential difference would be a difference in the ratio of components found in the SHE blend.

While these results suggest components of *Reticulitermes* SHE function as primer pheromones, soldier secretions of other termite species have *bona fide* defensive functions. For example, *Coptotermes* soldiers produce latex to defend against predators (Prestwich, 1983, 1984; Abe et al., 2000). This proposed primer pheromone function for *Reticulitermes* head chemicals is supported by a study reported by Zalkow et al. (1981), who assayed a number of *R. flavipes* and *R. virginicus* soldier head chemicals against the native fire ant, *Solenopsis geminata*. Their results indicated the ants had not been sprayed with an irritant or toxicant and that the soldier head chemicals have non-defensive functions.

No evidence was obtained in the present study to suggest that the chemicals are expelled from soldiers. One explanation for the soldiers having a large amount of putative primer

pheromone in their heads is to serve as a recruiting mechanism after an individual soldier is killed. For example, if a soldier is killed when defending the colony, the chemicals acquired while disposing of the body may signal nestmate workers to differentiate into soldiers. Since workers also contained small amounts of cadinene and cadinenal, another possibility is that soldiers may absorb and sequester these compounds away from workers in order to suppress worker differentiation. For example, live soldiers suppress worker JH titers and inhibit presoldier formation (Park and Raina, 2004; Mao et al., 2005). Future research efforts will test hypotheses relating to impacts of live and dead soldiers in nestmate differentiation and terpene sequestration.

Of the soldier head terpenes identified in previous research, all but two significantly enhanced JH-induced presoldier formation when combined with JH III at a ratio of 1:2 (terpene:JH). When applied at the same concentration without JH III, however, none of the terpenes induced presoldier formation. This suggests that *Reticulitermes* have the ability to utilize an array of terpenes as cues to trigger soldier caste differentiation, provided that endogenous JH titers are above critical thresholds. Future research should determine what structural features of the terpenes are necessary for activity, and investigate ratios of blend components.

The regulation of termite caste differentiation is important in maintaining social structure and function, and therefore the disruption of termite caste differentiation / homeostasis may be able to be used as a control method. By using the termite's own chemistry (i.e., soldier-derived terpenes), it may be possible to develop a specific termiticide that causes a large proportion of worker termites to molt into soldiers. Because soldier termites cannot feed themselves, this would likely cause the termite colony to starve or at least have a severe effect on the colony. For

example, in this study (unpublished results), mortality was greatest in replicates in which a high proportion of worker termites molted into presoldiers.

In summary, the findings presented here verify a role beyond defense for the soldier caste within termite societies, as initially proposed by Henderson (1998). These results indicate that non-JH terpenes from termite soldier castes can influence caste polyphenism in nestmates. The results presented here help identify part of the complex chemical communication system that termites utilize to maintain a balanced social environment.

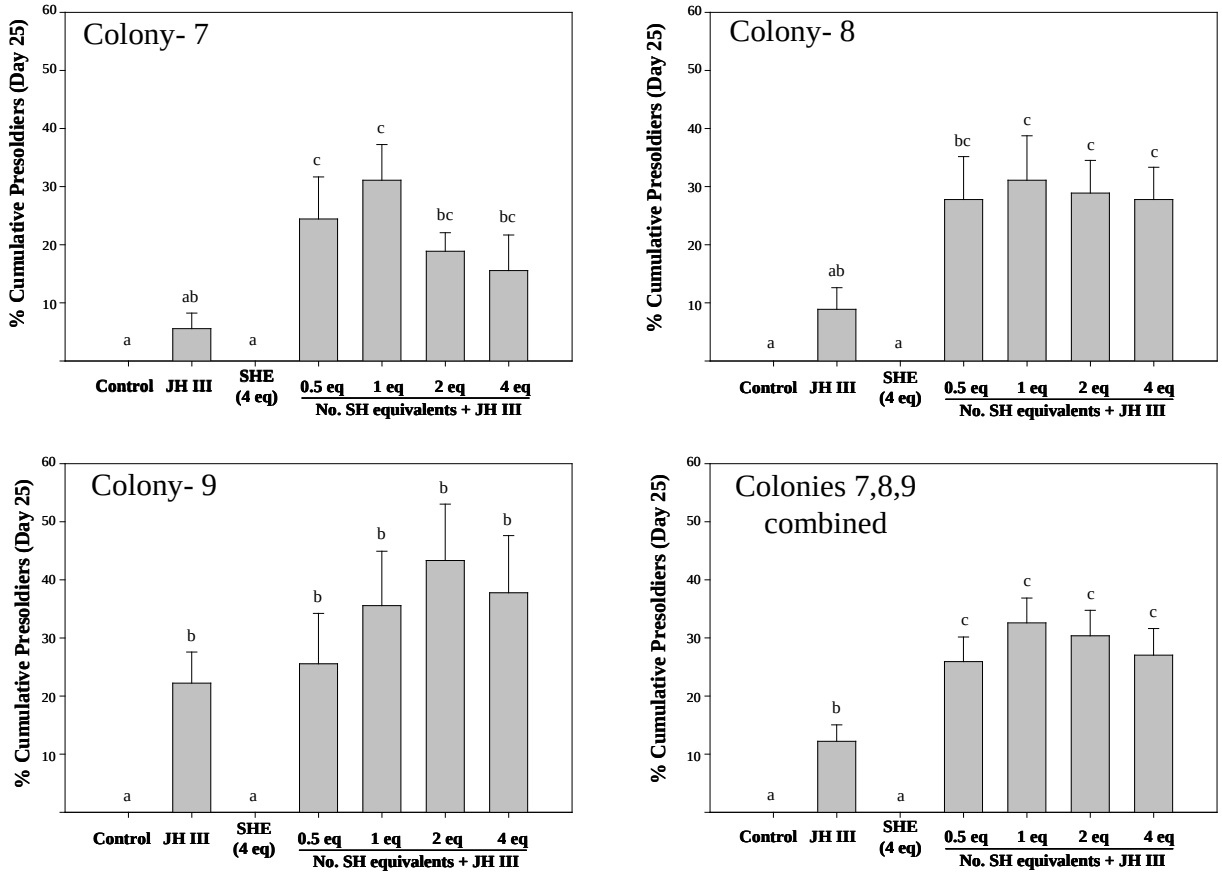

Figure 2-1**.** Soldier head extract (SHE) dose-response. Worker termites were exposed to different soldier head equivalents (eq) or control treatments for 25 days. SHE was prepared in acetone. Soldier head extract alone was applied at 4 head equivalents. The number of head equivalents tested in combination with JH III was 0.5, 1, 2 and 4. Each treatment was replicated six times on three different colonies (7, 8 and 9). The graphs for colonies 7, 8 and 9 show cumulative avg. ± std. error presoldier induction through assay day 25 for each of the separate colonies. The graph at the bottom right shows cumulative avg. ± std. error presoldier induction for the combined colony responses. Letters represent significant differences at $p<0.05$.

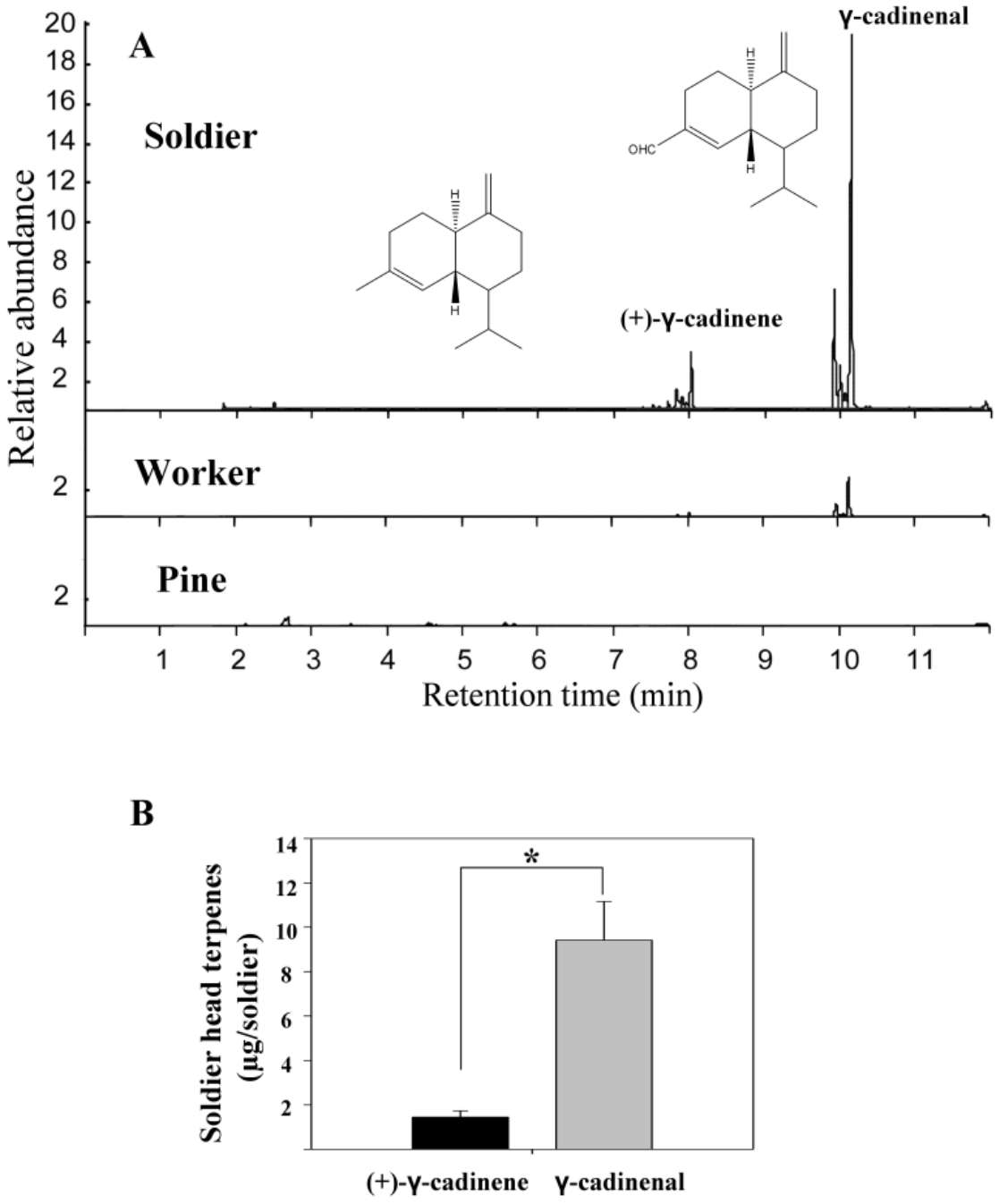

Figure 2-2. SHE chemistry. A) Gas chromatograms (flame ionization detection) of acetone extracts prepared from heads of 30 soldier (top) and worker (middle) *R. flavipes*, as well as 1.26 g of seasoned pinewood (bottom). Pinewood was seasoned and identical to that used to feed termite colonies. Chemical structures were putatively identified through NMR. B) Acetone extracts from individual soldiers were analyzed and the quantity of γ-cadinene and γ-cadinenal estimated by comparison to internal nonyl acetate standards. Bars represent avg. +/- std. error determined from 5 individuals. Asterisks (*) denote significant differences as $p<0.05$.

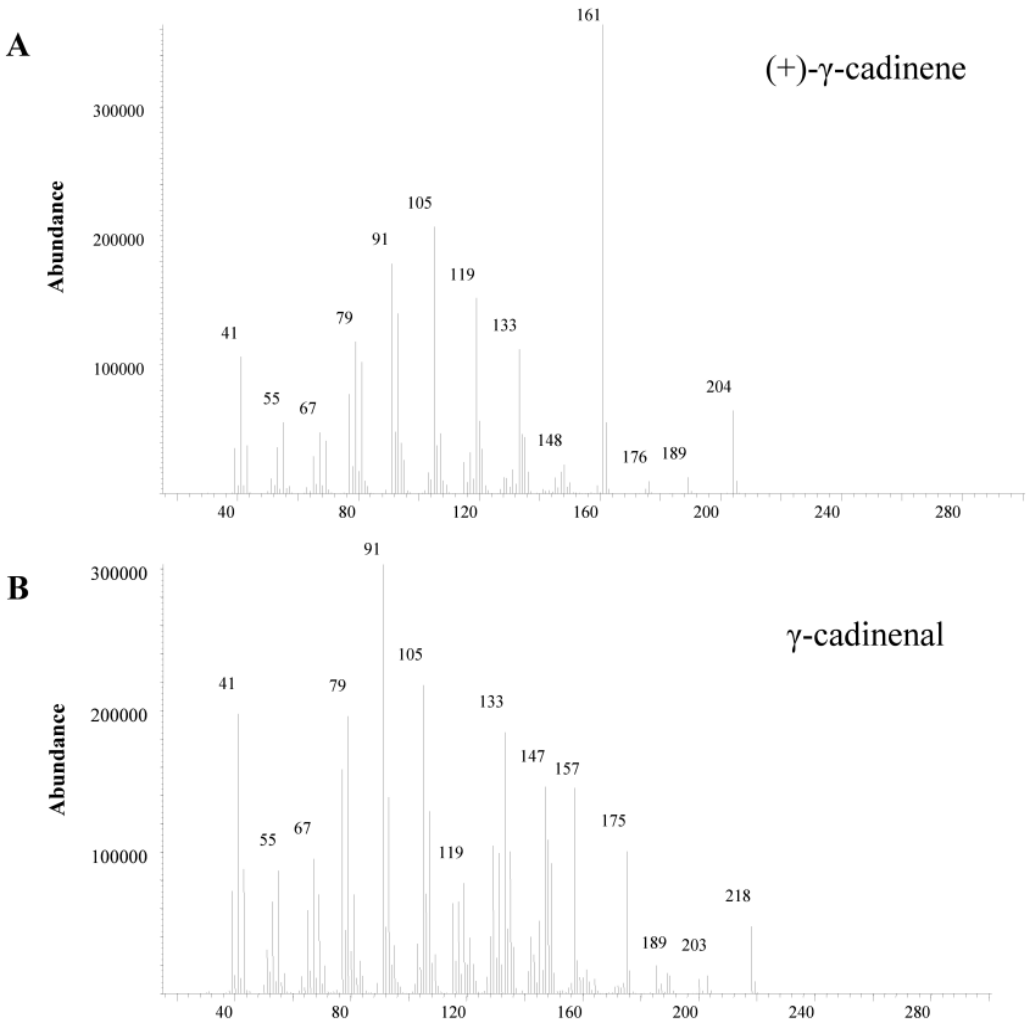

Figure 2-3. Analysis of soldier head extracts by mass spectrometry. Dominant compounds identified by gas chromatography were analyzed using mass spectrometry and were identified as γ-cadinene and γ-cadinenal.

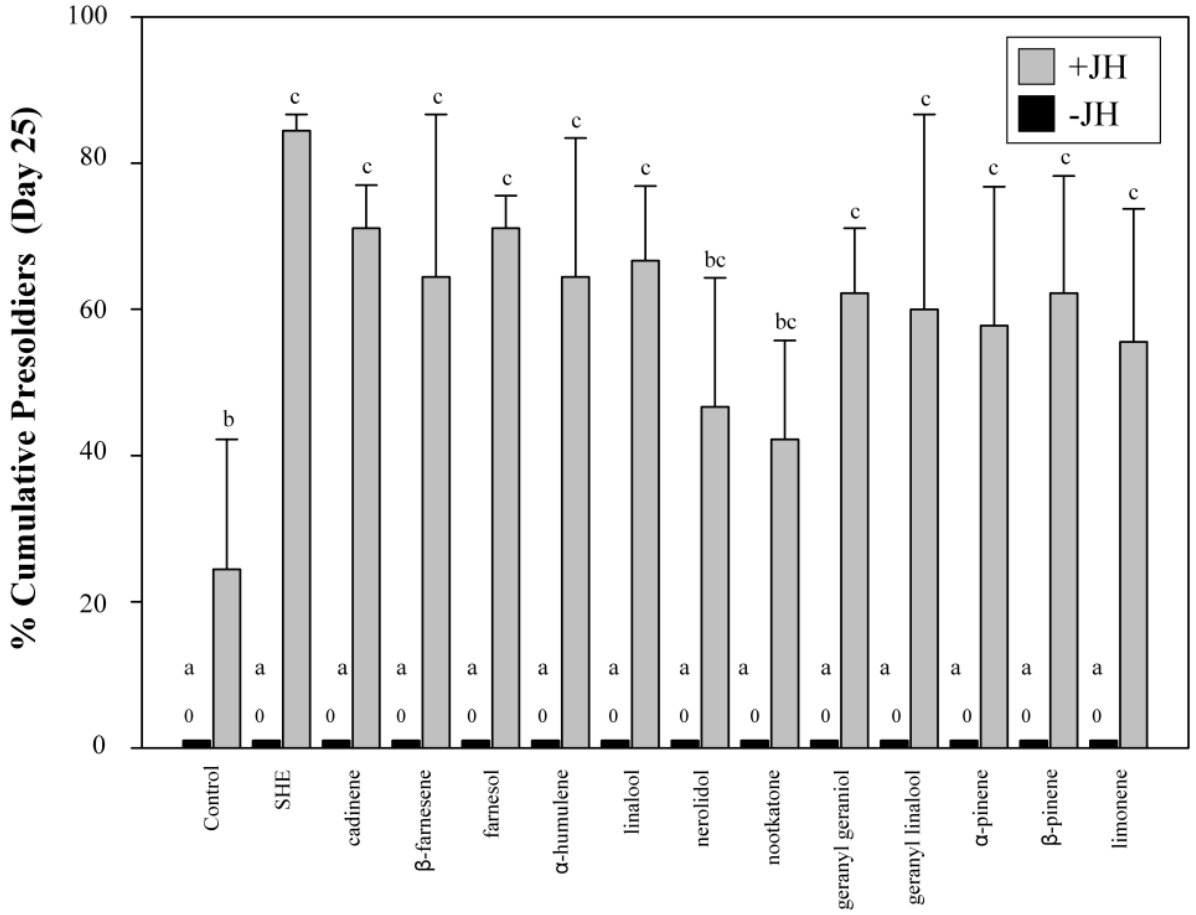

Figure 2-4. Previously described soldier-derived terpenes synergistically enhance JH-dependent presoldier differentiation. Twelve previously identified soldier chemicals (mono-, sesqui- and di-terpenes) were tested for their ability to induce presoldier differentiation, alone and in combination with JH III. Treatments included; negative controls (300 µl acetone), SHE (4 soldier head equivalents), humulene, β-farnesene, cadinene, geranyl linalool, linalool, farnesol, β-pinene, limonene, nootkatone, nerolidol, α-pinene, and geranyl geraniol. All soldier head chemicals were tested at 50 µg / dish, with and without JH III (150 µg). Each treatment was replicated three times. The graph shows cumulative avg. ± std. error presoldier induction through assay day 25. Letters represent significant differences at $p<0.05$.

CHAPTER 3
SEMIOCHEMICAL AND SOCIO-ENVIRONMENTAL EFFECTS ON CASTE DIFFERENTIATION AND GENE EXPRESSION IN *RETICULITERMES FLAVIPES*

Introduction

Phenotypic plasticity is described as a single genotype that has the ability to produce numerous different phenotypes, depending on various conditions encountered through its development (Nijhout, 1999). Phenotypic plasticity can be divided into two major types of responses to signals: reaction norms and polyphenisms. Reaction norms are phenotypically graded responses to environmental factors. Polyphenisms occur when two or more discrete alternative phenotypes are observed without intermediate forms (Nijhout, 2003).

Social insects have evolved to exhibit and use multiple phenotypes to accomplish different tasks within a colony. Castes are phenotypically and behaviorally discreet individuals that cooperate to perform colony tasks (Miura, 2004). Hemimetabolous social insects, such as the termites, utilize castes to meet various different needs within the colony. Most termite colonies are made up of three distinct castes; workers, soldiers, and reproductives. All termite eggs, except when a rare genetic component might be involved (Hayashi et al., 2007), are totipotent and differentiate based on a number of intrinsic and extrinsic factors.

Caste differentiation can proceed along two routes; the imaginal (winged) or the apterous (wingless). All castes are considered immature in lower termites except soldiers, alates and the three reproductive forms (Lainé and Wright, 2003). The first developmental branch is the point at which larvae differentiate into either workers or nymphs. Nymphs can either 1) regress into worker-like nymph/pseudergates, 2) differentiate into fully winged and eyed adult alates that disperse or 3) differentiate into winged and eyed non-dispersive second form reproductives that serve as supplemental reproductives within the colony. Workers can 1) undergo status quo worker-to-worker molts, 2) differentiate into a presoldier (immediately followed by a molt into a

soldier), 3) or differentiate into an apterous eyeless third-form reproductive (Buchli, 1958; Lainé and Wright, 2003).

The intrinsic and extrinsic factors that impact each of the developmental switches have yet to be fully understood. Essentially, the factors that affect the developmental switches in termites appear to be numerous environmental and social signals. Some of the signals that have been identified are temperature, food quality, juvenile hormone, instar, nestmates (soldiers and reproductives), nutrition, and season (Howard and Haverty, 1981; Waller and LaFage, 1988; Horiuchi et al., 2002; Fei and Henderson, 2002; Liu et al., 2005; Park and Raina, 2004; Mao et al., 2005; Scharf et al., 2007).

Phenotypic divergence from the worker-to-soldier caste has been shown to be regulated by multiple factors. For example, the application of juvenile hormone to workers causes them to molt into presoldiers (Park and Raina, 2004; Scharf et al., 2003). This alteration in the hormone titer presumably controls the developmental switch into a presoldier. Additionally, nestmates have also been shown to have an effect on soldier development. For example, the presence of soldiers inhibits the formation of new soldiers, thus implying that soldier termites produce an inhibitory pheromone that causes an elevated JH response threshold in nestmates (Park and Raina, 2003, 2004, 2005; Mao et al., 2005). This inhibition is presumed to be caused by a primer pheromone from the soldier termite (Lefeuve and Bordereau, 1984; Okot-Kotber et al., 1991; Korb et al., 2003). Alternatively, the putative primer pheromone may modulate hormone production within nestmates. Recently, soldier head extracts (SHE), in combination with juvenile hormone III (JHIII), have been shown to synergistically increase the number of presoldiers, compared to JHIII alone (Chapter 2). Interestingly, the SHE alone did not cause additional presoldiers to form (Chapter 2). SHE could contain components that act as a primer pheromone

that regulates caste differentiation. Primer pheromones are chemical messengers that are passed among individuals and trigger physiological responses in recipients (Wilson and Bossert, 1963). Two major components of SHE were identified to be γ-cadinene and its aldehyde version (γ-cadinenal). Thus, while the SHE blend is active, whether it is being actively released or absorbed has yet to be determined. Also the active component/s of the SHE blend has not been identified.

Functional genomics is described as the use of a holistic or global view of the entire or a large part of the system to elucidate gene function (Tittiger, 2004). One approach to determine the functions of semiochemicals (i.e., pheromones and hormones) and their production is to use functional genomics to identify and characterize the genes responsible for their action (Tittiger, 2004). Transcript levels generally correlate with the physical demand for the product they produce and the changes in transcript abundance can indicate which genes are most important in relation to a stimulus (Tittiger, 2004). By simultaneously observing changes in multiple transcript levels, we hope to better understand how genes work together and the networks they form. Such an approach has been used to help elucidate the chemical ecology of the bark beetle (*Ips pini*) (Seybold and Tittiger, 2003) and the honeybee (*Apis mellifera*) (Grozinger et al., 2003; Alaux et al. 2009). In bark beetles, research has shown that JHIII regulates pheromone production in male beetles through interactions in the midgut (Keeling et al., 2006). JHIII stimulates the *HMG-CoA* reductase gene, which plays a role in the mevalonate pathway (Tillman et al., 2004). Analysis by quantitative real-time PCR (qRT-PCR) of multiple genes in the mevalonate pathway indicated that feeding stimulated the pathway in male bark beetles and partially in females (Keeling et al., 2004). Through the use of functional genomics, pheromone biosynthesis along with multiple physiological processes in the bark beetle are now better understood. The use of functional genomics in the study of termites can help us better understand

potential primer pheromone function as well as the effects of internal and external factors on caste differentiation. Several functional genomics experiments have been completed for termites.

Miura et al. (1999) used differential-display and found one gene, *SOL1*, was expressed specifically in mature *H. sjostedti* soldiers. Hojo et al. (2005) identified a soldier –specific protein (Ntsp1) in the frontal gland of a nasute termite. The protein has homology with known insect secretory carrier proteins of the takeout-homologous gene family, which they suggested could be a carrier of JH.

Wu-Scharf et al. (2003) completed a study in *R. flavipes* identifying expressed sequence tags (ESTs), or partial cDNA sequences, to identify 88 high quality ESTs. Next, Scharf et al. (2003) used cDNA macroarrays to compare gene expression between polyphenic castes. This was the first experiment that provided a summary of caste-associated genes in termites. They found cellulase genes expressed in only workers and nymphs, genes relating to transcription regulation and signal transduction in soldiers, genes associated with musculature and cytoskeletal architecture in soldiers, genes encoding vitellogenin in presoldiers, and several unidentified genes present in some castes but not others. Scharf et al. (2005a) used the same approach to identify 34 nymph-biased genes. These genes had associations with vitellogenesis, nutrient storage, juvenile hormone sequestration, ribosomal translational and filtering mechanisms, fatty acid biosynthesis, apoptosis inhibition, and both endogenous and symbiont cellulases. Scharf et al. (2005b) used model bioassays to identify specific genes and hemolymph proteins that change expression during the worker-to-presoldier transition. Specifically, four hemolymph protein coding genes, two hexamerins and two vitellogenins, were differentially expressed between control and JH treatments (Scharf et al., 2005a).

Koshikawa et al. (2005) using fluorescent differential display, identified 12 upregulated genes expressed in developing *H. sjostedti* soldier mandibles. These genes included cuticle proteins, nucleic acid binding proteins, ribosomal proteins, and actin-binding proteins, which Koshikawa et al. inferred to be involved in caste-specific morphogenesis.

Zhou et al. (2006a,b) determined the change in expression of 17 genes in response to separation from the colony, the addition of JHIII, and to hexamerin RNAi silencing. Results indicated that multiple genes change expression during different treatments. This led the authors to hypothesize that hexamerins play an important role in regulating JH efficacy. Also, Zhou et al. (2006b) identified a number of new termite cytochrome P450 genes and showed that their expression patterns varied in response to JHIII and colony release treatments.

Termite caste polyphenism is regulated by a multitude of semiochemical and socio-environmental conditions. Given that most termites within a colony are highly genetically similar, caste differentiation is thought to be controlled by differential gene expression. Therefore, my hypothesis is that different semiochemical and socio-environmental conditions will be associated with the differential expression of key target genes. The two main objectives of this study were to 1) concurrently investigate the organismal and molecular impacts of semiochemical and socio-environmental conditions on totipotent termite workers, and 2) to test the impacts of the two major SHE components γ-cadinene and γ-cadinenal. The treatments tested in the first objective include: juvenile hormone III (JHIII), juvenile hormone III+ soldier head extract (JH+SHE), soldier head extract (SHE), and live soldiers (LS). In the second objective I tested JH, JH+SHE, JH+ γ-cadinene, and JH+ γ-cadinenal. The specific goals for the first objective were to 1) determine the impacts of JHIII, JH+SHE, SHE and live soldier conditions on caste differentiation and the expression of 49 candidate genes, and 2) identify genes that are

significantly differentially expressed among treatments. The specific goals in the second objective were to identify the impact of the major SHE components on caste differentiation and gene expression, in order to identify the active component of the potential primer pheromone blend. The findings below reveal the impact that multiple semiochemical and socio-environmental conditions have on phenotypic plasticity and gene expression of termite workers. I also identified the phenotypic effects of γ-cadinene and cadinene aldehyde on caste differentiation, and identified γ-cadinene as a significant active component and γ-cadinenal as inhibitory.

Experimental Procedures

Termites

R. flavipes colonies were collected from different locations near Gainesville, Florida USA. Termites were held in the laboratory for at least two months before use in bioassays. Colonies were maintained in darkness within sealed plastic boxes, at 22 ^{o}C. Experiments were replicated across three termite colonies. All colonies contained male and female neotenic reproductives. Termites were considered true workers if they did not possess any sign of wing buds or distended abdomens. Termites were identified as *R. flavipes* by a combination of soldier morphology (Nutting, 1990), and *16S* mitochondrial-ribosomal RNA gene sequencing (Szalanski et al., 2003). The partial mitochondrial *16S* sequences of the four test colonies were deposited, respectively, in Genbank under accession numbers: FJ265704 (colony "GB1"), FJ627943 (colony "K2"), FJ265705 (colony "A8") and GQ403073 (colony K5). Using the *16S* mitochondrial sequences, colony 1 was 99% identical to mitochondrial haplotypes F22 and F1 (EU259755, EU259734), colony 2 was 98% identical to haplotype F20 (EU259753), colony 3 was 96% identical to haplotypes F34, 28, and 21 (EU259767, EU259761, EU259754) and colony 4 was 98% identical to haplotype F20 (EU259753).

Bioassays

Bioassays were conducted at 27 ^{o}C as described previously (Scharf et al., 2003b; Chapter 2). Paired paper towel sandwiches were treated with acetone (controls), JHIII, or SHE treatments delivered in acetone. JHIII (75% purity; Sigma; St. Louis, MO) was provided at a rate of 112.5 µg per dish in a volume of 200 µl acetone in the first objective and 56 µg per dish in the second objective. These two JHIII rates were chosen based on maximal efficacy with minimal mortality observed in previous concentration range studies (Scharf et al., 2003b). An adjustment from 112.5 µg to 56 µg was based on a number of personal observations and a change in JHIII quality from the manufacturer (Sigma). After solvent evaporation, paper towel sandwiches were placed in 5 cm plastic Petri dishes and moistened with 150 µl of reverse osmosis water. Fifteen worker termites were placed in each assay dish. Live solider treatments consisted of holding two live soldiers with 15 workers from the same colony. Every five days, termites were counted, presoldier formation was noted, and water was added if needed. Each treatment was monitored for 25 days.

Solider Head Extracts

Soldier head extract was collected as described in Chapter 2. In brief, soldier head extract (SHE) was prepared by collecting soldiers from lab colonies, removing their heads, and homogenizing the heads (80-150) in acetone, using a Tenbroeck glass homogenizer. To remove particulate matter, the homogenate was fractionated by passing it through a glass Pasteur pipette filled with approximately 250 mg of silica gel (60-200 mesh) on top of a glass wool plug. The SHE was eluted with 10 column volumes of acetone. The fractionated SHE was then brought to 50 ml with acetone in a volumetric flask. SHE components used in the second objective were separated by preparative gas-chromatography as described below, and similar to Chapter 2.

Gas Chromatography (GC) and Mass Spectrometry (MS)

Soldier heads from two different colonies (colonies 3 and 4) were extracted as described above (acetone), in a volume of 50 ml and evaporated under N_2 to 400 µl. Samples were first analyzed by GC / MS (electron ionization, 70eV) to confirm the presence of the previously published predominant terpenoids, namely γ-cadinene and γ-cadinenal (Nelson et al., 2001), and then subsequently quantified using a 6890 gas chromatograph (Agilent; Santa Clara, CA) coupled to a flame ionization detector as described in full by Schmelz et al. (2001).

To quantify semiochemical levels found in individual soldier heads, five individual soldier heads were extracted in a similar manner as above. Individual extracts were in a final volume of 400 µl; an internal standard of 400 ng of nonyl acetate was added to each sample. Samples were then separated by GC. Peaks were analyzed and quantified by comparing to the nonyl acetate standard.

The two main peaks of the SHE were separated using preparative GC. Initial sample preparation of soldier head solvent extracts utilized vapor phase extraction at 80 ºC on polymeric adsorbent traps, followed by dichloromethane elution to remove less volatile contaminants (Schmelz et al., 2004). Micropreparative gas chromatography (GC) was accomplished using an Agilent (Santa Clara, CA) 6890 gas chromatograph (He carrier gas; 5.7 ml min-1; cool on-column injector set to track oven) with an DB-1 column (30 m long, 530 µm i.d., 0.50 µm film thickness) with the temperature programmed from 35 °C (2 min hold) at 10 °C min-1 to 260 °C (hold for 5.5 min). Recovery of separated GC fractions followed from Heath and Dueben (1998) with slight modification. Specifically, a glass press-fit splitter was used at the end of the DB-1 column, coupling a 0.5 m (150 µm i.d. fused silica) capillary to the flame ionization detector (FID) and a second 0.5 m (350 µm i.d. fused silica) capillary directed to the heated transfer line

and chilled glass capillary for sample collection. Under these conditions, the two predominant soldier head sesquiterpenes eluted at 16.1 and 18.9 min.

Phenotypic and Gene Expression Bioassays

For the first objective a total of five different treatments were tested including acetone controls (300 µl), JHIII (200 µl acetone containing 112.5 µg JHIII), JHIII+SHE (112.5 µg JHIII in acetone + 1.5 soldier head equivalents in acetone), SHE (1.5 head equivalents extracted in acetone), and live soldiers (two per assay replicate). Each treatment was replicated five times for colony 1 and six times for both colonies 2 and 3.

During the SHE bioassays additional replicates were included for gene expression studies. Three biological replicates were used for the colony 1 and four biological replicates were used for the colonies 2 and 3 per treatment. Samples of 15 termites were collected for destructive sampling at days 0, 1, 5, and 10. Collected samples were immediately frozen at -80 ^{o}C.

For the second objective procedures were similar as above except the treatments used were acetone controls, JHIII (56 µg), JHIII+SHE (56 µg JHIII in acetone +1.5 soldier head equivalents in acetone), JH+CAD (JHIII 56 µg + 1.136 µg of γ-cadinene), and JH+ALD (JHIII 56 µg +5.081 µg of γ-cadinenal). The quantity of γ-cadinene and γ-cadinenal were determined by relative quantification of approximately 1.5 soldier heads described in Chapter 2. Each treatment was replicated four times for colonies 3 and 4.

Additional replicates were included for gene expression studies. Four biological replicates were used for each colony per treatment. Samples of 15 termites were collected for destructive sampling at days 0, 1, 5, and 10. Collected samples were immediately frozen at -80 ^{o}C.

RNA Isolation and cDNA Synthesis

Total RNA was isolated from frozen samples using the SV total RNA Isolation System (Promega; Madison, WI) according to the manufacturer's protocol. Whole body RNA extracts

were isolated from the 15 worker termites included in each bioassay dish. The amount of RNA was quantified by spectrophotometry and equal amounts of RNA were used in cDNA synthesis reactions. First strand cDNA was synthesized using the iScript cDNA synthesis Kit (Bio-Rad; Hercules, CA) according to the manufacturer's protocol.

Gene Expression

Quantitative real-time PCR (qRT-PCR) was performed using an iCycler iQ real-time PCR detection system (Bio-Rad) with SYBR-green product tagging from cDNA (similar to Scharf et al., 2003a; Zhou et al., 2006). Gene specific primers are listed in Table 3-1. During the first objective forty nine genes were chosen based on their homology to developmental genes identified in other organisms (Scharf et al., 2003, 2005; Zhou et al., 2006, 2007), homology to JH biosynthetic and/or metabolism genes, and developmental genes from recent *R. flavipes* sequencing projects (Tartar et al., 2009).

In the first objective there were eleven total biological replicates tested for qRT-PCR (three from colony-1, and four from colonies 2 and 3). Average Ct values of three technical replicates were used for the analysis of each biological replicate. Ct values were collected at the end of each qRT-PCR run.

For the second objective only genes identified to be significantly differentially expressed between the JH and JH+SHE treatments were tested. In the second objective there were a total of eight biological replicates tested for qRT-PCR (four form colony 3 and four from colony 4). Average Ct values of three technical replicates were used for the analysis of each biological replicate. Ct values were collected at the end of each qRT-PCR run.

Reference Gene Selection

To select appropriate reference genes all of the Ct values across all colonies, treatments, biological reps, and technical reps for each gene were analyzed to identify genes with the least

amount of variation in expression. Three genes with the lowest standard deviation were chosen for use as reference genes in the first objective (Table 3-2) (analysis similar to Zhou et al., 2006a, 2006b). For the second objective only one reference gene was used (*Stero-1*). Stero-1 was was selected based on results from the first objective that showed this gene had low variation between the treatments tested in this experiment thus, indicating that *Stero-1* was a suitable control gene.

Data and Statistical Analyses

Relative expression of target genes was calculated by comparing the average of the three technical replications first normalized to the reference genes and then normalized to the control treatment using the $2^{-\Delta Ct\Delta Ct}$ method (Livak and Schmittgen, 2001). Normalized expression values ($2^{-\Delta Ct\Delta Ct}$) from all colony replicates were analyzed using the microarray visualization software ArrayStar (DNASTAR, Inc, Madison, Wisconsin, USA). To identify potential gene networks, genes with significant differential expression were clustered hierarchically using euclidean distance metrics and centroid linkage for each day (1, 5 and 10) using ArrayStar.

To determine significantly differentially expressed genes, CT expression values for target genes were normalized to the CT values from the reference genes (ΔCT). A two-way ANOVA, with adjusted least squares (LS) means and false discovery rate (FDR) correction was used to separate significant genes using JMP statistical software (SAS Institute, Cary, NC, USA) (**Object Table-3-3**). Tukey's HSD tests were used for separating means by treatment for each gene. An FDR correction was not used for ANOVA's associated with Objective #2 (Table 3-4) because of the lower number of ANOVAs that were performed.

Object 3-1. Table 3-3(.pdf 297 kb)

Results

Phenotypic Responses of Objective #1

Results from the phenotypic bioassays were similar to previously described work showing that the combination of JHIII + SHE significantly increases presoldier development when compared to JHIII alone (Chapter 2). As in previous work, no presoldiers formed in the acetone treated control, SHE, or live soldier treatments (Figure. 3-1). A two-way ANOVA and adjusted LS means were used for analysis (whole model $F=24.092$, $df=14$, $P<0.0001$; treatment $F= 54.32$, $df=4$, $P<0.0001$; colony $F=24.140$, $df=2$, $P<0.0001$; treatment*colony $F=11.513$, $df=8$, $P<0.0001$). Variation was observed between the different colonies tested, with Colony 1 showing the greatest responsiveness to JHIII (40%) and JHIII+SHE (80%). But, as seen in previous research, the overall trend was the same in that JHIII+SHE increased presoldier development compared to JHIII alone (Chapter 2).

Reference Gene Selection

To accurately determine relative gene expression in totipotent workers, we chose three reference genes that had stable expression across all treatments and colonies (*Stero-1*, *LIM*, and *Mev-1*). These reference genes were selected by comparing the standard deviation of the raw Ct values for all 49 genes across treatments (Table 3-2). This determination is important because it allows normalization of target genes (n=46) to reference genes (n=3) that have stable expression across all treatments and colonies (similar to Zhou et al., 2006a,b).

Gene Expression Results of Objective #1

Changes in the expression patterns of multiple genes across several days in response to JH, JH+SHE, SHE and live soldier treatments were determined via qRT-PCR. Two-way ANOVAs with adjusted LS means and FDR correction on normalized CT (ΔCT) values were used to identify significantly differentially expressed genes across treatments (**Object Table-3-3**, Table

3-5, 3-6, 3-7). For a large proportion of the genes tested colony effect was significant. These results are to be expected because there was also a significant colony effect in the phenotypic bioassay and the colonies are different mitochondrial haplotypes. Colony effect was compensated for by using adjusted LS means.

To easily visualize gene expression responses, genes showing significant expression changes across treatments were organized into heat maps separated by Day (Figure. 3-2a,b,c). Genes with similar expression profiles were clustered together. By clustering genes we were able to identify groups of genes that 1) respond similarly and 2) putatively belong to the same gene networks.

Day 1

As shown in the Day 1 heat map (Figure 3-2a), 17 out of the 46 genes tested showed differences in their expression in response to the different semiochemical and socio-environmental conditions tested (Table 3-5). Day 1 receives focus here because we presume Day 1 responsive genes to be important immediate-early responders. Three main clusters of genes were identified, with subgroupings of genes in some clusters. Genes in group IIB were significantly affected by SHE and live solder treatments, with IIB2ii genes *Carbx-1*, *Myosin*, *Bactin*, *Btube*, *R-Pro*, *ATPase*, and *HMG* all being down-regulated with live soldiers, while group IIB1, *NADH* and *nanos*, were up-regulated in live soldier treatments. Group IIB2i genes *Hex-2* and *18s* were down-regulated in SHE treatments. The P450 protein coding genes in group IIA, *CYP15F1*, *CYP4C48*, *CYP6.G*, and *CYP4C47* were down regulated with JHIII and JHIII+SHE treatments, while group I genes, *CYP4C46* and *CYP4U3*, were up-regulated with JHIII. These Day 1 results reveal a number of early responder genes from totipotent workers that are both up and down-regulated in response to the different treatments. Perhaps most

importantly, a number of P450 genes that may play roles in semiochemical or hormone processing were differentially expressed among treatments at this early time point.

Day 5

Five days into assays, 23 genes showed significant differential expression among the five treatments (Figure 3-2b, Table 3-6). A larger number of genes showed significant variation in expression at this point compared with Days 1 and 10, with the majority of the genes showing a down-regulation in response to most treatments. Genes in group IIB2iib3, *CYP4C44v1*, *broad*, and *APO* had a slight expression increase with JH, while being down-regulated with SHE and live soldier treatments. Group IIB2iib2 genes, *CoxIII*, *HSP*, and *Shp* displayed an up-regulation with live soldier treatment. Genes *SH3*, *NADH* and *CYP15F1*, in group IIB2iib1, were down-regulated with JH+SHE and SHE treatments. Group IIB2iia genes, *Famet-2*, *Carbx-1*, *CYP4U3*, *Carbx-2*, and *To-F* were all down-regulated with live soldier treatments. *Bic* and *nanos*, in group IIB2i, were down-regulated with JHIII, JHIII+SHE and SHE. Genes that clustered into group IIB1, *Hex-2*, *Hex-1*, and *CYP4C46* were up-regulated with JHIII and JH+SHE treatments. Finally two hemolymph protein coding genes, *Vit-1* (IIA) and *Vit-2* (I) were up-regulated with JHIII and JH+SHE and down-regulated with SHE and live soldier treatments. Five days into assays is in the middle of the worker-to-solder differentiation process (Scharf et al., 2005a). Therefore, genes identified at this time point could be playing intermediate roles in the caste differentiation cascade. The hemolymph protein coding genes *Vit-1*, *Vit-2*, *Hex-1* and *Hex-2*, have been linked to caste differentiation in past research in termites and honey bees (Scharf et al., 2007, Zhou et al., 2006a,b; Zhou et al., 2007, Page et al., 2006; Amdam et al., 2003; Bloch et al., 2002, Nelson et al., 2007; Antonio et al., 2008, Denison and Raymond-Delpech, 2008). Thus, their differential expression during the worker-to-presoldier differentiation process was expected, and serves as a positive control that validates this approach.

Day 10

On the last day investigated (day 10), nineteen genes showed significant variation across treatments (Figure 3-2c, Table 3-7). The group II genes *Epox-1* and *Vit-2* were up-regulated with JHIII and JH+SHE treatments. Genes in group IB3iib *CYP15F1*, *Shp*, and *Tro-1* were down-regulated with JH+SHE treatment, while *Hex-1* and *To-F* (IB3iia) were down regulated with JHIII and JH+SHE treatments. The putative ribosomal RNA coding *18s* gene was down-regulated with live soldier treatment (IB3i). Group IB2ii genes *CYP4U3*, *28s*, and *CYP4C46* were up-regulated with JHIII but down-regulated with JH+SHE treatment, while genes in group IB2i, *Lprs*, *Famet-1*, and *NADH* were down-regulated with JHIII. Genes that clustered in group IB1, *Myosin*, *APO*, and *broad* were up-regulated with JH+SHE treatment. Finally group IA genes, *Carbx-1* and *SH3*, were down-regulated with JH+SHE treatment. These results from Day 10 revealed a number of late responding genes that are both up and down-regulated in response to the different treatments. Thus, these late responding genes likely are part of multiple pathways that are involved in the later stages of the worker-to-presoldier differentiation process.

Also, across all three days tested four genes, *CYP15F1*, *CYP4C46*, *CYP4U3*, and *NADH* were found to have significant differential expression on all treatment days. This suggests that these four genes could be of broad general importance in worker-to-soldier caste differentiation or caste regulation / homeostasis.

GC-MS Analysis and GC Separation of Soldier Head Chemicals

GC-FID analyses of SHE produced similar results to those obtained in Chapter 2, that identified the two major peaks as γ-cadinene (CAD) and γ-cadinenal (ALD). The approximate amount of γ-cadinene and γ-cadinenal was estimated to be 0.75 and 3.38 ug per soldier. Amounts are less than reported in Chapter 2, (1.44 ± 0.29 μg γ-cadinene and 9.42 ± 1.75 μg γ-cadinenal)

but is probably due to seasonal or colony variation. The SHE blend was then separated using a micropreparative GC to be used in phenotypic and expression studies for Objective #2.

Phenotypic Results from Objective #2

The two major components γ-cadinene (CAD) and JH+ γ-cadinenal (ALD) were assayed for their impacts on worker caste differentiation and gene expression. The combination of JH+SHE and JH+CAD treatments caused the greatest number of PS to form compared with JH+ALD and control treatments (Figure 3-3c) (whole model $F=8.5678$, $df=9$, $P<0.001$; treatment $F=6.3768$, $df=4$, $P=0.0008$; colony $F=35.167$, $df=1$, $P<0.0001$; treatment*colony $F=4.1089$, $df=4$, $p=0.009$). Because the JH+CAD and the JH+SHE each had a similar impact on worker-to-soldier differentiation, this suggests that γ-cadinene is the active component of the SHE blend. Also, JH+ALD had less of an impact, although not significant, compared to JH alone and JH+SHE, suggesting that γ-cadinenal is not the active component, and perhaps could be inhibitory (Figure 3-3c).

Gene Expression Results from Objective #2

For Objective#2 we only tested genes that were found to be significantly differentially expressed between the JH and JH+SHE treatments from Objective #1. This was done to narrow our focus and thus, potentially identify the genes that had a similar synergistic effect between JH and SHE as seen in the phenotypic bioassays (Chapter 2). This resulted in testing only one gene on Day 1, two on Day 5, and eleven genes on Day 10. Throughout all three days tested (1,5, and 10) only four genes were significantly differentially expressed between treatments; *Btube* at Day 1 and *CYP15F1*, *NADH*, and *Myosin* at Day 10 (Figure 3-4). Two genes *Btube* and *Myosin* had similar expression patterns between JH+SHE and JH+CAD, further supporting the idea that CAD is the active component in the SHE blend.

Discussion

Phenotypic plasticity is an important evolutionary adaptation that allows organisms to rapidly respond to changing environmental conditions. Social organisms, specifically hemimetabolous termites, utilize phenotypic plasticity to develop into different castes. Because all termite colony members share essentially the same genetic background, they rely on differential gene expression to differentiate among the castes (Miura, 2004). The functionality of the colony is dependent upon the cooperative effort of all castes. The development of a termite along a continuous caste pathway is regulated by a number of different internal and external factors (Scharf et al., 2007).

This study identifies clear phenotypic effects of SHE and its main components' and describes patterns of gene expression correlated with these phenotypic effects. Changes in expression were detected in several genes having homology to other well-characterized developmental and metabolic genes when termites were subjected to different treatments. Several genes and apparent gene networks important in caste differentiation and social interactions were also identified.

Our bioassay system induces changes in phenotype, gene and protein expression, and has been used repeatedly to monitor and elucidate mechanisms of caste differentiation, specifically the worker-to-soldier transition (Scharf et al., 2003, 2005, 2007; Zhou at al., 2006a, 2007; Chapter 2). Here, we investigated the effects of specific semiochemical and socio-environmental conditions (JHIII, JHIII+SHE, SHE, live soldiers, γ-cadinene, and γ-cadinenal) on soldier caste differentiation and gene expression of termite workers. Although there are certainly many other semiochemical and socio-environmental conditions that could play a role in worker-to-soldier differentiation, we focused on the components listed above.

Phenotypic assay results were similar to past findings in that JHIII induced presoldier formation, JHIII+SHE synergistically increased presoldier development, and SHE alone had no effect on PS development (Chapter 2). The addition of live soldiers to the bioassay did not increase soldier formation. However, this experimental design did not allow for determination of any inhibitory soldier effects. This suggests that SHE, or a component of it (as discussed later), acts with JH as a primer pheromone to help regulate caste differentiation within the termite colony. As with past research, inter-colony variation was observed; a common occurrence in insect sociobiology research (Bourke and Franks, 1995; Hahn, 2006). Colony variation could be a result of multiple factors, such as colony age, caste composition, maternal effects, nutrition, or multiple others.

In this research, we monitored phenotypic effects in concert with the expression patterns of multiple genes. This was accomplished with destructive sampling of some assay replicates for RNA isolation, while allowing other to proceed without disturbance. Because caste differentiation is mostly a result of differential gene expression, we hypothesized that genes responsible for caste differentiation should be differentially expressed. The typical worker-to-presoldier differentiation process takes approximately 15 days. To capture potential expression changes up to ecdysis, gene expression levels were monitored at 1, 5, and 10 days post treatment, which are considered early, middle and late time points, respectively. A total of forty-nine genes were investigated across three replicate colonies. Statistically significant genes that passed the FDR cutoff were clustered together based on expression pattern (Figure 3-2a,b,c). As discussed below three main groups of genes were identified as having potential roles in caste differentiation: 1) chemical production / degradation, 2) hemolymph protein coding, and 3) developmental.

Chemical Production / Degradation Genes

Chemical producing / degrading proteins are responsible for the production and / or degradation of many chemicals within an organism. Cytochrome P450s, JH production / degradation, and mevalonic pathway protein coding genes are included in this group. Many of the genes tested in this group were differentially expressed when treated with different semiochemical and socio-environmental treatments, suggesting the production and degradation of numerous chemicals are important in the caste differentiation process.

Cytochrome P450s are known for their role in the oxidation of endogenous and xenobiotic substrates including hormones, pheromones, insecticides, and secondary plant compounds (Andersen et al., 1997; Feyereisen, 2005). Specifically, P450s have been shown to play a role in the biosynthesis and metabolism of morphogenic hormones (JHIII, ecdysone) and terpenoids (Andersen et al., 1997). On Day 1, two groups of P450s were identified. The first group of P450s (IIA), *CYP15F1*, *CYP4C48*, *CYP6.G* and *CYP4C47* were down-regulated with JHIII and JHIII+SHE treatments, while group I, *CYP4C46* and *CYP 4U3* were up-regulated with JHIII and JHIII+SHE treatments. This opposite expression profile of the different P450s suggests they have different functions, likely acting on multiple substrates. However, it is still unknown whether their role is to degrade or produce specific chemicals, or what substrates are utilized or produced.

Past research has identified P450s that play significant roles in JH biosynthesis and degradation. In the cockroach, *Diploptera punctata*, *CYP15a1* directly epoxidizes methyl farnesoate to form JHIII (Helvig et al., 2004). In the present study, those P450s that were down-regulated with JH treatment (*CYP15F1*, *CYP4C48*, *CYP6.G* and *CYP4C47*) could have a similar function. Insect P450s have also been shown to play a role in the degradation of JHIII, as is the case with *CYP4C7*, which converts JHIII to 12-trans-hydroxy JHIII in *Diploptera punctata*

(Sutherland et al., 1998, 2000). The group of P450s (I) (*CYP4C46* and *CYP4U3*) that were up-regulated in the present study could be playing this role and / or the group of genes that were down-regulated could be inactivated, potentially blocking the worker-to-soldier transition.

A number of the P450s tested were differentially expressed at Days 5 and 10. Cytochrome P450s *CYP15F1*, *CYP4U3*, *CYP6.G* and *CYP4C44v1* were down-regulated with live soldier treatments on Day 5. This observation is opposite to those made at Day 1 where most P450s were up-regulated by JHIII treatment. Perhaps this indicates that P450s act on multiple substrates, or treatments at Day 5 have downstream effects relative to those at Day 1. Finally, on Day 10, *CYP15F1* was down-regulated with JH+SHE, and *CYP4U3* and *CYP4C46* were up-regulated with JH, similar to Day 1. Functional characterization studies must be undertaken to determine the exact role of each P450.

Juvenile hormone metabolism is also potentially mediated by JH esterases and epoxide hydrolases (Roe et al., 1996). Three genes having homology to JH esterases and epoxide hydrolases displayed significant expression differences between treatments. First, a potential JH esterase, *Carbx-1*, was down-regulated with live soldier treatments at Days 1 and 5, then down-regulated at Day 10 with JHIII and JH+SHE treatments. Although the carboxylesterase-1 of *R. flavipes* (Accession No. GQ180944) shares homology with the JH-esterase of the wood-feeding beetle *Psacothea hilaris* (BAE94685) (Munyiri and Ishikawa, 2007), it is shortened and missing the JH-esterase motif identified by Mackert et al. (2008). Because it lacks this motif, it may be degrading one of many lignin carboxylesters found in the termite diet (Geib et al., 2008; Wheeler et al., 2009) instead of metabolizing JH. *Carbx-2* (Accession No. GQ180944), which has homology to the JH esterase of the sawfly *Athalic rosae* (BAD91555), was down-regulated with live soldiers and SHE treatments at Day 5 only. Similarly, *Carbx-2* is truncated and apparently

missing the JH-esterase motif, suggesting it could also be degrading lignin and/or hemicellulose carboxyl esters (Wheeler et al., 2009).

The next group of candidate JH metabolism enzymes is the epoxide hydrolases, which break down JH by hydrolyzing the JH epoxide. *R. flavipes* epoxide hydrolase has sequence homology to that of *Aedes aegypti* (XP_001651935, evalue: 7e-55). *Epox-1* was significantly up-regulated at Day 10 with JH and JH+SHE treatments. If *Epox-1* is acting as a JH epoxide hydrolase, then the termite hydrolase could be up-regulated degrade or inactivate any exogenous of endogenous remaining JH prior to apolysis or ecdysis.

The production of JH and other sesquiterpenes is important to termite colony success, not only for development and caste differentiation, but also for production of defensive chemicals and pheromones possessing a sesquiterpene backbone (Seybold and Tittiger, 2003, Belles et al., 2005). Research has shown that up- and down-regulation of genes in the mevalonic pathway leads to the production of JH and pheromones (Tillman et al., 2004, Keeling et al., 2004). In the present study, five mevalonic pathway genes were investigated *Famet-1*, *Famet-2*, *Famet-3*, *Mev-1*, and *HMG*. Two genes homologous to farnesoic acid methyl transferase (*Famet-1*, *Famet-2*) were differentially expressed. *Famet-1* was up-regulated at Day 10 with JH and SHE treatments, and *Famet-2* was down-regulated with live soldiers on Day 5. Farnesoic acid methyl transferase methylates farnesoic acid, producing methyl farnesoate in the mevalonate pathway (Belles et al., 2005). RNAi-mediated knockdown of this gene in *Tribolium castaneum* lead to lower JH levels subsequently causing precocious molting (Minakuchi et al., 2008). *R. flavipes Famet-1* shares strongest homology to hymenoptera *Melipona scitellaris FAMet* (AM493719, evalue 2e-41) (Vieira et al., 2008). These results reveal that JH causes increased *Famet-1* expression. If this gene was acting as a farnesoic methyl transferase, increased expression would

raise JH titers causing a worker-to-soldier molt. These results also reveal that the presence of live soldiers down-regulated the *Famet-2* gene in workers. If the *Famet-2* gene is acting as a farnesoic acid methyl transferase, its down-regulation would lead to lower JH production and result in decreased worker-to-soldier molts. Past research and research performed in Appendix D show that live soldier inhibit the formation of additional soldiers (Park and Raina, 2004; Mao et al., 2005).

Another potential JH production gene, *HMG* of *R. flavipes* has homology to the HMG-CoA-reductase gene found in the German cockroach *Blattella germanic*a (p54960, evalue: 0.0), and the bark beetle *Ips pini*, (AAL09351, evalue: 9e-165) (Keeling et al., 2006) and was up-regulated at Day 1 with JHIII and down-regulated live soldier treatments, respectively. HMG-CoA-reductase is part of the bark beetle mevalonate pathway responsible for the production of JH and other sesquiterpenoids (Seybold and Tittiger, 2003; Belles et al., 2005). Overall, these results suggest that JHIII causes up-regulation of the mevalonate pathway, while live soldiers are suppressive. An up-regulation of the mevalonate pathway would likely result in increased downstream products, such as JH. An increase in JH, as shown here and in past research, would increase internal hormonal levels resulting in a worker-to-soldier molt.

Hemolymph Protein Coding Genes

Four hemolymph protein coding genes, *Hex-1*, *Hex-2*, *Vit-1*, and *Vit-2*, were found to be significantly differentially expressed throughout my experiments. These four genes are important in caste differentiation and regulation for a number of social insects. Much research has shown that these genes play major roles in insect sociobiology; therefore, it was not surprising they were again identified in the present study. The termite hexamerin genes have been shown to act as a socio-regulatory mechanism that affects the activity of JH, possibly limiting its availability (Zhou et al., 2006a). RNAi silencing of two hexamerins resulted in an increase in presoldier

development, suggesting hexamerins sequester JH, thereby preventing differentiation by worker termites to presoldiers and soldiers (Zhou et al., 2006a,b; Zhou et al., 2007). The extrinsic and intrinsic factors of temperature and nutrition have also been shown to affect hexamerin proteins and modulate caste differentiation (Scharf et al., 2007). Similar to past research, we observed increased *Hex-2* transcripts in JHIII-treated termites, supporting the notion that *Hex-2* may bind excess JH, thus preventing worker-to-soldier molts (Zhou et al., 2006b). Also, there was a slight increase in the *Hex-1* transcript in the JHIII+SHE treatments, similar to the increased presoldier differentiation results in the JHIII+SHE treatment when compared to JHIII alone. This indicates that *Hex-1* was more responsive to the JHIII+SHE signal than to JHIII alone, possibly serving to inhibit the transition from worker-to-soldier.

Vit-1 and *Vit-2*, two other hemolymph protein genes that were up-regulated with JHIII and JHIII+SHE treatments at Day 5, while only *Vit-2* was differentially expressed at Day 10. Throughout the experiment, both *Vit-1* and *Vit-2* genes displayed a high amount of variability among treatments and replicates. One explanation for this observation is the inclusion of both sexes of worker termites in the assay. In most insects, vitellogenin (*Vg*) serves as a female yolk precursor protein in oocyte development. However, *Vg* has also been shown to play a role in social insect caste regulation. For example, *Vg* in honeybee workers, which are female, has been shown to interact with JH. Both higher JH levels and lower *Vg* levels increased the transition from nursing to foraging behavior by worker bees (Page et al., 2006; Amdam et al., 2003; Bloch et al., 2002), while a reduction of JH delayed the onset of foraging (Schulz et al., 2002). Honeybee workers with RNAi-suppressed *Vg* levels performed foraging behaviors earlier than untreated workers (Nelson et al., 2007; Antonio et al., 2008). Nutrition has also been shown to affect *Vg* and JH, regulating transition from nursing to foraging (Schulz et al., 1998; Toth et al.,

2005; Toth and Robinson, 2005). Finally, *Vg* has been shown to affect queen honeybee longevity by interacting with insulin signaling cascades (Corona et al., 2007). Together, these factors suggest that the *Vg* protein in honeybees has been co-opted for an additional role as a regulator of caste polyethism (Denison and Raymond-Delpech, 2008). These results suggest that the same may be true for termite polyphenism. The *Vg* transcripts *Vit-1* and *Vit-2* were up-regulated in termites treated with JHIII and JHIII+SHE, and down-regulated with SHE and live soldier treatments. The addition of JHIII causes workers to develop into soldiers, molting from an immature worker to an adult soldier form. Potentially, *Vg* acts as a signal to modulate behavior and physiological caste differentiation.

Developmental Genes

The dramatic morphological change that occurs as worker termites become soldiers requires significant rearrangement of the termite body plan (Miura, 2004). The soldier termite's large structural mandibles and their associated muscles represent a large change from the relative small head of a worker termite (Miura and Matsumoto, 2000; Koshikawa et al., 2003; Ishikawa et al., 2008). Thus, it is likely that multiple genes must be needed to reorganize the termite body plan (Koshikawa et al., 2005). Six developmental genes were significantly differentially expressed throughout these experiments. Two main groups of developmental genes were identified, the cytoskeletal / structural and the body-plan genes.

The cytoskeletal gene, *Myosin*, was down-regulated in live soldier treatment at Day 1. Myosin proteins are actin-based motor proteins that convert chemical energy from ATP into mechanical force (Harrington and Rodgers, 1984). The *R. flavipes Myosin* gene has highest homology to a *Drosophila Myosin* gene (AAA28687, evalue: 8e-32). Down-regulation at Day 1 suggests that live soldiers inhibit the formation of new soldiers. On Day 10, expression of the muscle related *Myosin* gene was up-regulated in the JH+SHE treatment. Previously, this

treatment caused the greatest presoldier formation, suggesting that *Myosin* is used in the worker-to-soldier molt.

The cytoskeletal/ structural protein coding gene, *β-tubulin*, β*tube,* was also significantly differentially expressed at Day 1. *R. flavipes β-tubulin* has homology to that of many other insects which are thought to provide cytoskeletal structure (Dettman et al., 2001) and was previously found to have significant increased expression in *R. flavipes* soldiers (Scharf et al., 2003). *β-tubulins* are also hormone-dependent and perhaps play a role in the production of ecdysteroids in *Manduca sexta* (Rybczynski and Gilbert 1995, 1998). *α-* and *β-tubulin* genes were identified in *Bombyx mori* EST libraries linked to imaginal wing disk metamorphosis and 20-hydroxyecdysone (Kawasaki et al., 2003, 2004). The authors suggested that the expression change was due to the large restructuring needed in adult wing formation. *R. flavipes* β*tube* shares homology with *B. mori β-tubulin* (NP_001036964, evalue: 2e-35). The soldier termite has numerous muscles driving its enlarged mandibles in comparison to those of the worker caste; perhaps influence by live soldiers down-regulates these structural genes as a soldier-inhibitory mechanism. Or, *R. flavipes* β*tube* could be participating in ecdysteroid hormone production similar to *M. sexta*, as it shares homology with *M. sexta β-tubulin* (017449, evalue: 4e-36).

Three developmental/ body plan genes were also differentially expressed. First, *broad* (BTB/POZ), homologous to the broad (*br*) transcription factor of the hemimetabolous insect *Oncopeltus fasciatus* (ABA02191, evalue: 2e-33) and holometabolous *T. castaneum* (XP_973299, evalue 2e-43), was up-regulated at Day 5 with JH and JH+SHE treatments and at Day 10 with JH+SHE treatment. Erezylimaz et al. (2006) successfully silenced the *br* gene in *O. fasciatus*, resulting in an additional immature molt. They suggested that *br* is required for morphogenesis, and that its expression is regulated by JH. RNAi silencing of *br* in *T. castaneum*

had similar results (Parthasarathy et al., 2008). If *br* is acting in the same manner in termites, up-regulation of the gene by JH+SHE would enable the worker-to-soldier transition. This mechanism is in agreement with phenotypic bioassay showing a greater number of worker-to-soldier molts in the JH+SHE treatment.

In *Drosophila*, two transcription factors, *bicaudal* and *nanos*, are important in embryonic pattern formation. In *R. flavipes* the *bicaudal* and *nanos* homologs, *Bic* and *nanos*, were up-regulated with live soldier treatments; *nanos* at Day 1 and *bic* and *nanos* at Day 5. *Bicaudal* has been shown to regulate expression of the posterior determinant gene, *nanos* (Bull, 1966; Markesich et al., 2000). The similar expression patterns shown here suggest these genes cooperate and perhaps play similar roles in body plan regulation.

Defining Chemical Ecology Through Gene Expression

Transcriptional profiling clearly can lead to a better understanding of chemical ecology (Tittiger, 2004). By using gene expression patterns to identify active components of a pheromone blend, one can eliminate, or narrow down the amount of bioassays needed to identify an active component. Here, I correlated the response of worker gene expression profiles and worker-to-soldier differentiation of the SHE blend to each of its components (γ-cadinene and γ-cadinenal). Bioassay results indicated that γ-cadinene increased PS formation while γ-cadinenal reduced PS formation, suggesting it might be inhibitory. However, the expression profiles of 14 target genes did not give a clear view to which component was the active one. Only two genes *Btube* at Day 1 and *Myosin* at day 10 shared similar expression patterns when comparing JH+SHE and JH+CAD, potentially suggesting the γ-cadinene is the active component. JH+ALD transcription profiles did not share any significant matches with JH+SHE profiles. Possibilities why there were not as may genes with similarities among the treatments could be because of a small sample size of genes and associated statistical power. A larger sample size or a whole transcriptome

would certainly give a clearer picture. Another possibility could be that the two major components of the SHE blend that we tested may not be the active components, or the phenotypic effects observed may result from a specific ratio of the two components (Roelofs and Jurenka, 1996). Additional research using genome arrays or whole transcriptome comparisons would provide a larger number of genes to sample, and likely clearer results.

Conclusions

The research presented here demonstrates for the first time, the effects semiochemicals, influence of other castes, and internal hormones have on phenotype and gene expression of totipotent *R. flavipes* workers (Figure 3-5). Factor such as JHIII, JH+SHE, and JH+CAD all increased worker-to-presoldier differentiation, while live soldiers and JH-ALD inhibited presoldier formation. SHE by itself did not have any observable phenotypic effects (Figure 3-5a). Results suggest the influence of nestmates, specifically the soldier caste, helps to regulate caste differentiation of other nestmates. Phenotypic results also suggest that γ-cadinene could be the active component of the SHE blend. Semiochemical and socio-environmental factors modulated gene expression patterns for a number of genes. Significantly responsive gene networks identified here include chemical production/ degradation, hemolymph protein coding, and developmental genes (Figure 3-3b). The JHIII and live soldier treatments had a significant influence on a number of genes homologous to those responsible for JH biosynthesis / degradation and development. However, the responsive genes had an opposite reaction to JHIII and live soldier treatments. Past research using a larger group format demonstrated the presence of live soldiers inhibited additional soldier formation (Mao et al., 2005; Appendix D). This suggests that live soldiers act as part of a negative feedback loop, inhibiting new soldier formation by regulating the expression of genes important for caste differentiation (Figure 3-5b) (Henderson, 1998; Mao et al., 2005; Park and Raina, 2005).

Still unknown is the biological role that potential SHE primer pheromones play within the termite colony. Extracts from the soldier head, when combined with JHIII, caused an increase in presoldier development. However, SHE caused differing gene expression patterns when compared to live soldier treatments. This finding reveals that SHE terpenes act differently than live soldiers in the termite colony, thus, these findings suggest two hypotheses: 1) either soldiers are "sponging" chemicals (or some other still unidentified element) from the colony, inhibiting nestmate worker-to-soldier molts (Henderson, 1998), or 2) soldiers are producing SHE terpenes, which are used to trigger new soldier replacements when current soldiers expire (Chapter 2). When the two major components of the SHE blend were tested separately, γ-cadinene produced similar phenotypic results as the SHE blend. γ-cadinenal caused a reduction in the number of PS formed. If the soldier termites can somehow control the ratio between the γ-cadinene and the γ-cadinenal, then they could be able to both promote PS formation and inhibit PS formation depending on the need of the colony. This could explain past research that showed that SHE was inhibitory to PS formation (Lefeuve and Bordereau, 1984; Okot-Kotber et al., 1991; Korb et al., 2003). Further research using RNAi to silence key responsive genes, whole genome expression profiles, and GC quantification of soldier head chemicals in response to proximate and socio-environmental conditions is needed to resolve the roles of soldiers and soldier-derived terpenes in termite caste regulation.

Table 3-1. Gene list and primers

	Gene	Abbreviation	Accession No.	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
1	bicaudal (Rfb-NAC)	bic	AY258589	GAGGCAAGTACGGATTGGAG	CTCTTCATGGTAACAACCAG
2	nanos (RF PDL)	nanos	BQ788190	CCACTGACTAAATGTATGGG	TTCAAGCCTCAACACTCTGT
3	broad (Rf BTB POZ)	broad	AY258590	CTGGACCAGCATCTACATCTTC	GATGGTGTTCTGTCGTGGAG
4	Cop-9 Signalsome Subunit 5	Cop9	DN792518	CTCGATCAGGAGGCACACTC	TTGCTGCCTCAATGTATGCT
5	LIM (legs incomplete & malformed)	LIM	CB518301	GTGCTTCAAGTGTGGCATGT	GTCCATGCTGAGACAACCAG
6	18S rRNA-like (Senescence Protein)	18S	AY572860	TATCGATCCTTTTGGCTTGG	TCGCAATGATAGGAAGAGCC
7	28S RRNA-like (Integral Membr. Prot.) (transporter)	28s	CK906357	GCGAATGATTAGAGGCCTTG	ACAGCGCCAGTTCTGCTTAC
8	SH3 Domain Kinase	SH3	CB518513	CGTGTTGCCAATGAGTTGAG	ACAATCCTATTCGGCCATCC
9	GTPase Activating Protein	Gtpase	BQ788178	TTCCAACAGCACAAGAGCAC	TAACTGGTTGCGACAGGCAC
10	Malonyl Co-A Decarboxylase	MalcoA	AY572861	GCTACCGGCGACTCTTAATC	GAGGACACGCTGATTCCTTC
11	Apoptosis Inhibitor	APO	CK906364	CGTACATGTGTGAGCAGGTG	ATCACCATCAGGTGGCAGAG
12	ATP-ase	ATP	BQ788171	TCAGGAAGTCTTGGATTCGG	TACGAACTCTGGTGCGTCTG
13	Larval Cuticle Protein	LCP	DN792534	CGTCGACACCGACTACGAC	GGTCAGCGGTGTACTCGAC
14	Troponin I (Rf1 form) wup	Tro-1	CB518302	CGACCTAGAATACGAAGTGG	TTCTTCTCCTTGTCCTCCTCC
15	Troponin I (Rf2 form) wap	Tro-2	CB518303	GAAGAGTTGAAGAAGGAGCAGG	TTGTTCACCAGGCTATTCAGG
16	Hexamerin-1	Hex-1	AY572858	GATCCATTCCACAAGCACG	ACATTCTCCACCGTCACTCC
17	Hexamerin-2	Hex-2	AY572859	ACGGAAGACGTTGGACTCAG	GAGGACCTGCTGGATCTTGT
18	Vitellogenin-1	Vit-1	BQ788169	CCTACATGCGTTGTTGATGG	TGACGACTATGCACTCCAGC
19	Vitellogenin-2	Vit-2	CB518311	AGCGGTTATGCACCTCTCTG	ACCTGCAACTGTTGTTGTGC
20	B- Actin	Bactin	DQ206832	AGAGGGAAATCGTGCGTGAC	CAATAGTGATGACCTGGCCGT
21	NADH- dh	NADH	BQ788175	GCTGGGGGTGTTATTCATTCCTA	GGCATACCACAAAGAGCAAAA
22	HS p70	HSP	BQ788164	AGAACCAAGTGGCCATGAAC	CCAATGCTTCATGTCTGC
23	COX III	CoxIII	DQ001073	GATCAACCTTCTTCATAGCC	GCTGCTGCTTCAAATCCAA
24	R-Protein Ribonucleoprotein SMD3	R-Pro	CK906360	CTGTGCTATCGAGTGAAGGC	TTCATATTGTCCTCCGCCTC
25	Intronic Ribosomal intronic protein	Intro	CK906359	ATATTGCGCGCCACCGTAAG	TCTCTGATCTGCGTTGCTTG
26	CYP4U3	Cyp4U3	DQ279461	ACGTCTGGCATTTGTTTCAC	ATCCGTGTAGGTGGCATCA
27	CYP4C43v1	CYP4C43v1	DQ279463	AGGAGAAGGCGTACCAGGAG	TCATTCAAAGTTCTTCCAACACC
28	CYP4C4v1	CYP4C4v1	DQ279465	GAAGTGCCTTGAGAGGGTCA	TGGGAACTGGACAGGGTTC
29	CYP4C45v1	CYP4C45v1	DQ279467	CTTCAAACATCTCCTGGACCA	TCTCCTTAATTACTCGCTCCAAA
30	CYP4C46	CYP4C46	DQ279469	TGAAGTACCTTGAGAGGGTCATT	TTGAATTGGGTCCCGATAG
31	CYP4C47	CYP4C47	DQ279470	GCATTTCATGGGCTATGTACTTG	GAGCTTCCTTGATAACCTGCTC
32	CYP4C48	CYP4C48	DQ279471	TGACCACTCTGTGACCATGAA	TGGGTACTGTCATTATCACTCCA
33	Deviate	To-F	BQ788174	AGGCGAGTTGGTGACCATAG	GTAGAAGGCAGGCCAGTACG
34	Cyp15F1	Cyp15F1	FJ792773 /FL638893, FL636088, FL636262, FL636256, FL640637, FL640773, FL635527	CGGCCTCAACATTCACAGAA	CTTCCCACAACTGCATCCAA
35	B-Tubulin	Btube	CB518304	CAGATCGGTGCTAAGTTCTGG	TATGGCACGCGGTACATATT
36	Myosin	Myosin	CB518305	CAAGGAGTTGAGCTTCCA	TCTTCCAGTTCCTGCTGTGC
37	SHp- PIP kinase	Shp	CK906365	AATTCTGCTGCCTTCTCTGG	ACCTTGCTTGGTCAACCATC
38	Farnesoic methyl transferase 1	Famet-1	FL638251, FL637991	CCACTTCTGCATAACCACAGAG	CAGGGCACATAAGAGGCATT
39	Cyp6.G	Cyp6.G	FL637360	GTCCCAATGTCTCTCGGAATAG	GTCCATTGTCATACCAGCAGAG
40	Carboxyl Esterase-1	Carbx-1	FL636973, FL640151, FL638979	GGCACCGATAAAGGCATAGA	GGTCCGTTCCTGCGTTAATA
41	Mevalonate kinase-1	Mev-1	FL639092	CTGAGGTCACGGTTCCTATACA	TGAACCACTGAATGCTCACC
42	Farnesoic methyl transferase 2	Famet-2	FL639748, FL638947	ACTGCACATTGAGGTTCGTG	GCCACTCTATCAAGAAGCGACT
43	Steroid binding protein 1	Stero-1	FL639110, FL636382, FL635522	TTGGACTGTGGACCTTAAGAGG	CCCTTAGCAACGCAGACAAT
44	Epoxide hydrolase-1	Epox-1	FL640608, FL636393, FL635113	ATACAGACGTTCACAGCGACAG	CACTCCCTCAATTGGCAGAT
45	cJun transcription factor-1	cJun-1	FL638224	GGGTCCATGACATGCTCAATAC	GGGCTGGCAACAAAGTTCTA
46	Carboxyl Esterase-2	Carbx-2	FL638686	GCCAGAATTCAAGCTGCTGT	TGTCCTTGTCTTGCTGTGTCTC
47	Farnesoic methyl transferase-3	Famet-3	FL636743	TGGAGGACACGACAAAGATG	TCACTTGCAGTCTGCCACAT
48	Lipophorin receptor	Lprs	FL635452,FL636727, FL636380, FL637727, FL636288	ACCAGTACCAGCCACAGAGAAT	TACCACTTTGAGCGATGCAC
49	HMG-CoA-Reductase	HMG	FL638074, FL637763, FL638896, FL640394, FL638646	CTCCTGTTGGATGGGTGTCT	GTTCTGAGGTTCCTGCATCC

Table 3-2. Meta analysis of all genes over all treatments and days

	Gene	N	Max [CT]	Min [CT]	Average	SD
1	Stero1*	495	24.18	19.00	20.47	0.70
2	Lim*	494	20.89	15.96	17.55	0.79
3	Broad	495	28.88	21.74	23.65	0.80
4	Mev-1*	495	27.01	20.78	22.75	0.80
5	JHest2	494	29.25	21.10	22.99	0.86
6	Intro	495	25.29	18.71	21.09	0.87
7	Lprs	495	25.99	19.89	22.55	0.91
8	ATP	495	26.41	17.64	19.99	0.92
9	Tro-1	492	22.80	17.86	20.06	0.92
10	Famet-3	495	35.14	20.57	24.41	0.92
11	Btube	495	24.64	16.09	19.23	0.93
12	Myosin	494	21.61	15.18	18.22	0.94
13	28s	495	13.89	8.66	10.76	0.94
14	Tro-2	490	23.46	17.40	19.46	0.96
15	CYP4C43v1	494	28.16	21.90	24.67	0.96
16	GTP	495	29.78	22.14	25.76	0.96
17	Famet2	495	26.74	19.99	21.71	0.97
18	Cop9	495	27.67	20.68	22.68	0.98
19	cJun-1	493	35.82	20.86	23.38	1.00
20	CoxIII	492	19.25	14.14	16.71	1.01
21	R-Pro	494	26.49	20.07	22.88	1.01
22	Cyp15F1	494	26.69	18.76	21.26	1.03
23	HSP	628	26.47	17.81	19.98	1.05
24	Famet-1	495	36.69	18.90	21.12	1.06
25	CYP4C44v1	495	30.79	23.79	26.17	1.09
26	CYP4c45v1	495	32.21	22.21	25.04	1.09
27	TO-F	495	24.51	17.63	20.25	1.12
28	18s	494	15.06	8.71	9.77	1.12
29	APO	495	32.59	22.45	24.97	1.14
30	CYP4C47	494	30.55	22.51	25.03	1.15
31	CYP4C48	495	30.70	21.96	24.30	1.23
32	MalcoA	489	16.08	8.10	11.50	1.23
33	Hex-2	495	20.81	13.69	16.90	1.28
34	HMG	312	23.32	31.66	25.77	1.29
35	Cyp6.1	495	32.58	23.68	25.92	1.32
36	CYP4C46	457	43.60	36.62	40.00	1.37
37	Hex-1	495	26.24	16.13	19.28	1.38
38	Bactin	648	25.75	16.76	20.88	1.42
39	Jhest-1	495	33.40	21.76	24.10	1.59
40	Nanos	494	32.06	22.57	28.56	1.61
41	NADH	626	30.96	22.08	26.07	1.65
42	Shp	494	41.80	30.49	35.57	1.71
43	Cyp4U3	495	32.38	21.62	27.72	1.79
44	Sh3	493	36.73	22.37	26.96	2.59
45	Epox1	494	34.47	17.06	25.41	2.64
46	Bic	495	37.03	19.89	23.65	3.05
47	Vit-1	494	43.10	21.79	30.51	3.36
48	Vit-2	494	43.37	21.42	33.62	3.97
49	LCP	495	37.59	14.08	23.56	4.14

Table 3-4. Objective #2 ANOVA table

Gene	Day					Gene	Day					Gene	Day				
Btube	Day 1	Source	DF	F ratio	p value	28s	Day 5	Source	DF	F ratio	p value	NADH	Day 5	Source	DF	F ratio	p value
		Whole model	9	8.800	<0.0001			Whole model	9	18.370	<0.0001			Whole model	9	20.700	<0.0001
		Colony	1	42.835	<0.0001			Colony	1	155.770	<0.0001			Colony	1	165.900	<0.0001
		Treatment	4	6.570	0.0006			Treatment	4	0.920	0.4625			Treatment	4	2.500	0.0631
		Colony*Treatment	4	2.500	0.0631			Colony*Treatment	4	1.461	0.2386			Colony*Treatment	4	2.590	0.0563
		Error	30					Error	30					Error	30		
		Total	39					Total	39					Total	39		
Gene	Day					Gene	Day					Gene	Day				
Cyp15F1	Day 10	Source	DF	F ratio	p value	Shp	Day 10	Source	DF	F ratio	p value	Tro-1	Day 10	Source	DF	F ratio	p value
		Whole model	9	6.242	<0.0001			Whole model	9	32.494	<0.0001			Whole model	9	10.169	<0.0001
		Colony	1	41.210	<0.0001			Colony	1	272.495	<0.0001			Colony	1	85.311	<0.0001
		Treatment	4	2.837	0.0416			Treatment	4	2.162	0.0975			Treatment	4	0.313	0.8672
		Colony*Treatment	4	9051.000	0.4735			Colony*Treatment	4	2.825	0.0422			Colony*Treatment	4	1.240	0.3151
		Error	30					Error	30					Error	30		
		Total	39					Total	39					Total	39		
Gene	Day					Gene	Day					Gene	Day				
Cyp4U3	Day 10	Source	DF	F ratio	p value	Cyp4-5	Day 10	Source	DF	F ratio	p value	NADH	Day 10	Source	DF	F ratio	p value
		Whole model	9	42.577	<0.0001			Whole model	9	5.442	0.0002			Whole model	9	39.359	<0.0001
		Colony	1	374.081	<0.0001			Colony	1	36.186	<0.0001			Colony	1	327.174	<0.0001
		Treatment	4	1.339	0.2783			Treatment	4	2.160	0.0977			Treatment	4	4.396	0.0065
		Colony*Treatment	4	0.938	0.4556			Colony*Treatment	4	1.037	0.4044			Colony*Treatment	4	2.367	0.0751
		Error	30					Error	30					Error	30		
		Total	39					Total	39					Total	39		
Gene	Day					Gene	Day					Gene	Day				
Myosin	Day 10	Source	DF	F ratio	p value	28s	Day 10	Source	DF	F ratio	p value	APO	Day 10	Source	DF	F ratio	p value
		Whole model	9	7.429	<0.0001			Whole model	9	58.650	<0.0001			Whole model	9	7.301	<0.0001
		Colony	1	45.969	<0.0001			Colony	1	524.746	<0.0001			Colony	1	54.564	<0.0001
		Treatment	4	5.141	0.0028			Treatment	4	0.447	0.7738			Treatment	4	2.144	0.0998
		Colony*Treatment	4	0.081	0.9875			Colony*Treatment	4	1.322	0.3299			Colony*Treatment	4	0.642	0.6368
		Error	30					Error	30					Error	30		
		Total	39					Total	39					Total	39		
Gene	Day					Gene	Day										
broad	Day 10	Source	DF	F ratio	p value	SH3	Day 10	Source	DF	F ratio	p value						
		Whole model	9	11.018	<0.0001			Whole model	9	0.808	0.6123						
		Colony	1	87.012	<0.0001			Colony	1	0.557	0.4614						
		Treatment	4	1.902	0.1360			Treatment	4	1.623	0.1942						
		Colony*Treatment	4	1.497	0.3589			Colony*Treatment	4	0.057	0.9937						
		Error	30					Error	30								
		Total	39					Total	39								

Table 3-5 Objective #1 Day 1 relative expression

Day 1			Relative Expression					ANOVA Results				
Gene	**Description**	**Control**	**JH**	**JH+SHE**	**SHE**	**LS**	**Whole model**	**Colony**	**Treatment**	**interaction**		**q-value**
9	Carbx-1	1	1.275	1.004	1.008	0.573	<0.0001	0.1220	<0.0001	0.000	1	0.0011
14	Cyp15F1	1	0.459	0.430	1.097	1.033	<0.0001	<0.0001	<0.0001	0.735	2	0.0022
19	CYP4C46	1	2.369	3.985	1.024	1.208	<0.0001	<0.0001	<0.0001	0.355	3	0.0033
20	CYP4C47	1	0.323	0.364	1.226	1.600	<0.0001	0.0178	<0.0001	0.174	4	0.0043
21	CYP4C48	1	0.436	0.577	1.213	1.050	<0.0001	0.0017	<0.0001	0.163	5	0.0054
22	Cyp6.G	1	0.518	0.588	1.375	0.905	<0.0001	0.0003	<0.0001	0.301	6	0.0065
36	Myosin	1	1.062	0.809	1.029	0.680	<0.0001	<0.0001	<0.0001	0.004	7	0.0076
37	NADH	1	1.293	1.125	1.091	1.863	<0.0001	<0.0001	<0.0001	<0.0001	8	0.0087
5	Bactin	1	1.050	0.886	0.806	0.641	<0.0001	<0.0001	0.0003	0.000	9	0.0098
29	Hex-2	1	1.726	1.335	0.686	0.927	0.0076	0.0606	0.0020	0.244	10	0.0109
39	R-Pro	1	1.417	1.304	1.091	0.914	<0.0001	<0.0001	0.0046	0.611	11	0.0120
8	Btube	1	1.277	0.847	0.901	0.994	0.0024	0.0019	0.0055	0.194	12	0.0130
15	Cyp4U3	1	4.112	4.454	0.966	1.303	<0.0001	<0.0001	0.0065	0.005	13	0.0141
1	18s	1	1.304	1.415	0.737	1.098	<0.0001	<0.0001	0.0097	0.012	14	0.0152
38	nanos	1	0.992	0.853	1.178	2.332	0.0002	0.0003	0.0124	0.005	15	0.0163
30	HMG	1	1.403	1.347	1.019	0.599	0.0035	0.0008	0.0134	0.174	16	0.0174
4	ATP	1	1.340	1.265	1.077	0.887	<0.0001	<0.0001	0.0159	0.391	17	0.0185
41	Shp	1	1.128	1.382	1.217	2.129	<0.0001	<0.0001	0.0201	0.034	18	0.0196
45	Vit-1	1	0.779	0.622	1.074	5.288	0.0072	0.3387	0.0201	0.007	19	0.0207
7	broad	1	1.009	0.937	0.900	0.761	0.0002	<0.0001	0.0221	0.106	20	0.0217
46	Vit-2	1	1.099	0.916	1.815	3.901	0.0180	0.2060	0.0253	0.027	21	0.0228
16	CYP4C43v1	1	0.731	0.849	1.146	1.013	<0.0001	<0.0001	0.0352	0.055	22	0.0239
43	Tro-1	1	1.402	1.343	1.456	1.350	<0.0001	<0.0001	0.0418	0.060	23	0.0250
33	LCP	1	3.540	0.412	0.228	1.008	0.1302	0.0498	0.0586	0.740	24	0.0261
25	Famet-2	1	1.170	1.016	1.074	0.895	0.0037	0.0010	0.0734	0.073	25	0.0272
42	To-F	1	1.132	0.701	0.897	0.795	0.0004	<0.0001	0.0829	0.038	26	0.0283
23	Epox-1	1	1.937	0.643	1.063	1.474	0.1246	0.4697	0.0851	0.216	27	0.0293
10	Carbx-2	1	0.892	0.843	0.857	1.015	0.0017	<0.0001	0.1049	0.120	28	0.0304
13	CoxIII	1	1.197	1.293	0.940	1.216	<0.0001	<0.0001	0.1266	0.075	29	0.0315
18	CYP4C45v1	1	0.656	0.834	1.130	1.084	0.0004	<0.0001	0.1413	0.383	30	0.0326
12	Cop9	1	1.249	1.042	1.148	1.420	<0.0001	<0.0001	0.1458	0.389	31	0.0337
11	cJun-1	1	0.982	0.967	0.799	1.021	<0.0001	<0.0001	0.1546	0.279	32	0.0348
31	HSP	1	1.175	1.293	0.997	0.934	<0.0001	<0.0001	0.1584	0.799	33	0.0359
3	APO	1	1.088	0.866	1.153	0.930	0.0153	0.0017	0.1609	0.164	34	0.0370
44	Tro-2	1	1.144	1.043	1.273	1.156	<0.0001	<0.0001	0.1637	0.164	35	0.0380
40	SH3	1	0.738	0.911	0.782	0.839	<0.0001	<0.0001	0.1963	0.002	36	0.0391
17	CYP4C44v1	1	0.881	1.035	1.279	0.969	0.0049	<0.0001	0.2522	0.421	37	0.0402
27	Gtpase	1	0.970	0.956	1.028	1.344	0.0069	0.0002	0.2761	0.147	38	0.0413
34	Lprs	1	1.299	1.206	1.022	1.132	0.0349	0.0004	0.3106	0.790	39	0.0424
35	MalcoA	1	1.366	1.816	1.331	1.436	0.0650	0.4334	0.3155	0.027	40	0.0435
24	Famet-1	1	0.978	0.915	0.868	1.044	0.0006	<0.0001	0.3542	0.899	41	0.0446
32	Intro	1	1.293	1.293	1.221	1.203	<0.0001	<0.0001	0.4097	0.650	42	0.0457
28	Hex-1	1	1.010	0.805	0.774	0.917	<0.0001	<0.0001	0.4943	0.097	43	0.0467
6	Bic	1	1.589	1.215	1.378	1.626	<0.0001	<0.0001	0.5019	0.899	44	0.0478
26	Famet-3	1	1.050	1.219	0.884	1.034	0.5042	0.0577	0.5034	0.798	45	0.0489
2	28s	1	1.168	1.114	0.916	1.193	<0.0001	<0.0001	0.5102	0.023	46	0.0500

Table 3-6. Objective #1 Day 5 relative expression

Day 5			Relative Expression					ANOVA Results				
Gene	**Description**	**Control**	**JH**	**JH+SHE**	**SHE**	**LS**	**Whole model**	**Colony**	**Treatment**	**interaction**		**q-value**
2	28s	1	2.115	1.006	1.013	1.383	<0.0001	<0.0001	<0.0001	<0.0001	1	0.0011
25	Famet-2	1	1.070	1.096	1.082	0.648	<0.0001	0.0021	<0.0001	<0.0001	2	0.0022
28	Hex-1	1	2.079	2.466	0.741	0.863	<0.0001	0.0004	<0.0001	0.697	3	0.0033
29	Hex-2	1	2.614	3.361	0.748	0.890	<0.0001	0.0004	<0.0001	0.036	4	0.0043
46	Vit-2	1	26.483	126.862	0.452	1.336	<0.0001	0.0049	<0.0001	0.354	5	0.0054
7	broad	1	1.155	1.445	0.950	1.071	<0.0001	0.2624	0.0001	0.000	6	0.0065
22	Cyp6.G	1	0.940	0.706	0.898	0.548	<0.0001	<0.0001	0.0001	<0.0001	7	0.0076
6	Bic	1	0.564	0.955	0.508	1.289	<0.0001	<0.0001	0.0005	0.000	8	0.0087
17	CYP4C44v1	1	1.795	1.439	0.846	0.853	<0.0001	<0.0001	0.0005	0.243	9	0.0098
45	Vit-1	1	2.088	6.702	0.216	0.487	0.0005	0.0007	0.0014	0.239	10	0.0109
38	nanos	1	0.830	0.630	0.569	1.787	<0.0001	<0.0001	0.0020	0.011	11	0.0120
40	SH3	1	0.776	0.656	0.966	1.292	<0.0001	<0.0001	0.0028	0.003	12	0.0130
13	CoxIII	1	1.080	1.021	1.241	1.313	<0.0001	<0.0001	0.0037	0.000	13	0.0141
10	Carbx-2	1	1.162	1.105	0.942	0.744	0.0016	0.0328	0.0042	0.008	14	0.0152
37	NADH	1	1.394	0.671	0.854	1.138	<0.0001	<0.0001	0.0060	0.001	15	0.0163
3	APO	1	1.194	1.148	0.670	0.923	0.0093	0.0137	0.0067	0.188	16	0.0174
41	Shp	1	1.447	1.261	1.463	1.358	<0.0001	<0.0001	0.0067	0.002	17	0.0185
9	Carbx-1	1	0.709	1.061	0.688	0.612	0.0080	0.0087	0.0089	0.250	18	0.0196
31	HSP	1	1.255	1.214	1.148	1.453	<0.0001	<0.0001	0.0129	0.002	19	0.0207
14	Cyp15F1	1	1.100	0.754	0.767	1.036	<0.0001	<0.0001	0.0133	0.002	20	0.0217
19	CYP4C46	1	1.734	2.397	1.650	1.167	0.0031	0.0006	0.0134	0.409	21	0.0228
42	To-F	1	0.841	0.985	1.436	0.856	0.0085	0.0008	0.0156	0.722	22	0.0239
15	Cyp4U3	1	1.003	1.037	0.763	0.603	<0.0001	<0.0001	0.0214	0.254	23	0.0250
39	R-Pro	1	1.010	1.375	0.904	1.239	<0.0001	<0.0001	0.0262	0.012	24	0.0261
5	Bactin	1	0.796	0.813	1.115	0.869	<0.0001	<0.0001	0.0282	0.428	25	0.0272
27	Gtpase	1	1.143	1.138	0.767	1.198	<0.0001	<0.0001	0.0287	0.032	26	0.0283
8	Btube	1	1.339	1.295	0.930	1.418	0.0015	0.0007	0.0290	0.039	27	0.0293
20	CYP4c47	1	1.717	1.238	1.109	0.920	0.1407	0.3122	0.0303	0.342	28	0.0304
32	Intro	1	1.393	1.125	0.932	1.137	<0.0001	<0.0001	0.0315	0.439	29	0.0315
16	CYP4C43v1	1	1.066	0.959	1.001	1.396	<0.0001	<0.0001	0.0335	0.000	30	0.0326
18	CYP4c45v1	1	1.220	1.332	1.137	0.775	<0.0001	<0.0001	0.0539	0.139	31	0.0337
34	Lprs	1	0.975	0.890	0.907	1.350	0.0164	0.9784	0.0567	0.006	32	0.0348
24	Famet-1	1	1.174	1.205	1.146	1.176	<0.0001	<0.0001	0.0602	0.015	33	0.0359
21	CYP4c48	1	0.865	0.582	1.101	0.899	0.0620	0.0037	0.0616	0.917	34	0.0370
26	Famet-3	1	1.211	1.030	0.963	1.238	0.0005	0.0008	0.0630	0.003	35	0.0380
30	HMG	1	1.163	1.188	1.221	0.974	0.0258	0.0028	0.0957	0.315	36	0.0391
43	Tro-1	1	1.026	0.890	1.202	1.295	0.0016	<0.0001	0.1102	0.642	37	0.0402
44	Tro-2	1	1.095	0.793	0.765	0.940	0.0044	<0.0001	0.1205	0.821	38	0.0413
23	Epox-1	1	0.777	0.610	2.739	1.726	0.3676	0.5118	0.1530	0.562	39	0.0424
12	Cop9	1	0.864	0.988	0.813	1.167	0.0225	0.1035	0.1837	0.016	40	0.0435
36	Myosin	1	0.954	0.912	1.037	0.905	<0.0001	<0.0001	0.2202	0.069	41	0.0446
35	MalcoA	1	1.183	1.552	1.751	0.930	0.0008	0.7037	0.2431	0.000	42	0.0457
33	LCP	1	1.002	3.655	7.032	1.412	0.0490	0.5181	0.3202	0.029	43	0.0467
4	ATP	1	1.110	1.175	0.964	1.133	<0.0001	<0.0001	0.4134	0.005	44	0.0478
1	18s	1	0.961	1.075	1.013	1.119	0.0859	0.0043	0.7108	0.026	45	0.0489
11	cJun-1	1	0.644	1.216	1.161	1.169	0.1557	0.0153	0.7824	0.349	46	0.0500

Table 3-7 Objective #1 Day 10 relative expression

Day 10			Relative Expression			ANOVA Results					
Gene	**Description**	**Control**	**JH**	**JH+SHE**	**SHE**	**Whole model**	**Colony**	**Treatment**	**interaction**		**q-value**
2	28s	1	2.055	0.898	1.059	<0.0001	<0.0001	<0.0001	<0.0001	1	0.0011
15	Cyp4U3	1	2.200	0.621	1.603	<0.0001	<0.0001	<0.0001	0.089	2	0.0022
36	Myosin	1	1.028	1.890	1.107	<0.0001	<0.0001	<0.0001	0.006	3	0.0033
46	Vit-2	1	7.331	28.136	0.869	<0.0001	0.0013	<0.0001	0.007	4	0.0043
14	Cyp15F1	1	0.892	0.525	0.892	<0.0001	<0.0001	0.0003	<0.0001	5	0.0054
23	Epox-1	1	2.604	8.965	0.859	0.0143	0.4477	0.0008	0.239	6	0.0065
34	Lprs	1	1.376	1.341	1.380	0.0003	0.0118	0.0020	0.002	7	0.0076
41	Shp	1	0.982	0.582	1.161	<0.0001	<0.0001	0.0020	0.160	8	0.0087
9	Carbx-1	1	0.425	0.328	1.010	0.0025	0.0070	0.0021	0.082	9	0.0098
24	Famet-1	1	1.337	1.165	1.346	<0.0001	<0.0001	0.0022	0.002	10	0.0109
7	broad	1	1.082	1.525	1.216	0.0066	0.0547	0.0029	0.126	11	0.0120
28	Hex-1	1	0.650	0.583	1.409	0.0053	0.0131	0.0035	0.155	12	0.0130
37	NADH	1	1.835	1.136	1.504	<0.0001	<0.0001	0.0041	0.002	13	0.0141
40	SH3	1	1.021	0.260	0.889	0.0018	0.0012	0.0084	0.065	14	0.0152
1	18s	1	0.766	0.726	0.622	<0.0001	<0.0001	0.0086	0.000	15	0.0163
3	APO	1	0.919	1.644	1.009	0.0066	0.0138	0.0094	0.074	16	0.0174
19	CYP4C46	1	1.589	0.794	1.033	<0.0001	<0.0001	0.0114	0.000	17	0.0185
42	To-F	1	0.764	0.841	1.365	0.0032	0.0006	0.0153	0.320	18	0.0196
43	Tro-1	1	1.099	0.765	0.857	<0.0001	<0.0001	0.0160	0.006	19	0.0207
39	R-Pro	1	1.399	1.194	1.393	<0.0001	<0.0001	0.0238	0.022	20	0.0217
5	Bactin	1	1.116	1.681	1.287	<0.0001	<0.0001	0.0262	0.256	21	0.0228
22	Cyp6.G	1	0.706	0.702	0.841	<0.0001	<0.0001	0.0361	0.002	22	0.0239
16	CYP4C43v1	1	1.221	0.982	1.103	<0.0001	<0.0001	0.0374	0.000	23	0.0250
29	Hex-2	1	1.102	1.585	1.327	0.0023	0.0155	0.0396	0.008	24	0.0261
20	CYP4C47	1	1.356	0.835	0.939	0.0108	0.0221	0.0419	0.060	25	0.0272
11	cJun-1	1	1.330	1.399	1.269	<0.0001	<0.0001	0.0518	0.749	26	0.0283
32	Intro	1	1.364	1.310	1.083	<0.0001	<0.0001	0.0559	0.006	27	0.0293
33	LCP	1	4.384	28.946	4.600	0.1465	0.0911	0.0623	0.615	28	0.0304
8	Btube	1	1.111	1.555	1.102	0.0051	0.0066	0.0648	0.025	29	0.0315
25	Famet-2	1	0.949	1.093	1.234	0.0162	0.0033	0.0725	0.269	30	0.0326
10	Carbx-2	1	1.234	0.991	1.149	<0.0001	<0.0001	0.0852	0.017	31	0.0337
17	CYP4C44v1	1	0.879	0.765	0.964	<0.0001	<0.0001	0.1239	0.086	32	0.0348
44	Tro-2	1	1.327	1.059	0.978	0.0054	0.0211	0.1257	0.013	33	0.0359
45	Vit-1	1	3.670	4.766	1.169	0.0095	0.0476	0.1277	0.021	34	0.0370
27	Gtpase	1	1.368	1.259	1.385	<0.0001	<0.0001	0.1338	0.291	35	0.0380
13	CoxIII	1	1.173	0.928	1.043	<0.0001	<0.0001	0.1614	0.036	36	0.0391
38	nanos	1	1.377	0.766	1.347	0.0007	<0.0001	0.1752	0.165	37	0.0402
4	ATP	1	0.843	1.251	1.029	0.0851	0.0070	0.2348	0.514	38	0.0413
18	CYP4C45v1	1	1.786	1.472	1.332	<0.0001	<0.0001	0.2730	0.195	39	0.0424
30	HMG	1	1.307	1.332	1.489	0.5523	0.1275	0.2969	0.946	40	0.0435
12	Cop9	1	1.044	1.092	1.177	0.0034	<0.0001	0.4195	0.325	41	0.0446
31	HSP	1	1.019	1.071	1.231	0.0182	0.0019	0.4423	0.119	42	0.0457
35	MalcoA	1	1.256	0.960	1.576	0.4381	0.0702	0.4430	0.804	43	0.0467
6	Bic	1	1.016	1.154	1.075	<0.0001	<0.0001	0.8464	0.089	44	0.0478
26	Famet-3	1	0.977	0.903	0.989	0.5240	0.0520	0.8722	0.800	45	0.0489
21	CYP4C48	1	1.069	1.091	1.029	<0.0001	<0.0001	0.9434	0.009	46	0.0500

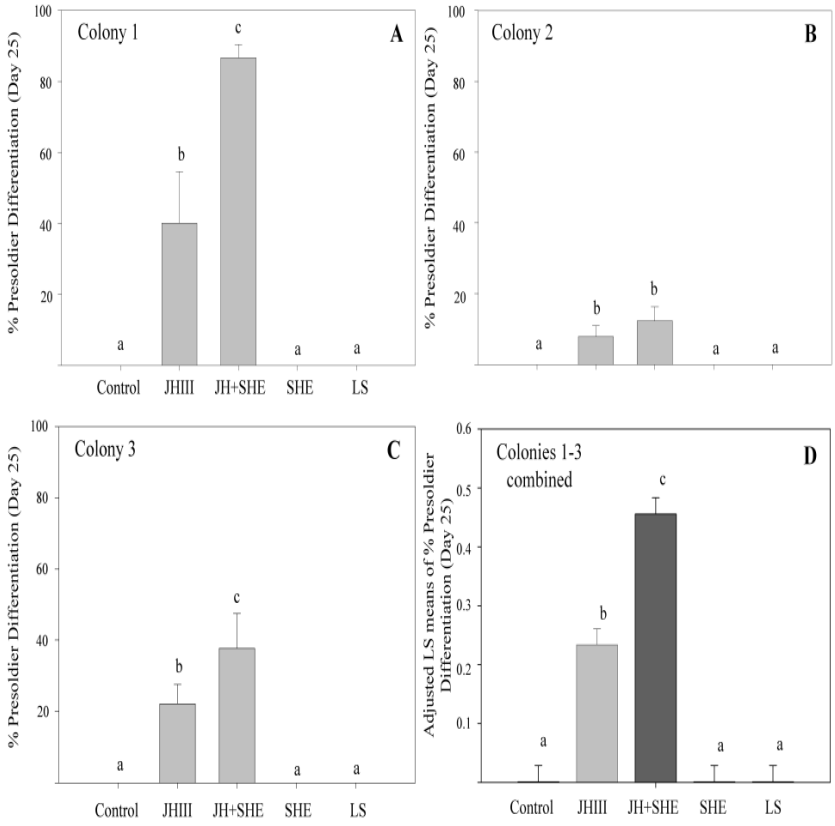

Figure 3-1. Impact of semiochemical and socio-environmental treatments on soldier caste differentiation**.** Results shown represent cumulative presoldier formation through Day 25 of assays that were conducted under five different treatments: control, JHIII, JHIII+SHE, SHE, and LS (live soldiers) for three different colonies (1,2,3). The three colonies were combined and an adjusted LS means are shown. Bars with the same letter are not significantly different $p<0.05$.

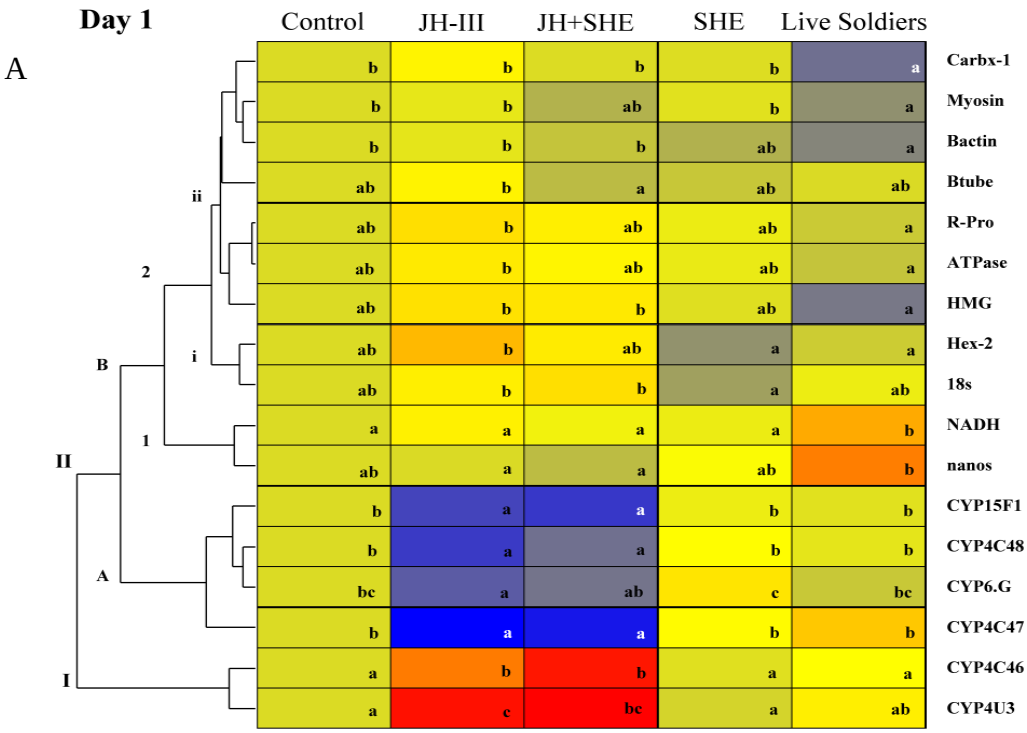

Figure 3-2. Expression changes for significant genes in termite workers in response to semiochemical and socio-environmental treatments after 1, 5, and 10 days. Results shown represent the relative expression values of significant differentially expressed genes under five different treatments: control, JHIII, JHIII+SHE, SHE, and live soldiers at three different days; (A) Day 1, (2b) Day 5 and (2c) Day 10. Blue boxes represent genes that are down-regulated while red boxes represent genes that are up-regulated. Boxes with the same letter are not significantly different (FDR). Caladograms at the left group genes by similar expression pattern.

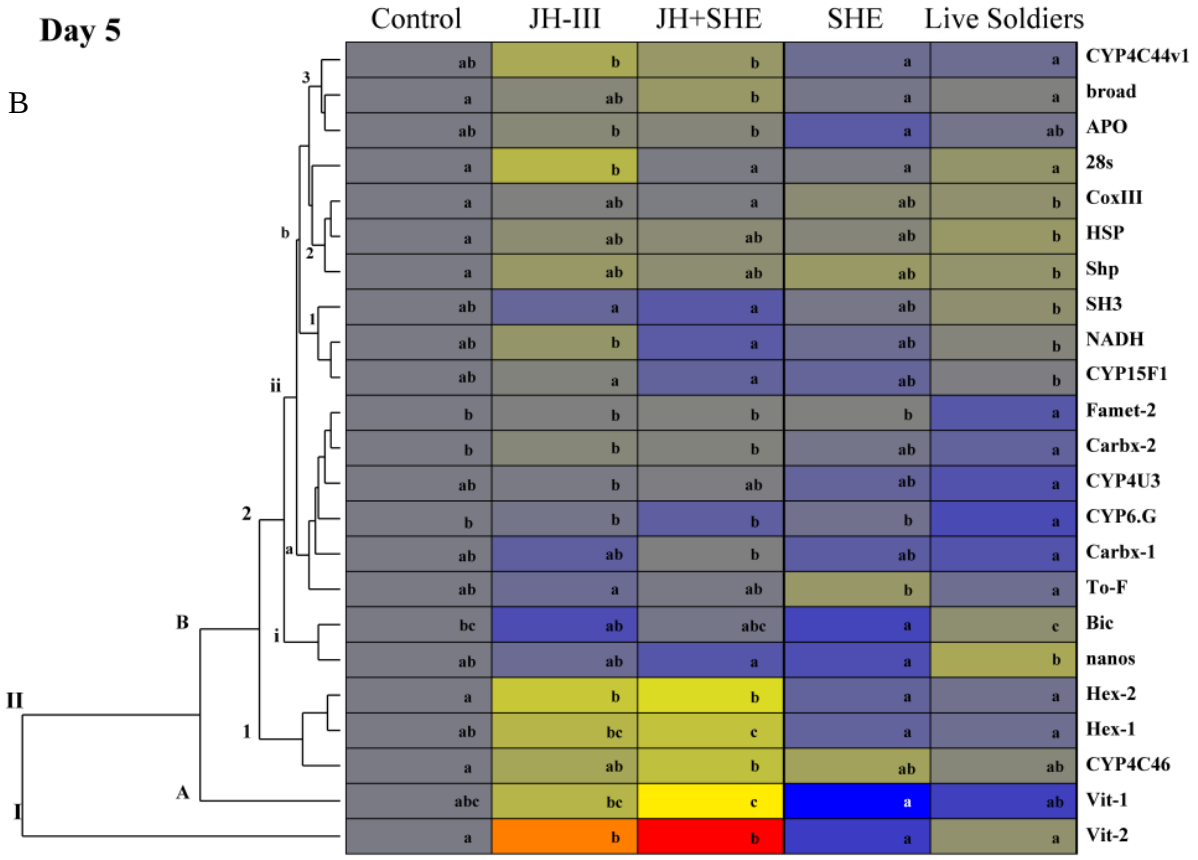

Figure 3-2 Continued.

C

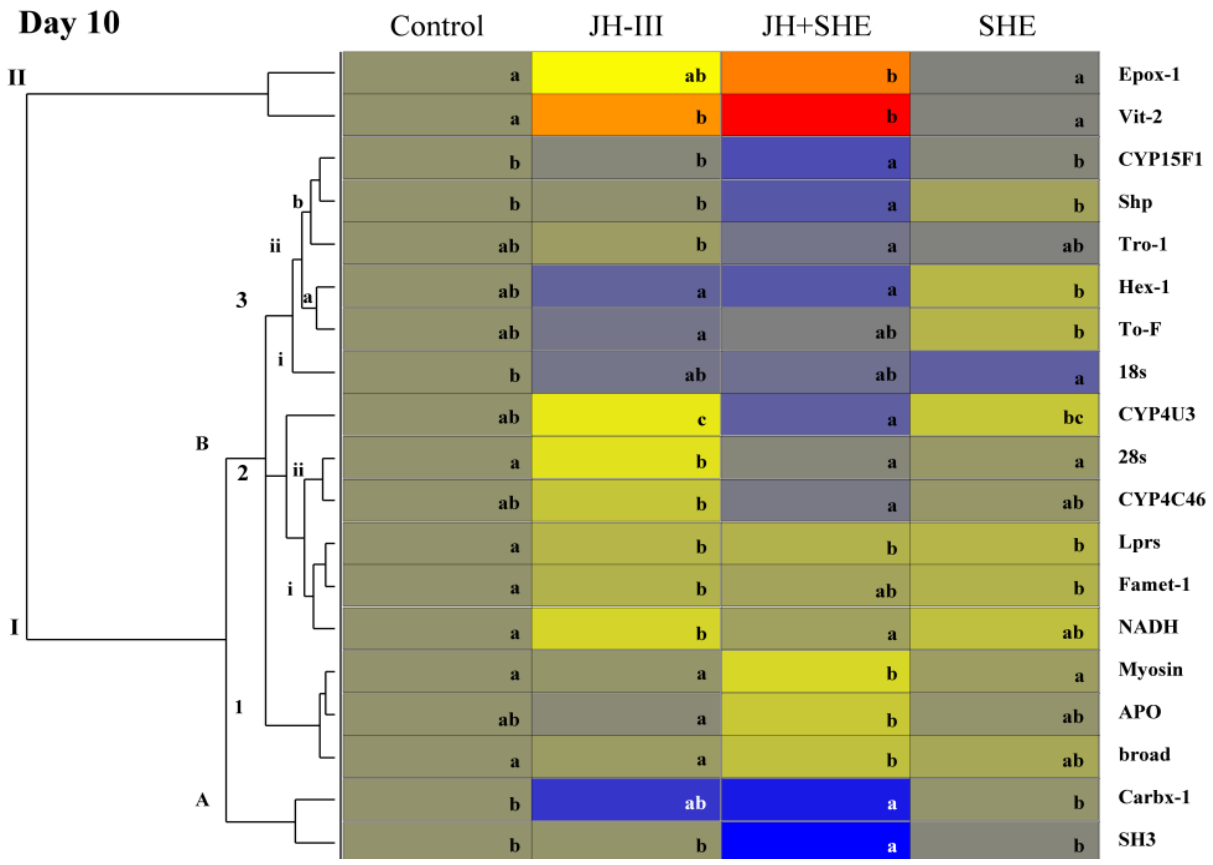

Figure 3-2 Continued.

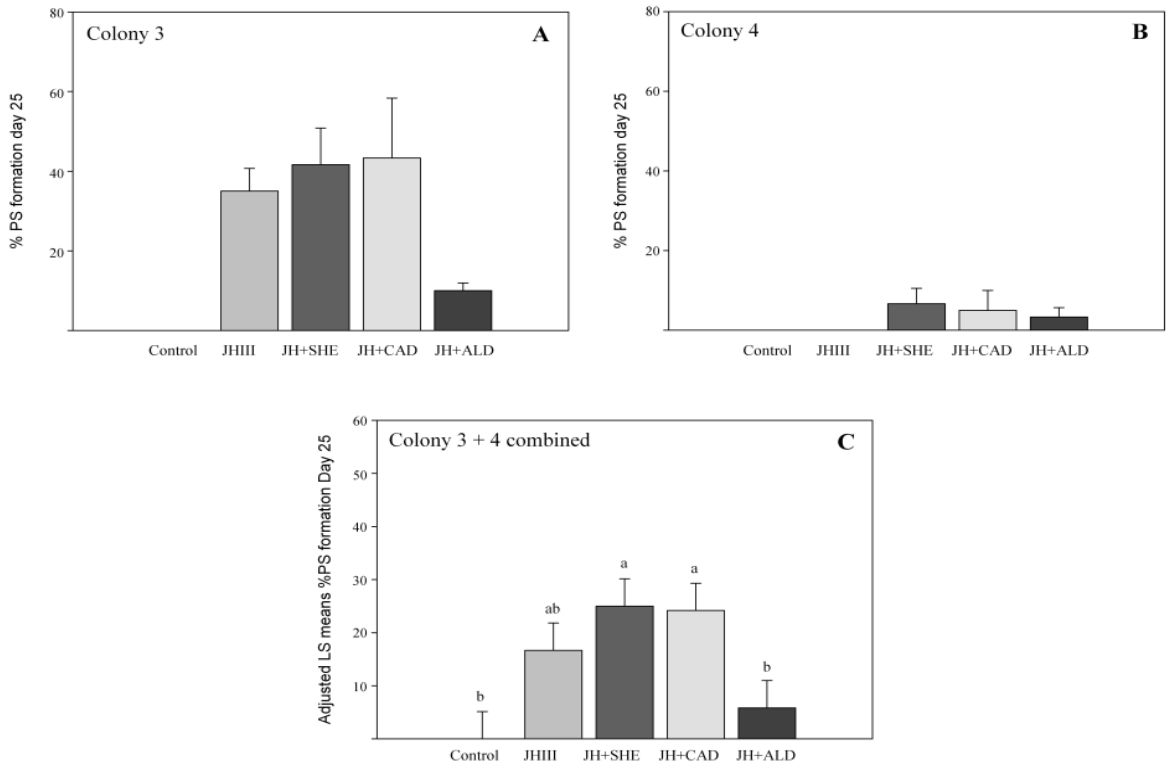

Figure 3-3. Impact of SHE blend and SHE components on soldier caste differentiation. Results shown represent cumulative presoldier formation through Day 25 of assays that were conducted under five different treatments: control, JHIII, JHIII+SHE, JHIII+ γ-cadinene (CAD), and JHIII+ γ-cadinenal (ALD), for two different colonies (3 (A) and 4 (B)). (C) The two colonies were combined and an adjusted LS means are shown. Bars with the same letter are not significantly different $p<0.05$.

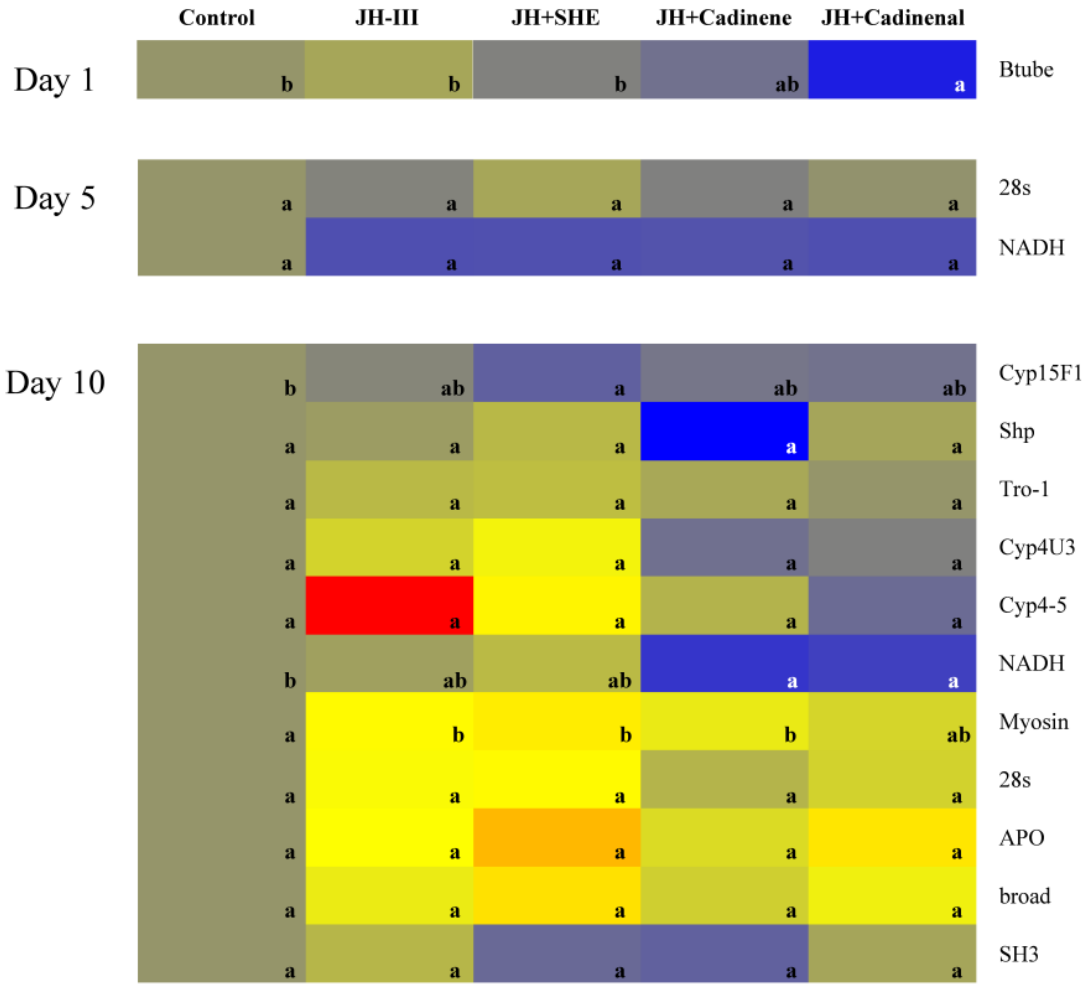

Figure 3-4. Expression changes for genes in termite workers in response to SHE blend and SHE components after 1, 5, and 10 days. Results shown represent the relative expression values of significant differentially expressed genes under five different treatments: control, JHIII, JHIII+SHE, JHIII+ γ-cadinene (CAD), and JHIII+ γ-cadinenal (ALD). Blue boxes represent genes that are down-regulated while red boxes represent genes that are up-regulated. Boxes with the same letter are not significantly different.

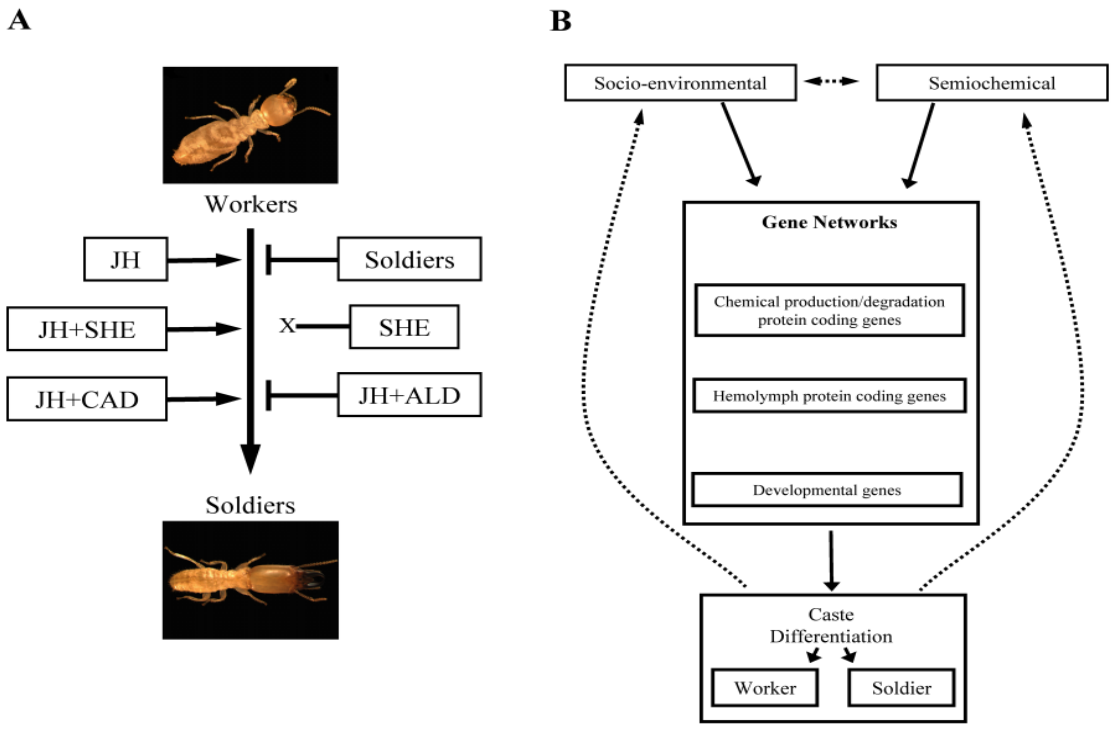

Figure 3-5. Diagrams summarizing the influence of socio-environmental and semiochemical factors on caste differentiation. A) Semiochemical and socio-environmental factors tested and their effects on worker-to-soldier differentiation. JHIII and JH+SHE caused an increase in soldier formation, while SHE had no effect on presoldier/soldier formation. Past research (Mao et al., 2005; Park and Raina, 2004; Appendix D) indicates that soldiers inhibit worker differentiation. SHE components γ-cadinene +JH caused an increase in soldier formation, while γ-cadinenal +JH resulted in a reduction in soldier formation. B) Diagram representing how socio-environmental and semiochemicals factors might modulate the expression patterns of multiple genes and caste differentiation. Networks including the following gene categories showed significant changes among treatments: chemical production/degradation, hemolymph protein coding, and developmental genes. Dotted lines represent the possible feedback loop when colony worker termites molt into soldiers, the increase in the soldier number consequently inhibits the formation of additional soldiers.

CHAPTER 4
FUNCTIONAL ANALYSES OF R. FLAVIPES CYTOCHROME P450 AND ESTERASE GENES LINKED TO JUVENILE HORMONE BIOSYNTHESIS AND DEGRADATION

Introduction

In animals, the control of the production and degradation of hormones is essential for proper development (Nijhout, 1994). One of the major morphogenic hormones in insects is the terpene, juvenile hormone (JH). Juvenile hormone has been shown to play diverse roles in insect biology including modulation of larval development, metamorphosis, diapause, migratory behavior, wing length, seasonal development, and eusocial caste determination (Hartfelder, 2000).

JH is produced in insects through the mevalonate pathway. The mevalonate pathway begins with acetyl-CoA and ends with JH as the end product. One of the final steps in the pathway, the epoxidation of methyl farnesoate to JH III, is carried out by cytochrome P450 enzymes (Bellés et al., 2005; Helvig et al., 2004). The "P450s" are a diverse family of oxidative enzymes found in almost all living organisms (Feyereisen, 2005). P450s are general mixed function oxidases that catalyze the transfer of oxygen to substrates, while in the process reducing oxygen to water. Cytochrome P450s are known for their diverse roles in the oxidation of endogenous and xenobiotic substrates including hormones, pheromones, insecticides, and secondary plant compounds (Andersen et al., 1997; Feyereisen, 2005). Specifically, P450s have been shown to play a role in the biosynthesis and metabolism of morphogenic hormones (JHIII, ecdysone) and other terpenoids (Andersen et al., 1997, Helvig et al. 2004; Rewitz et al. 2006). Helvig et al. (2004) reported that the cytochrome P450, *Cyp15A1* of the cockroach, *Diploptera punctata* is responsible for the last epoxidation step in JH biosynthesis. In the termite, Zhou et al. (2006b) identified a number of family 4 cytochrome P450 genes and showed that their expression patterns varied in response to JHIII and colony release (removal from colony)

treatments, suggesting that they might play a role in caste differentiation. However, no physiological roles of termite P450s are yet defined.

The degradation of JH within immature and adult insects is also an integral part of their development. For example, the removal of circulating JH from the insect hemolymph is necessary for an immature insect to molt into an adult (Nijhout, 1994). The degradation of JH has been shown to be controlled in part by JH esterases (Roe et al., 1996). Juvenile hormone esterases are usually synthesized in the fat body and secreted into the hemolymph, where they metabolize JH through hydrolysis of the ester linkage, converting it to JH acid (Goodman and Granger, 2005). Mackert et al. (2008) functionally identified a juvenile hormone esterase-like gene (*amjhe-like*) in the honey bee (*A. mellifera*). Comparison of published information on developmental JH titers and expression analysis of the gene indicates that *amjhe-like* transcript levels are positively correlated with JH titers (Mackert et al., 2008). RNAi mediated silencing of the *amjhe-like* gene resulted in an increase in JH levels. The authors concluded that the *amjhe-like* gene was a JH esterase responsible for the metabolism of JH within the honey bee (Mackert et al., 2008).

In social insects JH plays an important role in caste differentiation (Hartfelder, 2000; Scharf et al., 2007; Macket et al., 2008). Castes are phenotypically and behaviorally discreet individuals that cooperate to perform colony tasks (Miura, 2004). Termite colonies are composed of three main castes: workers, soldiers, and reproductives. Because all colony individuals share the same genetic background, termites castes develop in response to a combination of intrinsic and extrinsic factors (Koshikawa, 2005; Scharf et al., 2007; Zhou et al., 2007), except where a rare genetic component might be involved (Hayashi et al., 2007; Goodisman and Crozier, 2003).

In termites, juvenile hormone has been proposed to act as a possible primer pheromone (Henderson, 1998). Primer pheromones are chemical messengers that are passed among individuals and trigger physiological responses in recipients (Wilson and Bossert, 1963). Previous studies have shown that ectopic exposure of worker termites to JH III readily induces soldier caste differentiation (Howard and Haverty, 1979; Scharf et al., 2003b, 2005a, 2007; Zhou et al., 2006a,b, 2007), indicating that JH can act via exogenous exposure. Under natural conditions, high endogenous JH titers in worker termites cause differentiation into presoldiers, and then into soldiers (Park and Raina, 2004; Mao et al., 2005). Work in Chapter 2 identified two potential primer pheromones, located in the soldier termite head, which apparently work synergistically with JH to increase worker to presoldier differentiation. Soldier head extract (SHE) in *Reticulitermes flavipes* is comprised of two major components, γ-cadinene and γ-cadinenal. Further studies on the SHE blend, indicated that the γ-cadinene is the active component and γ-cadinenal might actually be inhibitory towards soldier development (Chapter 3).

The central objective of this study was to begin to understand the roles of two cytochrome P450 genes (*Cyp15F1*, *Cyp15A1*) and a putative JH esterase (*RfEst1*) in termite caste differentiation and/ or caste regulation. As discussed above, the rationale behind this work is that these genes may play roles in JH biosynthesis / degradation, and subsequently, termite caste differentiation and thus, they may serve as good pesticidal targets. To meet this objective, studies were conducted to 1) obtain the full length gene sequence of each of the three genes, 2) determine the baseline tissue distribution of expression for each of the three genes, 3) investigate gene expression changes in response to JH and JH+ soldier head extract (SHE), and 4) use RNAi to identify possible gene functions in caste differentiation or regulation.

Materials and Methods

Termites

R. flavipes colonies were collected from locations near Gainesville, Florida USA. Termites were identified as *R. flavipes* by a combination of soldier morphology (Nutting, 1990), and *16S* mitochondrial-ribosomal RNA gene sequencing (Szalanski et al., 2003). Termites were brought to the laboratory and held for at least two months before use in bioassays. Colonies were maintained in darkness within sealed plastic boxes at 22 ^{o}C. Termite workers were considered workers if they did not possess any sign of wing buds or distended abdomens.

Solider Head Extract

Soldier head extract was collected as described in Chapter 2. In brief, soldier head extract (SHE) was prepared by collecting soldiers from the colony, removing their heads, and homogenizing the heads (80-150) in acetone, using a Tenbroeck glass homogenizer. To remove particulate matter, the homogenate was fractionated by passing it through a glass Pasteur pipette filled with approximately 250 mg of silica gel (60-200 mesh) on top of a glass wool plug.

Gene Sequence Identification and Analyses

Two cytochrome P450s and one esterase were chosen from a set of ESTs previously sequenced from an *R. flavipes* normalized gut library (Tartar et al., 2009). Each gene was assembled from multiple ESTs to form contigs. To obtain additional sequence length and verify sequences, select library clones were picked and re-sequenced. Translated amino acid sequences were aligned using Megalign^{TM} in the Laser gene software package (Madison, WI). Hydropathy plots were made and signal peptides identified using Protean^{TM} in Laser gene. Virtual translations were made using standard genetic code with ExPASy translation tool.

Gene Tissue Distribution and JH and JH+SHE Response

Bioassays were conducted at 27 ^{o}C as described previously (Scharf et al., 2003b; Chapter 2). Paired paper towel sandwiches (Georgia Pacific) were treated with acetone (controls), JHIII, or JH+SHE treatments delivered in acetone. JHIII (75% purity; Sigma; St. Louis, MO) was provided at a rate of 56 µg per dish in a volume of 50 µl acetone and SHE was provided at 1.5 soldier head equivalents in acetone. After solvent evaporation, sandwiches were placed in 5 cm plastic Petri dishes and moistened with 60 µl of reverse osmosis water. Fifteen worker termites were placed in each assay dish. Each treatment was replicated three times. The three treated groups of fifteen worker termites were dissected into head, gut and carcass. Dissections were placed into PBS and immediately frozen at -80 °C. The experiment was repeated on two different colonies.

RNA Isolation and cDNA Synthesis

Total RNA was isolated from frozen samples using the SV total RNA Isolation System (Promega; Madison, WI) according to the manufacturer's protocol. Whole body RNA extracts were isolated from 15 termites from each bioassay dish. The amount of RNA was quantified by spectrophotometry and equal amounts of RNA were used for cDNA synthesis. First strand cDNA was synthesized using the iScript cDNA synthesis Kit (Bio-Rad; Hercules, CA) according to the manufacturer's protocol.

Gene Expression

Quantitative real-time PCR (qRT-PCR) was performed using an iCycler iQ real-time PCR detection system (Bio-Rad) with SYBR-green product tagging from cDNA (similar to Scharf et al., 2003a; Zhou et al., 2006). Four genes were tested, one control (*Stero-1*) and three target (*Cyp15F1*, *Cyp15A1*, *RfEst1*), gene specific primers are the same as used in the previous chapter.

Data and Statistical Analyses

For Day 0 tissue localization, relative expression of target genes was calculated by comparing the average of three technical replications first normalized to the reference gene (*Stero-1*) and then normalized to the body region that had the lowest expression or control treatment using the $2^{-\Delta Ct\Delta Ct}$ method (Livak and Schmittgen, 2001).

To determine significantly differentially expressed genes for the body region response to JH and JH+SHE CT expression values for target genes were normalized to the CT values from the reference gene (ΔCT) and then to the Day 0 control (ΔΔCT). A two-way ANOVA, with Tukey's HSD correction for multiple comparisons was used to separate significant genes using JMP statistical software (SAS Institute, Cary, NC, USA) (Table 4-1).

Esterase Native PAGE and Colorimetric Esterase Assays

In the first experiment, 15 termites per treatment were held on paper towel, glass microfiber filters (Whatman, Cat. No. 1823-042), or on paper towel treated with JH (56ug). After five days termites were dissected to collect hemolymph, gut, and carcass fractions. Hemolymph from fifteen individuals was isolated into 30 uL of 0.1 M potassium phosphate, pH 7.6 buffer. Dissected guts from 15 individuals were isolated in to 400 ul of potassium phosphate, and carcasses into 1000 ul of potassium phosphate.

In the second set of experiments, 15 termites per treatment were added and held for a time course of 0-5 days. The two treatments were control and siRNA injected. Control individuals were injected with 41.4 nl of RNase free H_20. siRNA treated individuals were injected with 41.4 nl of *Rfest1* siRNA at a concentration of 15 pg/nl (621 pg/insect).

To make the *Rfest1* siRNA, dsRNA was first synthesized using a commercially available kit (Silencer Ambion, Austin, TX) and eluted with H_20. dsRNA was then quantified using a spectrophotometer. For production of siRNA, dsRNA was digested into ~25-mer fragments

using RNAse III included with the Ambion kit. *Rfest1* dsRNA primers with T7 ends were F-TAATACGACTCACTATAGGGTGGTTTCAAAAGCCATGACA and R-TAATACGACTCACTATAGGGACATACACCTGGGAAGCGAC. At each time point, sets of individuals were dissected into hemolymph, gut, and carcass as above and immediately frozen at -80 °C.

Esterase Native PAGE

Methods followed an established protocol with slight modification (Scharf et al., 1998). Volumes of supernatant containing 5 µg of total protein were diluted 1:1 with Native PAGE sample buffer (Bio-Rad) and loaded onto native PAGE gels (7.5% resolving gels and 4% stacking gels). Electrophoresis was conducted in Tris-Glycine running buffer for 1 hr at 4 °C. After running, gels were incubated in 100 ml sodium phosphate buffer (0.1 M, pH 7.5) containing 1 ml of 60 mM α-naphthyl propionate (Sigma; St. Louis, MO) substrate in acetone (1 mM final conc.) for 15 min. To visualize bands, 20 mg of Fast Blue BB (Sigma) in 1 ml water (0.02% final conc.) was added. The Fast Blue BB solution was filtered through glass wool just before use to remove insoluble particles. Gels were fixed, destained and stored in 10% acetic acid before photographing.

Colorimetric Esterase Assays

Methods followed an established protocol (Scharf et al., 1998) with adaptation to a microplate format. The assay mixture consisted of 188 µl of 0.02 M sodium phosphate buffer (pH 7.0), 2 µl of 25 mM substrate solution, and 10 µl tissue homogenate. Reactions proceeded at room temperature for 10 min. Reactions were stopped with 50 µl of 0.3% Fast Blue BB dissolved in 3.5 % SDS in water. The assay plates were incubated at 30° C for 15 min before being read at 600 nm with a microplate reader. Formation of the naphthol product was

determined from α-naphthol standard curves (serial dilutions starting at 5µm), stained with the Fast Blue-SDS stop solution as above. Treatments were separated using a Student t-test ($p<0.05$).

Results

Gene Sequencing

Over 5,000 ESTs were previously sequenced from a normalized *R. flavipes* gut cDNA library (Tartar et al., 2009). Here we focused on three genes identified by Tartar et al. (2009) that encoded predicted proteins with significant homology to known proteins involved in JH biosynthesis and degradation from other insects; two cytochrome P450s, *Cyp15F1* and *Cyp15A1*, and one JH- like esterase, *RfEst1*. Each cDNA sequence was compiled from multiple ESTs to build continuous contigs. Library clones were also selected and re-sequenced to add additional length and verify sequence. Corresponding Genbank accession numbers for each gene *Cyp15F1*, *Cyp15A1*, and *RfEst1* are FJ792773, FJ792774, and GQ180944, respectively. Nucleotide sequences and amino acid translations, are provided in Figure 4-1. *RfEst1* was previously described in Wheeler et al. (2009).

Alignments

Cyp15F1 and *Cyp15A1* amino acid sequences were aligned and compared with seven homologous cytochrome P450s (Figure 4-1a,b). The alignment revealed a significant proportion of conserved amino acid residues, including five signature P450 motifs (Table 4-1). *Cyp15F1* contains three out of the five signature P450 motifs, but was missing the WxxR and GxE/DTT/S motifs. Each of these motifs is present, but with slight amino acid variation (Table 4-1). *Cyp15F1* also contains the N-terminus "PGPP hinge" necessary for proper heme incorporation in the mature protein (Figure 4-1a).

Cyp15A1 contains four of five signature P450 motifs, while missing the ExLR motif (Figure 4-1, Table 4-1). The ExLR motif is present but contains a substitution of Q for L.

Interestingly, *Cyp15A1* lacks the N-terminal PGPP hinge and a signal peptide. Re-sequencing of the 5' end of the cDNA was performed multiple times to ensure the missing PGPP hinge and signal peptide were not due to sequencing errors. Except for its missing signal peptide, *Cyp15A1* is a virtual ortholog of the *Diploptera punctata Cyp15A1*, which is involved in methyl farnesoate epoxidation (evalue 0.0) (Helvig et al., 2004). A preliminary phylogenetic tree shows the relationship between *Cyp15F1*, *Cyp15A1*, and the seven homologs, with *Cyp15A1* closely matching *D. punctata Cyp15A.*

RfEest1 motifs and alignments were previously described by Wheeler et al. (2009). Briefly, *Rfest1* contains a signal peptide, 30 N-glycosylation sites, a conserved JH esterase catalytic triad (GxSAG,E/D, and GxxHxD), and two substrate recognition motifs (RF, DQ) (Figure 4-2a). *RfEst1* shares homology with other insect JH esterases including isoforms from: *Psacothea hilaris* (BAE94685), *Tribolium castaneum* (XP_967137), *A. mellifera* (AAU81605), and *Athalia rosae* (BAD91555) (Wheeler et al., 2009) (Figure 4-2b). A preliminary phylogenetic tree shows the relationship between *RfEst1* and the four homologs, with *RfEst1* being most similar to the *A. rosae* JH esterase.

Tissue Localization

Baseline tissue expression levels in colony workers for all three genes were determined through qRT-PCR (Table 4-2). The *Cyp15F1* transcript was expressed most highly in the carcass followed by the head and then the gut. The *Cyp15A1* transcript was significantly more highly expressed in the head than the carcass or gut. Finally, under baseline conditions, *RfEst1* was significantly more highly expressed in the carcass than in the head or gut. These results indicate where each gene is predominantly expressed in workers under colony conditions: *Cyp15F1* and *RfEst1* in the carcass, and *Cyp15A1* in the head.

Localized Expression Response to JH and JH+SHE

Tissue expression profiles 24 hours after JH and JH+SHE treatment were measured by qRT-PCR (Figure 4-3). One day after treatment, *Cyp15F1* expression was lower with JH and JH+SHE treatment in all body regions; but was only significantly decreased in the gut and carcass (Figure 4-3a). *Cyp15A1* expression was reduced with JH and JH+SHE treatment only in the head region (Figure 4-3b). Finally, *RfEst1* expression was significantly increased in the gut with JH treatment (Figure 4-3c). These results suggest that *Cyp15A1* and *Rfest1* play physiologically significant roles in head and gut tissue, respectively.

Functional Characterization of *RfEst1*

Esterase activity was investigated through a combination of Native PAGE with naphthyl propionate staining, colorimetric microplate assays, and injection of *RfEst1* siRNA. First, esterase activity was compared between control, starved, and JH-treated termites after five days of JH exposure in dish assays, in the hemolymph, gut, and carcass (Figure 4-4a). An increase in esterase staining was evident in the carcass fraction with JH treatment, while a slight increase in intensity was observed in the hemolymph and gut fractions. Re-analysis of untreated versus JH-treated termites verified a significant increase in esterase activity in the carcass (Figure 4-4b).

Next, a five day time course evaluation of esterase activity after siRNA injection was performed. Protein knockdown of a single esterase band was observed in the gut and carcass only at five days after injection of *RfEst1* siRNA (Figure 4-5a). Gel imaging on gut esterases indicated a noticeable reduction with siRNA treatment 1 and 5 days after treatment (Figure 4-5b). Carcass esterases increased one day after siRNA treatment, but consistent with the carcass results, were reduced at Day 5 (Figure 4-5cC). Colorimetric naphthyl-propionate microplate assays indicated a reduction of esterase activity in the gut with JH treatment, but an increase in the carcass ($df= 3,7$; $F=6.19$; $p=0.0031$) (Figure 4-6a). Microplate assays on siRNA treated gut

and carcass fractions were similar to Native PAGE results showing a reduction in esterase activity ($df=7,11$; $F=49.40$; $p<0.001$) (Figure 4-6b). Overall, siRNA treatment allowed for the identification of a native esterase protein band that appears to be the *RfEst1* gene product.

Discussion

Three genes identified in Chapter 3 with homology to JH biosynthesis and metabolism protein coding genes were selected for further characterization. Two P450s, *Cyp15F1* and *Cyp15A1*, had significant translated amino acid homology to P450 proteins responsible for JH production (methyl farnesoate epoxidation), while *RfEst1* had significant translated amino acid homology to esterase proteins responsible for JH metabolism (via hydrolysis of the C12 ester linkage).

Juvenile hormone is a morphogenetic hormone produced by a paired neurosecretory gland, the corpus allatum, which has a broad range of developmental and physiological effects (Nijhout, 1994; Gilbert et al., 2000; Goodman and Granger, 2005). In insects, JH plays a role in the control of larval development and metamorphosis, but also has been shown to affect diapause, migratory behavior, wing length, seasonal development, and eusocial caste determination (Hartfelder, 2000).

In termites, JH demonstrates primer pheromone-like characteristics. Primer pheromones are chemical messengers passed among individuals which trigger physiological responses in recipients (Wilson and Bossert, 1963). At high JH titers, worker termites differentiate into presoldiers, followed by a molt into a soldier (Park and Raina, 2004, 2005; Mao et al., 2005). The role of JH in soldier termite development is apparently the opposite of the “normal” role of JH among insects, where it maintains immature features (Truman and Riddiford, 1999). Previous studies have shown that exposure of worker termites to various JH homologues including JH III induces soldier differentiation (Howard and Haverty, 1979; Scharf et al., 2003a). Therefore,

morphogentic hormones such as JH appear to play a direct role in soldier caste differentiation, although the trigger for JH production by the corpora allata is likely to be extrinsic.

In insects, the production of the sesquiterpene JH is accomplished via the mevalonate pathway (Figure 4-7a). The mevalonate pathway begins with the reductive polymerization of acetyl-CoA, a precursor of several isoprenoid compounds (Bellés et al., 2005). In vertebrates, a final product of this pathway is cholesterol; however, insects lack a number of enzymes used to synthesize the sterol branch of cholesterol at the end of the mevalonate pathway. One of the final steps in the production of JH is the epoxidation of methyl farnesoate to JH III (Figure 4-7b). The order of the final two steps is different in orthopteran insects where esterification occurs before epoxidation; in lepidopterans it is reversed (Bellés et al., 2005). Helvig et al. (2004) reported that the cytochrome P450 *Cyp15A1* of the cockroach, *Diploptera punctata* is expressed in the head and is responsible for the last epoxidation step in JH biosynthesis. Use of recombinant *Cyp15A1* in metabolism studies confirmed that *D. punctata Cyp15A1* has a high affinity for methyl farnesoate and it is able to catalyze the NADPH-dependent epoxidation of 2E,6E-methyl farnesoate (Helvig et al., 2004; Feyereisen, 2005). These results conclusively linked *D. punctata Cyp15A1* to methyl farnesoate epoxidation in the production of JHIII.

The two cytochrome P450s studied here (*Cyp15F1* and *Cyp15A1*) share highly significant homology to *Cyp15A1* in *D. punctata* (Figure 4-1a,b). *Cyp15A1* is highly expressed in the termite head and has an e-value of 0.0 indicating almost an exact match to *D. punctata Cyp15A1* (Table 4-1). However, *R. flavipes Cyp15A1* is missing a signal peptide, which is thought to be responsible for incorporation of the terminal end of the protein into the microsomal membrane. The lack of a signal peptide, although unprecedented among insect P450s, suggests that the *R. flavipes Cyp15A1* could have cytosolic expression like bacterial P450s such as P450-CAM

(Larson et al., 1991a). More likely is that the protein can still function and incorporate into microsomes without its signal peptide. For example, recombinant cytochrome P450s engineered without signal peptides and heterologously expressed in *E.coli* have been shown to incorporate into the membrane and still function (Larson et al., 1991a,b). Based on sequence homology and relatedness of cockroaches and termites, it is possible that both *R. flavipes* Cyp15s could share the same function of methyl farnesoate epoxidation in the production of JH III.

The degradation of JH is also important in insect physiology and development. The ability to clear the body of JH is an important factor in insect development during the molt from an immature to an adult. Juvenile hormone esterases are members of the carboxylesterase family (EC 3.1.1.1) which are synthesized in the fat body and secreted into the hemolymph where they degrade JH (Goodman and Granger, 2005). Juvenile hormone esterases degrade JH by hydrolyzing the ester linkage on the C12 end of the molecule (Figure 4-7b). The *R. flavipes* *RfEst1* gene characterized in this study shares translated identity with several putative JH esterases (Figure 4-2a,b) and it was found to be expressed in the carcass, which contains significant amounts of fat body (Table 4-2). Also Wheeler et al. (2009) proposed that the *RfEst1* was one of the microsomal permethrin degrading esterases previously isolated by Valles et al. (2001), based on size and large number of predicted glycosylation sites. It has been hypothesized that genes such as JH esterases could participate in caste differentiation by regulating the titers and timing of JH and other semiochemicals and terpenes (Henderson, 1998, Wheeler et al., 2009).

Understanding the location of gene expression can also suggest potential functions. Although all the genes characterized here were first identified through sequencing of a gut EST library, their expression was also detected elsewhere in the termite body. Tissue localization

studies revealed expression distributions throughout the entire *R. flavipes* body for all three genes. Under baseline conditions, *Cyp15F1* was predominately expressed in the carcass, *Cyp15A1* was most highly expressed in the head, and *RfEst1* was most highly expressed in the carcass. However with JH treatment (i.e., feeding on JH-treated paper), *Rfest1* expression increased most substantially in the gut. Wheeler et al. (2009) focused on a suite of gut carboxyl-esterases genes including *RfEst1*, and identified the site of expression within the gut to be mostly in the mid and hindgut.

Production of semiochemicals, by social insects is integral for colony survival. In bark beetles, pheromone production was shown to be regulated through an interaction with JHIII and the midgut in male beetles (Keeling et al., 2006). JHIII stimulates the mevalonate pathway gene, *HMG-CoA* reductase (Tillman et al., 2004). Analysis by quantitative real-time PCR (qRT-PCR) of multiple genes in the mevalonate pathway indicated that feeding stimulated the JH and pheromone producing pathway in male bark beetles, and partially in females (Keeling et al., 2004). The identification of multiple JH responsive and JH production/ degradation genes in *R. flavipes* suggests that termites could be producing semiochemicals and communicating through the gut and trophallaxis, which may implicate *RfEst1*. Although *Cyp15F1* was constitutively expressed throughout the termite body, it was slightly higher in the carcass. *Cyp15F1* expression was reduced with JH and JH+SHE one day after treatment, which is similar to the expression pattern observed previously (Chapter 3).

Based on the similar JH and JH+SHE expression patterns, the potential primer pheromones contained in soldier head extracts do not appear to have any effect on the three genes studied here. These results suggest that the synergistic effect combining JH and SHE does not affect these three genes at the Day 1 time point. In previous experiments *Cyp15F1* was

differentially expressed between JH and JH+SHE only ten days after treatment (Chapter 3), thus future studies should examine gene expression changes of these genes at later time points.

Past results have shown that termite esterases can be visualized through native PAGE and naphthyl ester staining (Valles et al., 2001; Wheeler et al., 2009). In the current study native PAGE and esterase staining revealed termite esterases are JH inducible and that one esterase in particular was reduced with targeted *RfEst1* siRNA injection, suggesting the reduced band is the RfEst1 protein. If RfEst1 is a true JH esterase this is the first example of the Native PAGE being used for JHE visualization. Transcript levels were measured by qRT-PCR at multiple days after siRNA injection; however no detectable reduction in transcript level could be detected (Appendix E). These results disagree with native PAGE and esterase staining that clearly showed a reduction in an esterase at Day 5 (Figure 4-4a). It appears that there is some disconnect between transcript and protein levels that are not fully understood. Additional research is needed to further investigate the lack of detectable transcript reduction.

Results also suggest that five days were required before protein expression was reduced after *RfEst1* siRNA injection. A number of past studies using a combination of RNAi and JH have given inconclusive results (Appendix E). A possible reason why phenotypic bioassays and gene expression studies have yet to find any significant RNAi effect could be due to the delayed protein knockdown, and application of JH prior to protein attenuation.

This study investigated the activity and expression of three potential JH production/degradation genes in *R. flavipes*. The goals were to characterize the potential roles of these three genes in JH production and metabolism. Gene sequence, amino acid translation, and alignment with homologs were first completed. Two P450s, *Cyp15F1* and *Cyp15A1*, had significant translated amino acid homology to P450 proteins responsible for JH production

(methyl farnesoate epoxidation). *RfEst1* had significant translated amino acid homology to esterase proteins responsible for JH metabolism (ester hydrolysis). Each gene showed significant 24-hour responses to JH and JH+SHE treatments with an overall reduction in *Cyp15F1* expression, reduced *Cyp15A1* expression in the head, and increased *RfEst1* expression in the gut. Native PAGE and esterase staining revealed that termite esterases are JH inducible and one esterase in particular was reduced with targeted *RfEst1* siRNA injection. Results suggest that *RfEst1* is a JH esterase visible on Native PAGE gels with naphthyl propionate staining. This is potentially the first example of JH esterase identification by Native PAGE. Overall these findings suggest that these three genes play a role in JH production and degradation in *R. flavipes*, which potentially could have a direct impact on termite caste differentiation.

Table 4-1. Cyp15 signature motifs

			Motifs				
	S.P.	PGPP hinge	1	2	3	4	5
		PGPP	WxxxR	GxE/DTT/S	ExLR	PxxFxPE/DRF	PFxxGxRxCxG/A
Cyp15F1	Yes	PPGP	WVEQP	GGETMA	ELIR	PEVFRPERF	PFGAGKRVCIG
Cyp15A1	No	No	WQEQR	GSETTS	EVQR	PEVFRPDRF	PFGFGKRRCLG

Table 4-2. Day 0 Tissue distribution of three potential JH production / degradation protein coding genes.

		Tissue (±std. error)		
Gene		Head	Gut	Carcass
Cyp15F1	Average Δct	-0.06 (0.21) ab	0.62 (0.22) a	-0.61 (0.17) b
	Average ΔΔct	2.00 (0.72)	1.00 (0.00)	2.2 (0.55)
Cyp15A1	Average Δct	4.12 (0.15) b	7.57 (0.09) a	7.28 (0.27) a
	Average ΔΔct	11.34 (1.19)	1.00 (0.00)	1.39 (0.30)
RfEst1	Average Δct	3.09 (0.10) a	3.33 (0.24) a	2.33 (0.19) b
	Average ΔΔct	1.25 (0.17)	1.00 (0.00)	2.24 (0.48)

Data points within row with the same letter are not significantly different by ANOVA tukeys (*Cyp15F1* d.f.=3,17 F=5.7027, P<0.0091, treatment d.f.=2 F=8.5469, P<0.0038; *Cyp15A1* d.f.=3,17 F=75.5942, P<0.0001, treatment d.f.=2 F=112.41, P<0.0001; *RfEst1* d.f.=3,17 F=5.5295, P<0.0102, treatment d.f.=2 F=7.7793, P<0.0053).

Δct= the ct values normalized to the control gene *Stero-1* (Chapter 3)
ΔΔct= the ct values normalized to first the control gene *Stero-1* and then to the body region with the lowest level of expression.

Table 4-3. ANOVA table for gene localization.

Gene	Location					Gene	Location					Gene	Location				
Cyp15F1	Head	Source	DF	F ratio	p value	Cyp15F1	Gut	Source	DF	F ratio	p value	Cyp15F1	Carcass	Source	DF	F ratio	p value
		Whole model	5	2.190	0.123			Whole mode	5	6.131	0.005			Whole mode	5	20.613	<.0001
		Treatment	2	3.635	0.058			Treatment	2	11.345	0.002			Treatment	2	46.145	<.0001
		Colony	1	2.719	0.125			Colony	1	6.468	0.026			Colony	1	0.132	0.723
		Treatment*Colony	2	0.483	0.628			Treatment*C	2	0.748	0.494			Treatment*C	2	5.321	0.022
		Error	12					Error	12					Error	12		
		Total	17					Total	17					Total	17		
Gene	Location					Gene	Location					Gene	Location				
Cyp15A1	Head	Source	DF	F ratio	p value	Cyp15A1	Gut	Source	DF	F ratio	p value	Cyp15A1	Carcass	Source	DF	F ratio	p value
		Whole model	5	26.510	<.0001			Whole mode	5	1.003	0.457			Whole mode	5	2.305	0.110
		Treatment	2	58.883	<.0001			Treatment	2	0.880	0.440			Treatment	2	0.891	0.436
		Colony	1	2.377	0.149			Colony	1	1.857	0.198			Colony	1	8.117	0.015
		Treatment*Colony	2	6.203	0.014			Treatment*C	2	0.698	0.517			Treatment*C	2	0.814	0.466
		Error	12					Error	12					Error	12		
		Total	17					Total	17					Total	17		
Gene	Location					Gene	Location					Gene	Location				
RfEst1	Head	Source	DF	F ratio	p value	RfEst1	Gut	Source	DF	F ratio	p value	RfEst1	Carcass	Source	DF	F ratio	p value
		Whole model	5	1.721	0.204			Whole mode	5	4.052	0.022			Whole mode	5	1.845	0.178
		Treatment	2	1.251	0.321			Treatment	2	6.414	0.013			Treatment	2	2.539	0.120
		Colony	1	3.811	0.075			Colony	1	3.500	0.086			Colony	1	0.139	0.716
		Treatment*Colony	2	1.146	0.350			Treatment*C	2	1.967	0.182			Treatment*C	2	2.005	0.177
		Error	12					Error	12					Error	12		
		Total	17					Total	17					Total	17		

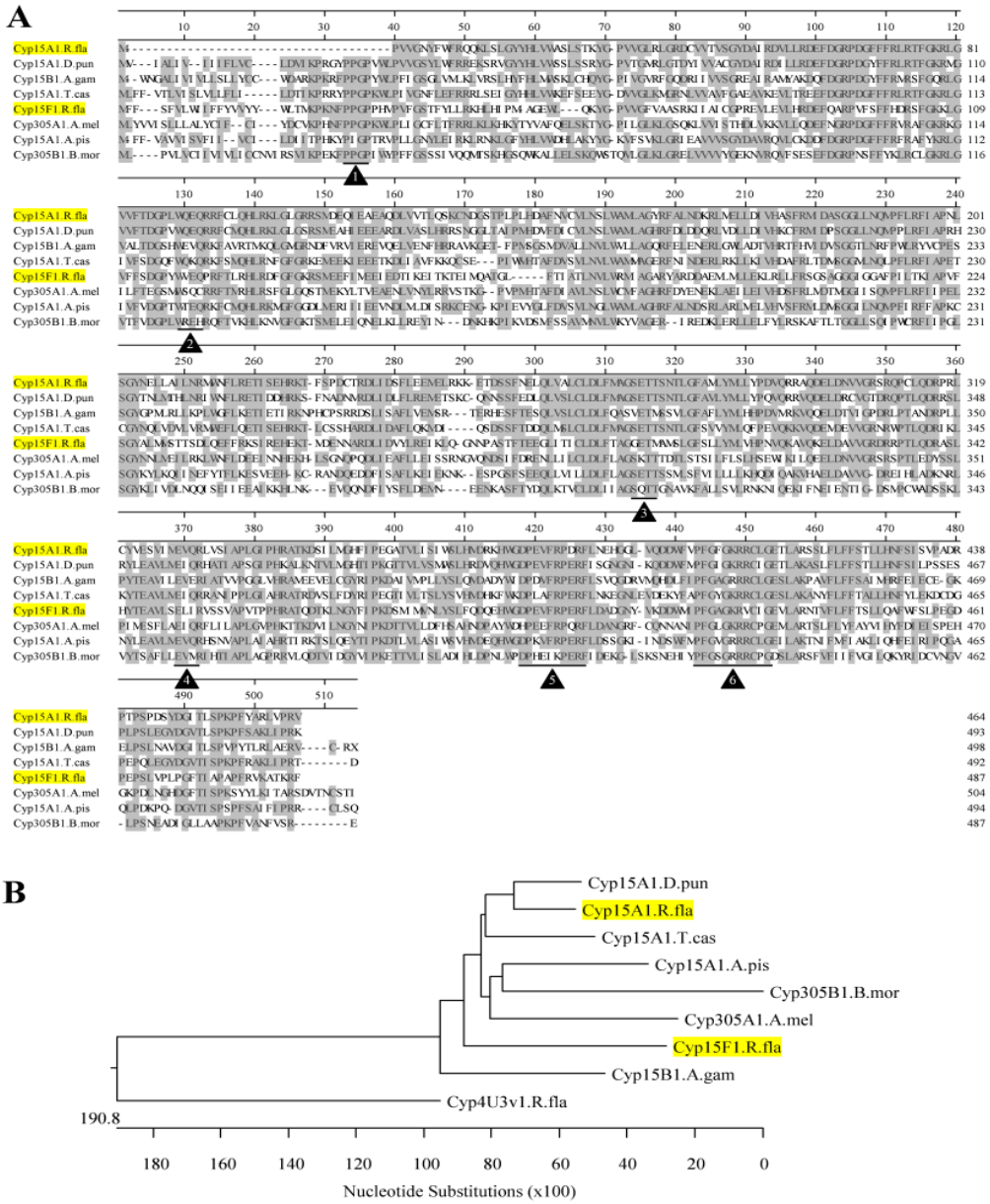

Figure 4-1. *Cyp15F1* and *Cyp15A1* sequences**.** (A) Clustal W alignments of translated amino acid sequences of Cyp15F1 and Cyp15A1 with other translated insect cytochrome P450s. Triangles (▲) indicate conserved P450 motifs. Shaded resides indicate shared amino acids. Species abbreviations are as follows: R. flav, *R. flavipes*; D. pun, *D. punctata*; A. gam, *A. gambiae*; T. cas, *T. castaneum*; A. mel, *A. mellifera*; A. pis, *A. pisum*; B. mor, *B. mori*. (B) Cyp15F1 and Cyp15A1 and related sequences aligned by Clustal W, and tree generated by Clustal W, with an out group of *R. flavipes Cyp4U3v1*.

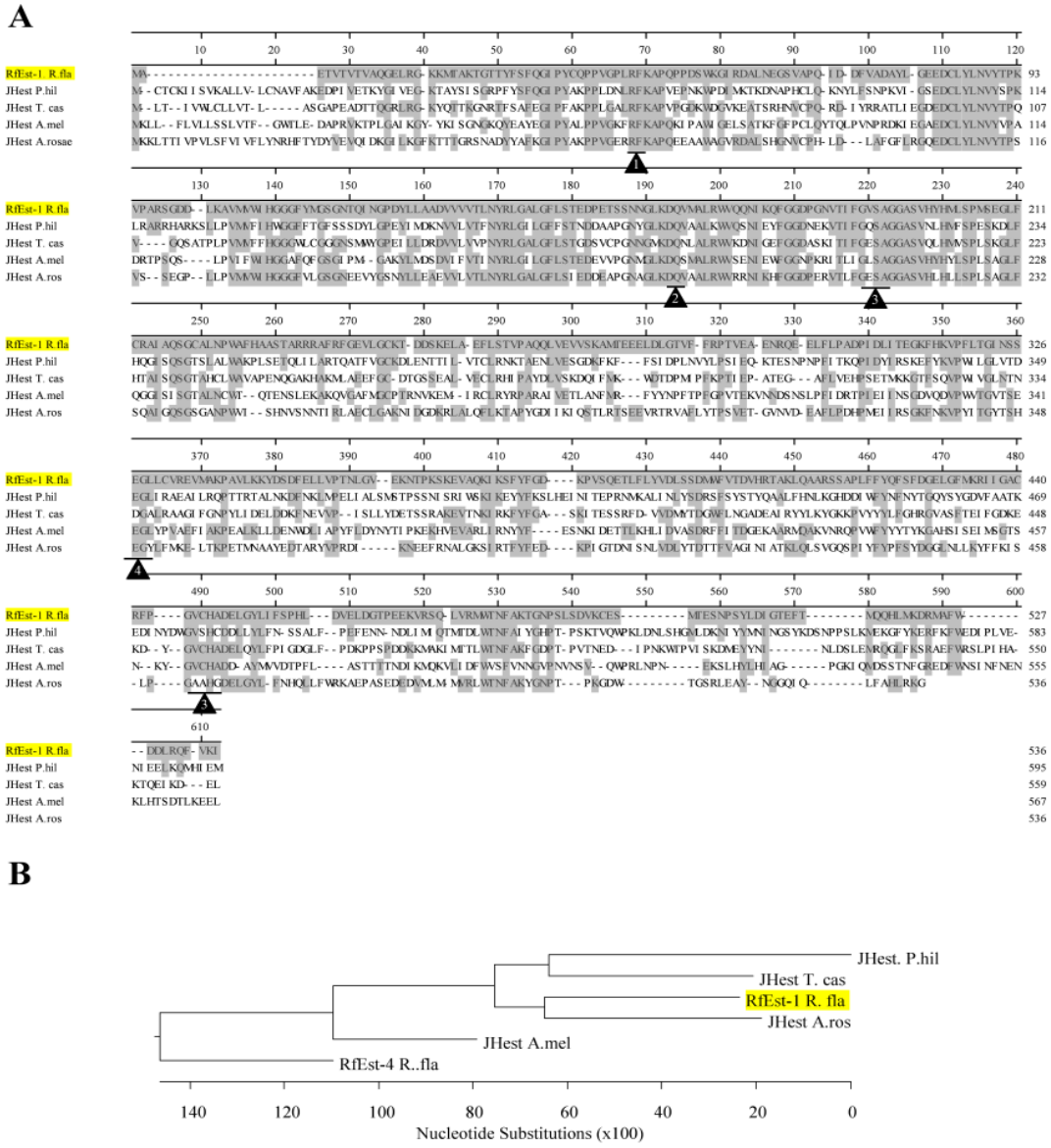

Figure 4-2. *RfEst1* sequences (A) Clustal W alignments of translated amino acid sequences for *R. flavipes* esterases RfEst1 with other insect JHE (juvenile hormone exterase)-like esterases. Triangles (▲) indicate conserved esterase catalytic site and substrate recognition motifs. Shaded residues are identical to the RfEst1 sequence. Additional species abbreviations not listed in Figure 1 are as follows: P.hil, *P. hilaris*; A. ros, *A. rosae*. (B) RfEst1 and related sequences aligned by Clustal W, and tree generated by Clustal W, with an out group of *R. flavipes* Rfest4.

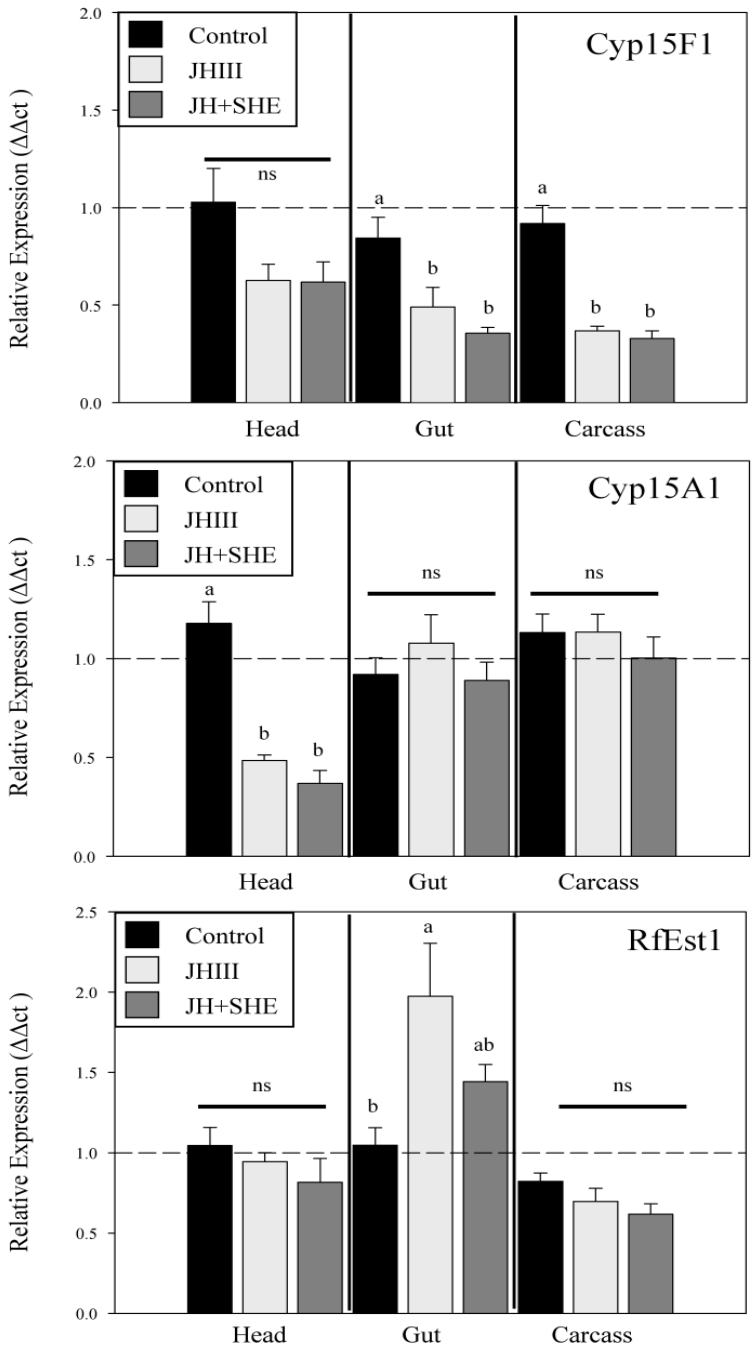

Figure 4-3. Analysis of gene expression in *R. flavipes* worker body regions for the three target genes, *Cyp15F1*, *Cyp15A1*, and *RfEst1*. CT expression values for target genes were normalized to the CT values from the reference genes and then to the target gene on Day 0 control (ΔΔCT). A two-way ANOVA (excluding Day 0), with adjusted LS means was used to separate significant genes using JMP statistical software (SAS Institute, Cary, NC, USA) (Table 4-3). Tukeys HSD tests were used for separating means by treatment for each body region.

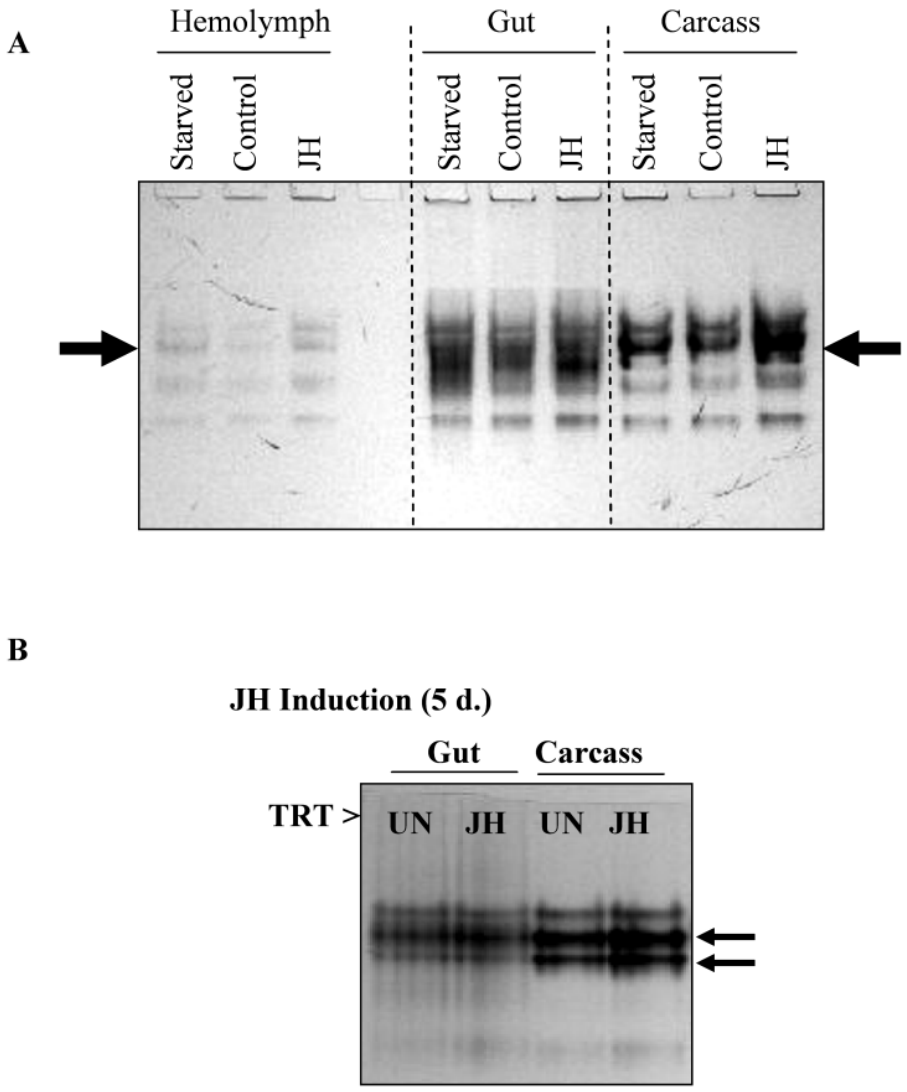

Figure 4-4. JH induction of *RfEst1* (A) Native PAGE gel showing esterase activity towards α-naphthyl propionate in *R. flavipes* worker hemolymph, gut and carcass, each for starved, control and JHIII treatment. (B) Native PAGE gel showing esterase activity towards α-naphthyl propionate in the gut and carcass for untreated vs. JHIII treated *R. flavipes* workers.

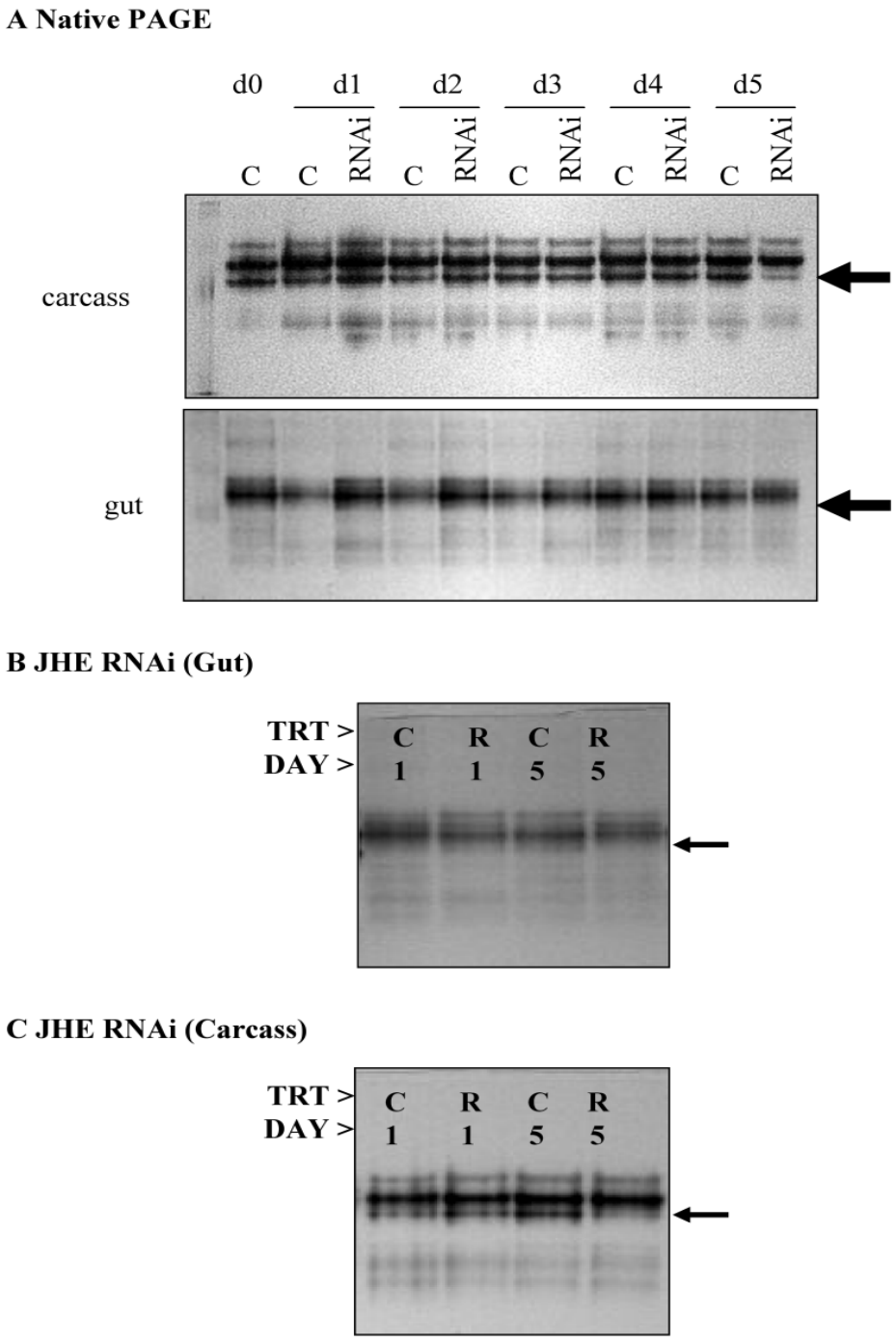

Figure 4-5. *RfEst1* Native PAGE (A) Gel showing esterase activity towards α-naphthyl propionate in *R. flavipes* worker carcass and gut over five days comparing control vs. RfEst1 siRNAi injections. (B,C) Side by side comparison of control vs RfEst1 siRNAi five days after treatment, showing a reduction in a single esterase band in both gut and carcass.

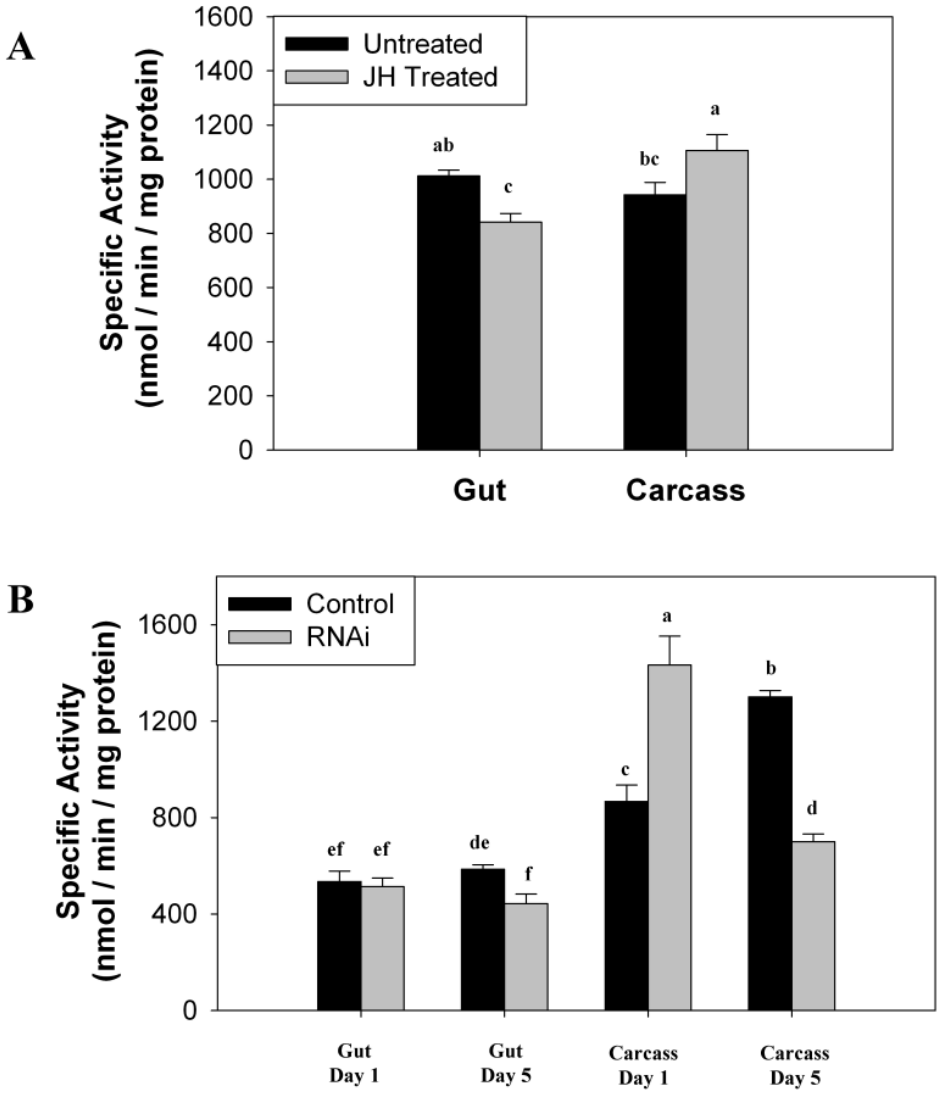

Figure 4-6. Colorimetric esterase assays. (A) Colorimetric esterase assays with α-naphthyl propionate comparing control vs. JHIII treatment in gut and carcass fractions. Bars with the same letter are not significantly different $p<0.05$. (B) Microplate esterase assays with α-naphthyl propionate comparing Days 1 and Days 5 gut and carcass, control vs. *RfEst1* siRNAi injected *R. flavipes* worker termites. Bars with the same letter are not significantly different $p<0.05$.

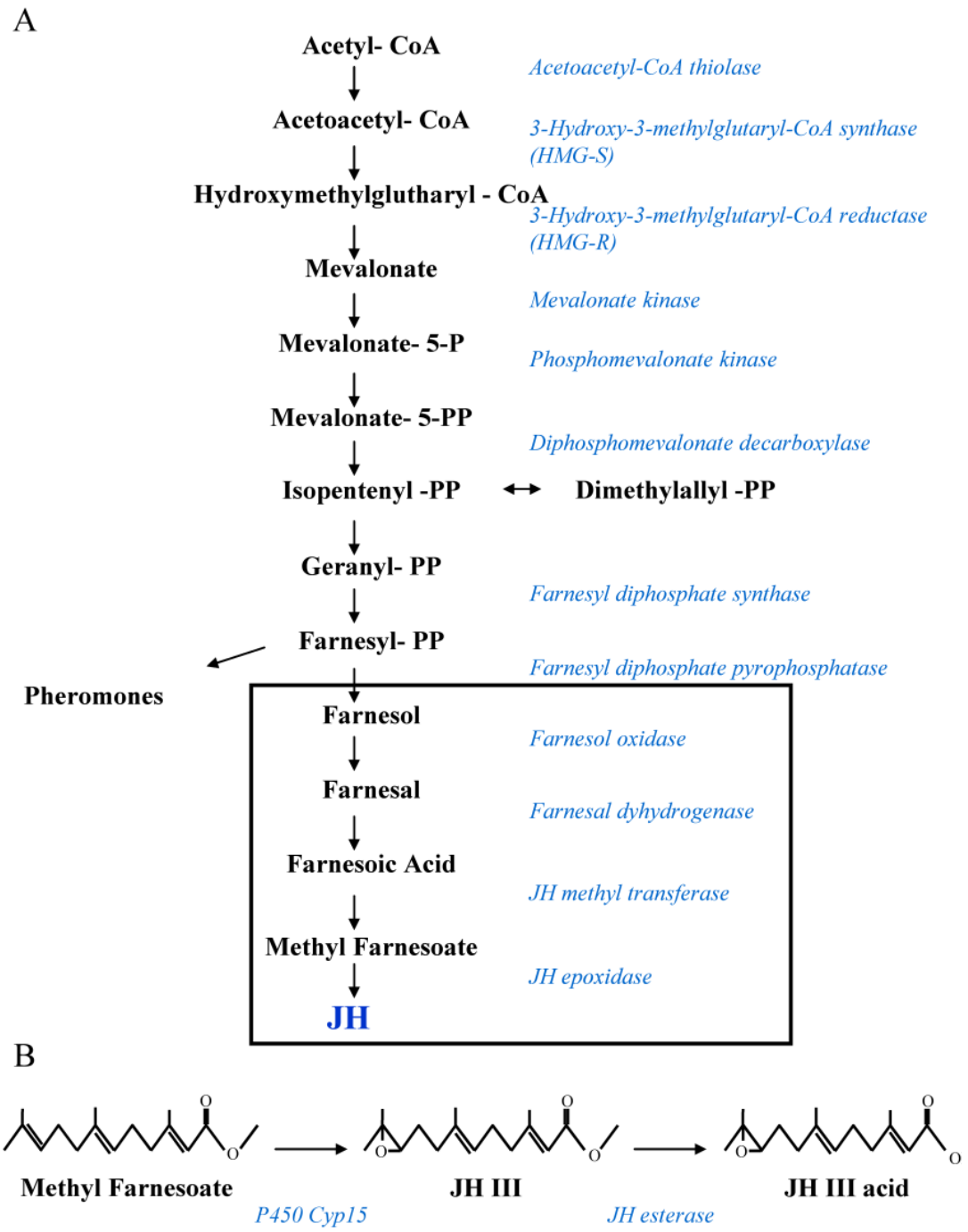

Figure 4-7. JH production and degradation (A) Diagram summarizing juvenile hormone production through the mevalonate pathway. (B) Conversion of methyl farnesoate to juvenile hormone III through a P450 and the metabolism of JHIII to JH acid by a JH esterase.

CHAPTER 5
OVERALL CONCLUSIONS

Conclusions

The broad goal of this research was to investigate a potential impact that soldier termites may have on nestmate caste differentiation. Specifically, studies were conducted to better understand the influence of soldier head chemicals on nestmate worker caste differentiation. The central hypothesis tested was that the chemicals produced by soldiers influence phenotype and gene expression of worker termites, and that responsive genes that are differentially expressed play a role in caste differentiation or regulation.

The first phase of this dissertation (Chapter 2) was to characterize and identify the phenotypic effect that soldier head extracts (SHE) have on worker caste differentiation. Results showed that soldier head extracts, when combined with JH, synergistically increased worker-to-presoldier (PS) formation in most colonies tested. Through gas chromatography (GC), mass spectrophotometry (MS), and nuclear magnetic resonance (NMR) the two major components of SHE were identified to be γ-cadinene and γ-cadinenal. In addition, other previously identified soldier head terpenes also had synergistic effects when tested in combination with JH.

The first goal of the second phase of this dissertation (Chapter 3) was to test the effects of JH, JH+SHE, SHE, and live soldier treatments concurrently on phenotypic caste differentiation and gene expression of worker termites at 1, 5, and 10 days of exposure. Through use of quantitative real-time PCR I investigated the expression pattern of 47 target genes. I identified 17, 23, and 19 genes with significant differential expression among treatments on days 1, 5, and 10 respectively. The three main groups of genes with significant differential expression were 1) chemical production/degradation protein coding genes, 2) hemolymph protein coding genes, and 3) developmental genes.

The second goal of the second phase of this dissertation was to test the individual effects of the two major components of SHE (γ-cadinene and γ-cadinenal) on phenotypic caste differentiation and gene expression of worker termites at three different days (1, 5, and 10). Phenotypic assays indicated that γ-cadinene was the active component of the SHE blend; whereas γ-cadinenal is apparently inhibitory. Next we monitored the expression of the genes that were significantly differentially expressed between JH and JH+SHE from Phase II Objective #1. Two genes, *βtube* and *myosin* had similar expression patterns between JH+SHE and JH+ γ-cadinene, supporting that γ-cadinene is the active component in the SHE blend. Clearly a larger number of genes need to be tested to better establish γ-cadinene as the active component of the SHE blend.

The last phase of this dissertation (Chapter 4) was conducted to further characterize three genes from the chemical production/ degradation group, identified in Chapter 3 (*Cyp15F1*, *Cyp15A2*, and *RfEst1*). Specifically these genes were chosen for investigation because their expression profiles from Chapter 3 and homology to previously published gene sequences that suggest *Cyp15F1*, *Cyp15A2*, and *RfEst1* have roles in JH production and degradation (Helivg et al., 2004; Mackert et al., 2008), suggesting they would likely have direct impacts on termite caste differentiation. The full length cDNA sequences and amino acid translations were analyzed and compared to their closest homologs. Both Cyp15's (*Cyp15F1*, *Cyp15A1*) have close homology to *Cyp15A1* of the cockroach *Diploptera punctata*, which was found to be responsible for the last epoxidation step in JH biosynthesis. *RfEst1* was found to be closely related to other insect JH esterases responsible for the breakdown of JHIII to JH acid. Finally *RfEst1* was putatively visualized with Native PAGE and esterase staining and was shown to be JH inducible and

silenced with targeted siRNA. Chapter 4 findings are not yet conclusive, but they do provide a solid foundation for additional experiments that are likely to provide conclusive answers.

Hypotheses and Caveats

Soldier Head Extracts

Past research has shown that different termites produce different amounts of terpene chemicals (Zalkow et al., 1981; Prestwich, 1983; Bagneres et al., 1990; Nelson et al., 2001; Quintana et al., 2003, Nelson et al., 2008); however these studies did not assay termites over a period of time to see if levels changed. I hypothesize that the termite soldiers can modulate the amount of terpene chemicals within the colony and therefore regulate the amount of soldiers within the colony. For example if additional soldiers are needed within the colony soldiers release γ-cadinene, causing additional soldiers to be formed. If there is an excess of soldiers within the colony the soldiers can inhibit additional soldiers by the primer pheromone γ-cadinenal.

JH Effects on Caste Differentiation

Over the last four years of my research I have noticed a fluctuation in the potency of commercially available JHIII in its ability to force the worker-to-soldier differentiation. Possible reasons for this include; 1) the quality and purity of the JHIII we obtain from Sigma-Alrich, 2) seasonal fluctuation of colony caste composition, 3) colony variation due to sampling (i.e. removing soldiers for SHE preparation disrupts the colony's endocrine balance).

Howard and Haverty, (1981) documented a natural rise in the number of soldiers during the early spring. This natural rise in soldier numbers coincides with alate production for swarming. The rise in soldiers was always hypothesized to be for the protection of the alates as they leave the colony. I believe that alate protection could be part of the reason for an increase in soldier numbers, but I feel that the rise in soldiers is needed to instead influence caste

composition, as hypothesized by Henderson (1998). Henderson (1998) hypothesized that the soldier caste was used as a sink for absorbing JH from the colony so that alates can form, therefore influencing nestmate caste differentiation. Mao et al. (2005) monitored JH levels and soldier formation of workers held with different number of soldiers. They found that soldier formation increased with lower initial soldier proportions (same as Appendix C) and found that JH levels were higher in those individuals, as compared to workers held with higher numbers of soldiers. They suggested that soldier caste proportions regulate JH levels which directly correlate to caste differentiation. My research directly supports this hypothesis that the soldiers influence nestmate caste differentiation, but through primer pheromones instead of sponging JH.

Gene Silencing Through RNAi

A number of experiments were performed with the goal of silencing gene transcripts through RNAi. Through a large number of attempts, genes, and methods have yet to give clear RNAi effect at the phenotypic level or transcript level (Appendix E). There are three main factors that could explain the lack of a result; 1) termite grouping, 2) termite variability, and 3) RNAi dosage. In my research I monitored groups of fifteen termites for each biological replicate. Perhaps when grouping all fifteen termites together I am missing the RNAi effect; for example the genes are being silenced, but high baseline variation between individuals, thus knockdown affects if any, are not detectible. I have attempted to monitor RNAi effect in individuals, but still I did not see a reduction in transcript level. Also, monitoring individual termites over multiple replicates across colonies is prohibitively expensive. Finally, I have been currently using the RNAi dosage published previously by Zhou et al. (2006a, 2008) and Korb et al. (2009). I hypothesize that each gene is different and would require a different amount of siRNA/dsRNA to obtain knockdown. A dose response to each siRNA fragment would be ideal, but again can be logistically impossible with siRNA injections.

On a positive note, in the final part of Chapter 4 the RfEst1 protein was visualized with native PAGE and esterase staining. The JH inducible esterase was silenced with corresponding *RfEst1* siRNA injections after five days; although no transcript reduction was observed. Results suggest that there is a possible disconnect between transcript and protein levels. A possible reason why I have yet to find any significant RNAi effect could be due to the delayed protein knockdown, and application of JH prior to protein attenuation. Additional research with combined transcript and esterase monitoring needs to be conducted.

Summary

In summary, this research has led to 1) a better understanding of the role termite soldier-derived chemicals play in worker caste differentiation, 2) the impact that JH, soldier head chemicals, JH+soldier head chemicals, and live soldiers have on nestmate gene expression, and 3) a better understanding of the potential function of three specific genes in caste regulation, or the mediation of termite worker-to-soldier caste differentiation. Results from this dissertation provide a better understanding of how soldier termites may manipulate or control caste differentiation, and provide a better understanding of termite biology, insect sociobiology, hemimetabolous insect development, and basic developmental biology. As a result of understanding how soldiers are formed, new and novel control methods of this serious structural pest can eventually be designed. By potentially causing all of the individuals within a treated colony to molt into soldiers, the feeding ability of a colony would clearly be reduced and theoretically the colony would starve. Such a control method would be highly termite-specific and therefore help reduce the current reliance on non-discriminatory pesticides. This dissertation represents a critical step toward development of more environmentally friendly, next-generation termiticides.

APPENDIX A
SOLDIER HEAD EXTRACTS PREPARED IN DICHLOROMETHANE (DCM, $MECL_2$) ALSO SYNERGISTICALLY INCREASE JH-INDUCED PRESOLDIER DIFFERENTIATION BY *R. FLAVIPES* WORKERS.

The overall goal of these experiments was to identify the effects different solvents have on the ability of soldier head extract to synergistically affect JH-induced soldier caste differentiation. Experiments were conducted as a continuation of Chapter 2 using identical materials and methods. Trends shown here using acidic dichloromethane (DCM) as a SHE extraction solvent are identical to trends identified when using acetone as the extraction solvent, as reported in Chapter 2. In initial experiments, DCM was used as an extraction solvent based on three previous studies (Lefevue and Bordereau, 1984; Okot-Kotber et al., 1991; Korb et al., 2003). DCM (Fisher Scientific) was of 99% purity and stored in its original amber glass bottle. The pH of the DCM was determined by mixing 1:1 with distilled water, shaking vigorously, then by measuring the pH of the upper water phase with a pH meter (pH = 4.0).

As shown in Figure A-1, four colonies were compared in their responses to SHE prepared in DCM. On average, presoldier induction significantly increased by 22% when termite workers were co-exposed to SHE + JH III, as compared to treatments of JH III alone ($p<0.05$). Controls treated with either acetone, DCM or SHE alone resulted in no presoldier formation. Presoldiers first appeared between days 10 and 15, and reached maximum levels by day 25 in both SHE + JH III and JH III-alone treatments. All four of the initially tested colonies responded similarly, but with variation. An ANOVA did not detect a colony effect ($p < 0.05$); thus results across colonies were pooled for mean separation testing. On average, treatment of workers with JH III alone resulted in 12.2% presoldier differentiation, whereas combining SHE + JH III treatments led to 34.4% presoldier differentiation ($df= 4,55$, $F=69.3094$, $p<0.05$)

Additional experiments were conducted as described in the Materials and Methods of Chapter 2, except that the JH III and SHE were extracted in different solvents (acetone or DCM) as reported in Table A-1 below. DCM (Fisher Scientific) was of 99% purity and stored in its original amber glass bottle. The pH of the DCM was determined by mixing 1:1 with distilled water, shaking vigorously, then by measuring the pH of the upper water phase with a pH meter (pH = 4.0).

Results show only one incidence of presoldier formation in untreated controls, SHE alone, or LS alone treatments. Since this is the only time that PS formation was observed in a control treatment in this entire dissertation, the 1.3% presoldier formation observed in one control experiment is likely the result of sampling a colony worker that was already becoming a presoldier. JH III diluted in acetone caused on average 25.6% presoldier formation, while presoldier formation was drastically reduced to 0.008% when JH III was in DCM. When JH III in acetone and SHE in either acetone or DCM were combined, 41.4% of workers on average became presoldiers. The combination of live soldiers and JH III in acetone resulted in no reduction in presoldier formation. These results suggest that (1) JH III is not stable in acidic DCM, but (2) SHE is, and (3) that live soldiers have no impact on JH III efficacy in our bioassay system. Also these results suggest that the results generated by past researchers showing SHE inhibited PS formation might be because of the DCM used in SHE extraction. However, as shown in Chapter 3, some SHE components may be inductive and others inhibitory; and thus variation in blend ratios also may dramatically impact soldier formation under bioassay conditions.

Table A-1. Effects of soldier head extraction solvents and live soldiers on juvenile hormone III (JH)-induced termite presoldier induction. Three separate experiments were performed on five different colonies.

	Solvent				% Presoldier Induction
Treatment	**Acetone**	**DCM**	**Experiment**	**Colony**	**(± Std. Error)**
Untreated controls	x		1	1	1.3 (1.3)
	x		1	6	0
	x		2	2	0
	x		3	7	0
	x		3	8	0
	x		3	9	0
JH alone	x		1	1	16.0 (5.0)
	x		2	2	45.3 (13.2)
	x		1	6	21.3 (5.3)
	x		3	7	40 (14.6)
	x		3	8	8.8 (3.7)
	x		3	9	22.2 (5.4)
		x	1	1	0
		x	1	6	1.3 (1.3)
		x	2	2	1.3 (1.3)
SHE alone	x		2	2	0
		x	2	2	0
	x		3	7	0
	x		3	8	0
	x		3	9	0
JH+SHE combinations	JH	SHE	1	1	52.0 (4.9)
	JH	SHE	2	2	25.3 (6.8)
	JH	SHE	1	6	32.0 (15.5)
	JH, SHE		2	2	44.0 (16.9)
	JH,SHE		3	7	86.7 (3.7)
	JH,SHE		3	8	12.2 (4.0)
	JH,SHE		3	9	37.8 (9.8)
		JH,SHE	1	1	6.7 (6.7)
		JH,SHE	1	6	13.3 (14.7)
live soldiers alone	x		2	2	0
	x		3	7	0
	x		3	8	0
	x		3	9	0
live soldiers + JH	x		2	2	34.6 (18.7)

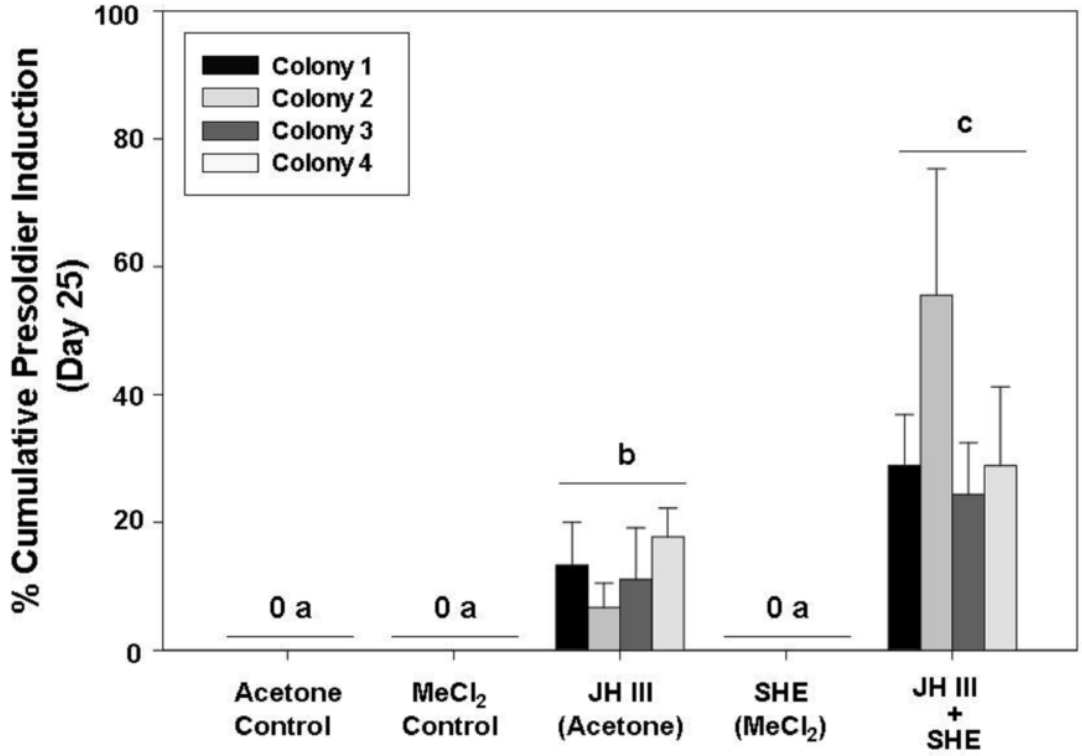

Figure A-1. Effects of soldier head extracts prepared in DCM on four *R. flavipes* colonies. Workers were isolated from colonies and exposed to five different treatments for 25 days. Soldier head extracts (SHE) were obtained by homogenizing soldier heads in methylene chloride (DCM – $MeCl_2$). The graph shows cumulative avg. ± std. error presoldier induction through assay day 25. Groups of bars with different letters indicate significant differences at $p<0.05$.

APPENDIX B
COMPARISON OF MULTIPLE RNA ISOLATION METHODS

Introduction

In order to generate accurate data, choosing the most efficient RNA isolation method that preserves maximal RNA quality is critical. The objective of this investigation was to compare multiple RNA isolation methods to determine the most efficient and effective method for isolation of total RNA from termites. Three isolation methods were examined that included trizol, trizol + DNase, and the Promega SV Total RNA Isolation System. Results were used to determine the best RNA isolation procedure for application in Chapter 3 and 4 experiments.

Materials and Methods

Termites

Workers termites used in these experiments were from a laboratory colony (Colony Y) held at a constant 22 ^{o}C in darkness and provisioned with brown paper towels and pine shims. Colony Y was collected on the University of Florida campus (Gainesville, FL, USA) approximately one year before use (colony started 7/7/06). Termites were identified as *Reticulitermes flavipes* by mitochondrial RFLP-PCR (Szalanski et al., 2003).

RNA Extraction

Total RNA was obtained by using three different methods; each method was tested using two different tissue concentrations (5 and 15 termites). In method #1, total RNA was isolated using trizol (Trisure, Bioline, Randolph, MA, USA) following the manufactures protocol.

In method #2 the method #1 trizol isolation was followed by DNase treatment (Promega, Madison, WI, USA) using the manufacturer's protocol for analyzing samples by gel electrophoresis.

In method #3, RNA was isolated using the Promega SV Total RNA Isolation System (Promega, Madison, WI, USA) following the manufacturer's protocol. Samples with 15 termites were divided into two isolations of 7 and 8 termites. This was done based on previous findings that the Promega SV kit becomes irreversibly plugged when attempting to isolate RNA from all 15 termites at once. *Future experience found that the SV kit can handle 15 termites at once, and that the mixture step of the lysis and ethanol is critical to prevent plugging*.

Sample concentrations were then determined by spectrophotometry (A260 method) using an Eppendorf BiophotometerTM and analyzed on both a 2% agarose gel and a 0.9% formaldehyde-agarose gel.

cDNA Synthesis

RNA samples were transcribed to cDNA *in vitro* using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Equal quantities of 0.5 µg total RNA were added to each of the cDNA reactions based on spectrophotometry readings.

To measure genomic DNA contamination, three RNA conditions were tested per isolation method: 1) trizol 15 termites, 2) trizol+DNase 15 termites, and 3) Promega 15 termites. The RNA was then separated into two aliquots. In the first aliquot 0.5 µg of RNA was treated normally for cDNA conversion. In the second aliquot the reverse transcriptase (RT) was omitted and an additional 1µl of water was added in its place.

Quantitative Real-Time PCR

Using the cDNA prepared above as a template, expression levels for four genes were measured by quantitative real-time PCR (qRT-PCR) using an iCycler iQ real-time PCR detection system with iQ SYBR Green Supermix (Bio-Rad). Ct values were determined for the four genes, Nicotinamide adenine dinucleotide-dehydrogenase (*NADH-dh*) (BQ788175), *B-actin*

(DQ206832), heat shock protein (*HSP-70*) (BQ788164), and hexamerin-1 (*Hex-1*) (AY572858). Ct values were used for analysis.

Results and Discussion

Three different RNA isolation methods (trizol, trizol+DNase, Promega SV) were tested using two different termite quantities per method (5 and 15 termites). Trizol isolation provided the greatest concentration and elution volume of total RNA followed by the Promega SV and Trizol +DNase methods (Table B-1). The trizol extraction alone does not remove trace amounts of genomic DNA, therefore a DNase step must be added to remove any leftover DNA. However, the additional DNase step results in a lower RNA yield. The Promega SV kit protocol includes a DNase step.

All samples with 15 termites provided more RNA than samples with only 5 termites. However, the Promega SV kit will can not handle 15 termites, so preparations must be divided into two samples and combined on the last elution step (*Later experience found the columns to be able to handled 15 termites. Fifteen termites per preparation were used in Chapter 3 and 4 experiments*).

RNA samples were viewed on both agarose and formaldehyde-agarose gels (Figure B-1). The RNA samples were equally visible on both gels indicating that the extra steps in running formaldehyde-agarose specific RNA gels are unnecessary. In fact, bands were less diffused on regular agarose gels.

After isolation, the RNA samples were transcribed *in vitro* to cDNA and tested in qRT-PCR reactions that amplified four separate genes (Figure B-2). Resulting Ct values indicated a difference between extraction methods with the trizol DNase extraction of 15 termites providing the lowest overall Ct values. Equal amounts of cDNA were added to each reaction so variations

in gene expression could be because of error in cDNA degradation, spectrophotometry readings, pipetting error, or contamination from genomic DNA.

RNA samples were then tested for genomic DNA contamination. By leaving reverse transcriptase out of cDNA synthesis reactions, the only thing that should amplify in PCR reactions would be contaminating genomic DNA. Ct values of treatments with and without reverse transcriptase suggested genomic DNA contamination with all three RNA isolation methods (Figure B-3). As seen earlier, all three methods provided similar trends in the data, but different absolute values. All RT treated samples were significantly different than the non-RT treated samples by pair-wise t-tests. *Nadh* RT and non-RT had the closest Ct values, followed by *β-actin*, *Hsp*, and *Hex-1*. The SV Promega kit provided the lowest overall p-values when comparing RT and non-RT treatments. These results suggest that the SV Promega isolation method has the least amount of genomic DNA contamination.

The overall goal of this study was to determine which RNA extraction method to use for the rest of my PhD thesis experiments (Chapters 3 and 4). From the results presented here, the Promega Total RNA SV kit provides the largest amount of RNA in the shortest amount of time with the smallest amount of genomic DNA contamination. As a result, the Promega SV Total RNA Isolation System was used for RNA isolations in Chapter 3 and 4 gene expression studies.

Table B-1. Spectrophotometer measurements of each of the RNA isolations

Treatment	# Termites	Sample*	A260	A260/280	Con (ng/µl)	Elution (µl)	RNA (µg)
Triazol	5	1	0.132	1.38	132.4	48	6.36
Triazol	5	2	0.103	1.49	102.6	48	4.92
Triazol	15	3	0.567	1.49	566.5	48	27.19
Triazol	15	4	0.786	1.58	786.4	48	37.74
Promega SV	5	5	0.048	1.91	48.3	75	3.60
Promega SV	15	6	0.083	1.69	83.0	150	12.45
Triazol Dnase	5	2a	0.038	1.75	37.5	23	0.87
Triazol Dnase	15	3a	0.034	1.82	33.8	23	0.78

* Lane positions on Figure B-1

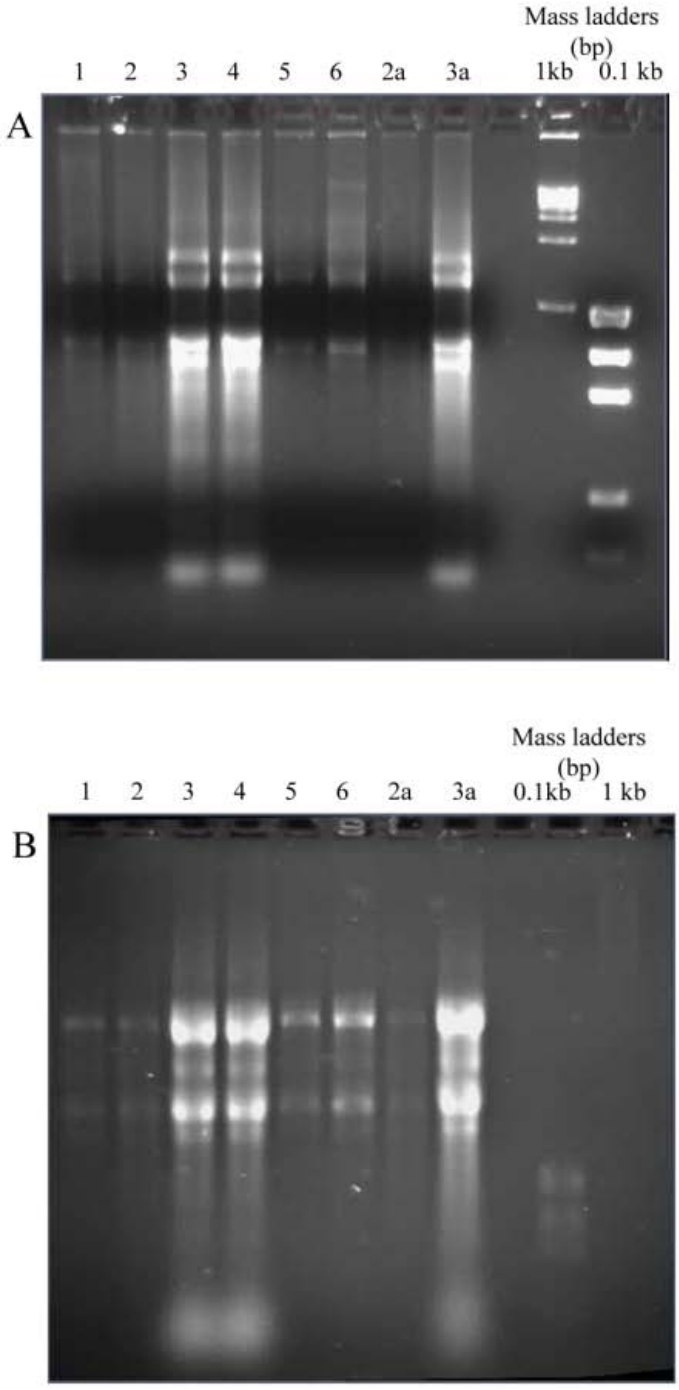

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Figure B-1. RNA samples viewed on both A) 2% agarose and B) 0.9% formaldehyde-agarose gels. Three µl of sample + 2µl of loading buffer were loaded into each lane. Lanes were loaded as follows: 1) trizol 5 termites, 2) trizol 5 termites, 3) trizol 15 termites, 4) trizol 15 termites, 5) Promega SV 5 termites, 6) Promega SV 15 termites, 2a) trizol+DNase 5 termites, 3a) trizol+DNase 15 termites, 1µl 1kb ladder, and 2.5 µl 0.1 kb ladder

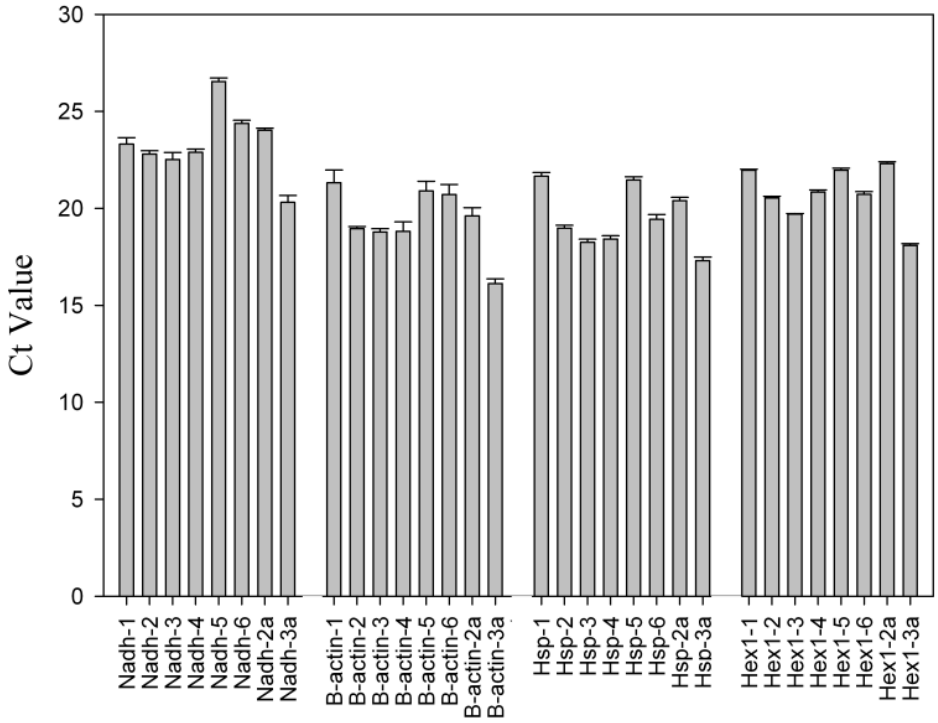

Figure B-2. Comparisons of gene expression levels of for four genes in *R. flavipes* workers determined from RNA isolation performed by different methods. RNA isolation treatments were as follows: 1) trizol 5 termites, 2) trizol 5 termites, 3) trizol 15 termites, 4) trizol 15 termites, 5) Promega SV 5 termites, 6) Promega SV 15 termites, 2a) trizol+DNase 5 termites, 3a) trizol+DNase 15 termites.

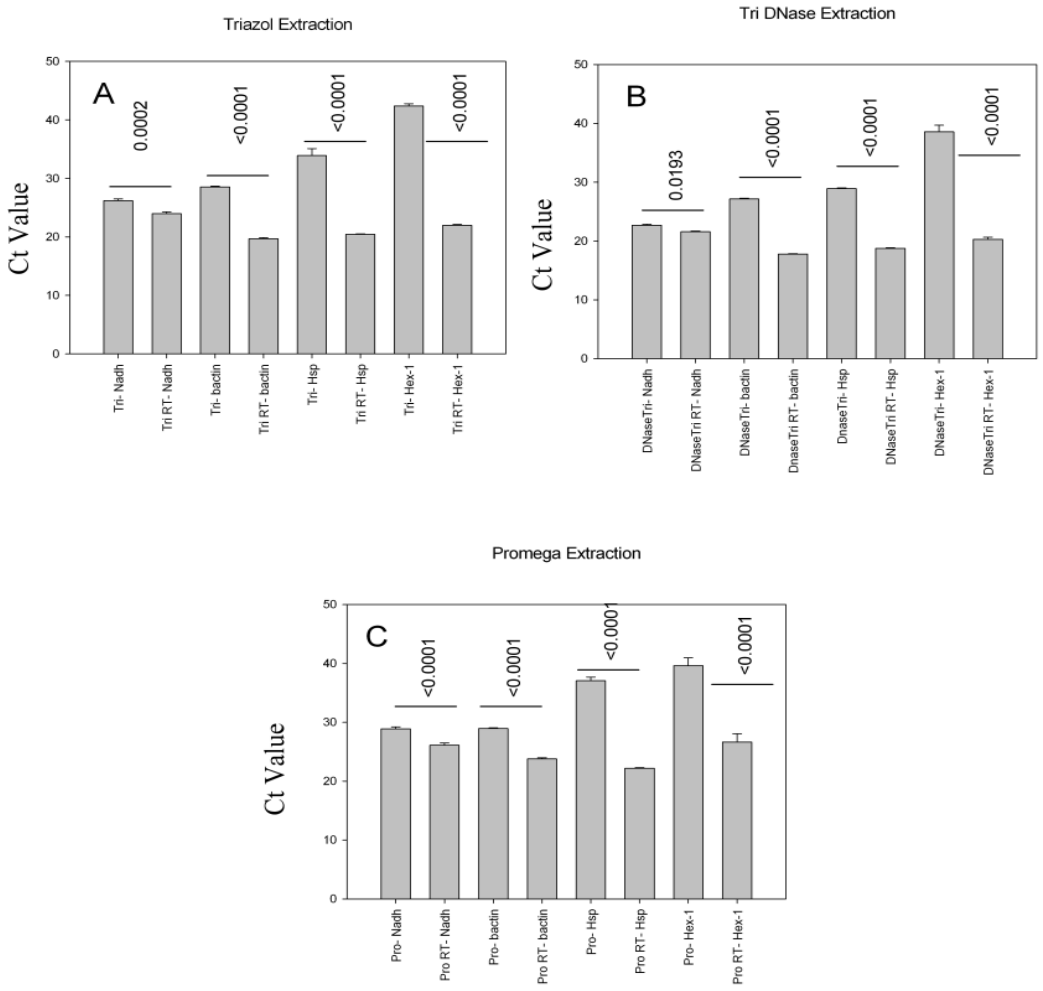

Figure B-3. Analysis of potential genomic DNA contamination in RNA preparations. Gene expression for four genes in *R. flavipes* workers from cDNA made from RNA with reverse transcriptase (RT) or without RT. By performing RT reactions without RT, no cDNA should be made; thus, any resulting PCR amplification should be from genomic DNA contamination. Three different RNA isolation methods were tested trizol, trizol+DNase, and Promega SV. ANOVA's between treatments within genes indicated a significant model effect ($p<0.0001$). Pair-wise t-test p-values between non-RT and RT treatments are listed.

APPENDIX C
SEQUENCES AND PHYLOGENETIC ANALYSES OF TERMITE GENES

Introduction

The following section is a collection of the gene sequences that I have obtained throughout this dissertation. The identity of these genes had to be verified through sequencing. Genbank accession numbers for corresponding genes are listed next to the gene name, where available. Sequences are listed in the forward direction (Sp6 sequenced from a vector) except when sequenced from the reverse direction, indicated by an "r", or "T7."

- Section 1- The first section lists 16s mitochondrial gene sequences corresponding to the termite colonies used in this dissertation. These sequences were used for species identification (Szalanski et al., 2003).
- Section 2- The next section reports the Hexamerin -2 sequences from eight different colonies. This analysis was used as a secondary method to assess relatedness of termite colonies.
- Section 3-Sequences of qRT-PCR products that correspond to EST sequences identified by Tartar et al. (2009). These genes were studied in Chapters 3 and 4.
- Section 4,5- Sequences from library clones identified from Tartar et al. (2009). These genes were targeted in Chapters 3 and 4.
- Section 6- Cloned high throughput array gene sequences used to make the macro arrays. Some of these genes were also targeted in Chapters 3 and 4.

Materials and Methods

Sequences from Section 1 were obtained by the following protocol. Genomic DNA was extracted using QuickExtractTM (Epicentre, Madison, WI) following the manufacturer's protocol. Termite genomic DNA was used as a template for PCR following the protocol of Szalanski et al. (2003) using SyberGreen PCR Master Mix (2xSensiMix Plus SYBR & Flourescein (Quantace, Norwood, MA)). PCR products were then purified by sodium acetate/ ethanol precipitation and viewed on agarose gels. *16s* bands were gel-extracted using the QIAquick gel extraction kit (Qiagen Valencia, CA) following the manufacturer's protocol.

Samples were submitted for sequencing at the UF-ICBR Genomics Core. All resulting 16s sequences were compared by BlastN against the NCBI nr database to confirm species identity. Resulting *16s* sequences were also trimmed, aligned, and used to generate phylogenetic trees within MegAlign Clustal W in the Lasergene software package.

Sequences from Section 2 and 3 were obtained by the following protocol. cDNA was reverse transcribed from total RNA as described in Chapter 3. PCR reactions were performed using SyberGreen PCR Master Mix with gene specific primers (Chapter 3, Table 3-1). PCR products were visualized on agarose gels and gel-extracted using the QIAquick gel extraction kit (Qiagen). Samples were submitted for sequencing at the ICBR Genomics Core. Section 2 sequences were trimmed, aligned, and used to generate phylogenetic trees within MegAlign Clustal W in the Lasergene software package.

Sequences from Section 4 and 5 were obtained by the following protocol. Select clones identified by Tartar et al. (2009) from a termite gut cDNA library were picked and grown up overnight at 37 °C in 3ml LB broth containing 3µl of (40 µg/ml) chloramphenicol. Samples were purified using the QIAprep Miniprep Kit (Qiagen Valencia, CA) following the manufacturer's protocol. Samples were submitted for sequencing at the UF-ICBR Genomics Core.

Sequences from Section 6 were obtained by the following protocol. First, cDNA was reverse transcribed from total RNA as described in Chapter 3. PCR reactions were performed using SyberGreen PCR Master Mix with gene specific primers (Chapter 3, Table 3-1). PCR products were then visualized on agarose gels and gel-extracted using the QIAquick gel extraction kit. Products were ligated overnight into the pGEM®-T Easy Vector (Cat.# A1360) (Promega, Madison, WI) following the manufacturer's protocol. Plasmids were transformed into JM109 competent cells (Cat.# L2001) (Promega) and plated on LB agar plates treated with Xgal,

IPTG, and ampicillin. After growing overnight at 37 °C, colonies were picked and colony PCR was performed to confirm positive insertion of the fragment, (gene specific primers are listed in Chapter 3, Table 3-1). Positive clones were picked and grown up overnight in 3ml LB with 100 µg/ml ampicillin at 37 °C with shaking. Glycerol stocks were made for a portion of each positive clone by adding 875 µl of LB liquid culture to 125 µl of sterile glycerol and frozen at -80 °C. Plasmid DNA was purified from the remaining using the QIAprep Miniprep Kit (Qiagen, Valencia, CA) following manufacturer's protocol. Samples were submitted for sequencing at the UF-ICBR Genomics Core.

Results and Discussion

Alignments and phylogenetic trees of *16s* mitochondrial gene sequences (Figure C-1,2) indicated the presence of genetic differences between the 15 termite colonies and one out group that were sequenced. Most of the *R. flavipes* colonies grouped together with the *Coptotermes formosanus* separating the *R. virginicus* samples. Interestingly colony K2 *16s* sequence was highly different from the other colonies. In Chapter 3 K2 colony (Colony 2) showed a reduced response to JH and JH+SHE compared to the two other colonies tested. Perhaps this result was because of the difference in relatedness.

Alignments and phylogenetic trees of *Hex-2* gene sequences (Figure C-3) revealed that there were minor nucleotide substitutions between the eight colonies tested, but larger differences between *R. flavipes* and *B.discoidalis* sequences. These results further support that genetic differences exist between termite colonies used in this research.

Overall, sequence variations could be indicative of genetic differences between termite colonies, and could be associated with variability observed between colonies in phenotypic and gene expression assays.

SECTION 1: 16s rDNA Sequences

>A8-16S- FJ265705
AACGAATATCTTACATCCAAATAAATGGCTCAGCAAATATAAATAAATATAACAACACAAAGGAGGG
GTTAAATAATATCCCTCCCATCACCCCAACAAAACATATTTGACAGCCCTACTGAACCCTCACAAACA
GAAAGACACCATACAAAATG

>A8-16S-R
CTTCCCCTAGTTTTTGAGTATGGCCTGCCCCTGACCTTGAATGTTGAAGGGCCGCGGTATTTTGACCGT
GCAAAGGTAGCATAGTCATTAGTTCTTTAATTGTGATCTGGTATGAATGGCTTGACGAGGCATAAGCT
GTCTTAATTTTGAATTGTTTATTGAATTTGGTCTTTGAGTTAAAATTCTTAGATGTTTTTATGGGACGAG
AAGACCCTATACAGTTTGGCATTTATTATGGTCTCTTTCTGTTTGTGAGGGTTCACCATGGCTGCCAAA
TC

>BI1-16S
CCGCATATACTCATCAAAAAATGGTTCAGCAAATATAAATAAATATAACAACACAAAGGAGGGGTTA
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GAGACCATAATAAATGTCAAACTCTATAGGGTCTTCTCGTCCCATAAAAACATCTAAGAATTTTAACTC
AAAGACCAAATTCAATAAACAATTCAAAATTAAGACAGCTCATGCCTCGTCAAGCCATTCATACCAGA
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AAGTCAGTGGGCAGGCCATACTTCAAAAACTAACGAGAAGAGATGTTTTTGATAAACAGGCGA

>GB1-16S- FJ265704
GTCACGGGGCTGGAGTTATATTGGGTCTGTTCGACCTTTAAAATCTTACATGATCTGAGTTCAAACCGG
CGTTCCTTGTGTAAC

>GB-16S-R
CCTTTCTAGTTTTTGAGTATGGCCTGCCCCTGACCTTGAATGTTAGAAGGGCCGCGGTATTTTGACCGT
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GCTTGATCCATTTATTTTGATTGTAAGATTAAATTACCTTAGGGATAACAGCGT

>GB-16S
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AAGAACAAATTCA

>IN-16S
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AAAGAGACCATAATAAATGTCAAACTCTATAGGGTCTTCTCGTCCCATAAAAACATCTAAGAATTTTA
ACTCAAAGACCAAATTCAATAAACAATTCAAAATTAAGACAGCTTATGCCTCGTCAAGCCATTCATAC
CAGATCACAATTAAAGAACTAATGACTATGCTACCTTTGCACGGTCAAAATACCGCGGCCCTTCAACA
TTCAAGGTCAGTGGGCAGGCCATA

>IN-16S-R
ACTCCTCTAGTTTTTGAGTATGGCCTGCCCCTGACCTTGAATGTTGAAGGGCCGCGGTATTTTGACCGT
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>K2-16S- FJ627943
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AGAGACCATAATAAATGTAAAACTCTATAGGGTCTTCTCGTCCCATAAAAACATCTAAGAATTTTAAC
TCAAAGACCAAATTCAATAAACAATTCAAAATTAAGACAGCTCATGCCTCGTGTGGCCATTCATACCA
GG

>K2-16S-R
CCTACCTTGTTTTTGAGTATGGCCTGCCCACTGACCTTGAATGTTGAAGGGCCGCGGTATTTTGACCGT
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GCTTGATCCATTTATTTTGATTGTAAGATTAAA

>NA-16S
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GACCATAATAAATGTAA

>NA-16S-R
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GTCTTAATTTTGAATTGTTTATTGAATTTGGTCTTTGAGTTAAAATTCTTAGATGTTTTTATGGGACGAG
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GCTTGATCCAA

>K4-16S
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GCGTAACCACACT

>K5-6S- GQ403073
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>K6-16S- GQ403074
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ACC

>NA_5-16S
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>NA6-16S
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>REGGIEIII-16S
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>REGGIEIII-16S-R
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>TR-16S
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>TR-16S-R
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>W10-16S
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>W11-16S
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>W18-16S
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AATTAAAGAACTAATGACTATGCTACCTTTGCACGGTCAAAATACCGCGGCCCTTCAACACCAAAGTC
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>W27-5-16S
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>Y-16S
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>Y-16S-R
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AAGACCCTATAGAGTTTTACATTTATTATGGTCTCTTTATGTTTGTGAGGGTTCACTAGGGCTGATTAA
TA

SECTION 2- Hexamerin-2 Sequences

>A8-HEX-2
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CAACCGTCGCGGTGAGCAGTTCTACTACAAAATCCAGCAGGTCCTACCTG

>A8-HEX2-R
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>GB-HEX-2
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>GB-HEX2-R
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>K2-HEX-2
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CGTCGCGGTGAGCAGTTCTACTACAAAACCCAGCAGGTCCTAAAAC

>K2-HEX2-R
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TGTAGTAGTAGTAGAGGTAGTACGTGCTGAGTCCAACGTCTTCCGTAACAG

>NA-HEX-2
CAGACCTCCTACCTCTATACTACTACAACTACCCCCGTTCTTCACAGCACCGATTACGGCGTTAATTTC
AACCGTCGCGGTGAGCAGTTCTACTACAAAACCCAGCAGGTCCTAAGCTTG

>NA-HEX2-R
TATGGCAGTACCGCTCCGCGCGGTCGAAAGAACGCCGTACTCGGTGCTGTTGAAGAACGTGGGGTAGT
TGTAGTAGTAGTAGAGGTAGTACGTGCTGAGTCCAACGTCTTCCGTAACCTCA

>IN-HEX-2
GGCAGCACTAATACCTCTATACTACTACAACTACCCCCGTTCTTAACAGCACCGATTACGGCGTTAATT
TCGACCGTCGCGGGGAGCAGTTCTACTACAAAACCCGGCAGGTCCTAAGCGGA

>IN-HEX2-R
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TTGTAGTAGTAGTAGAGGTAGTACGTGCTGAGTCCAACGTCTTCCGTAGGCTC

>REGGIE III- HEX-2
GTGAACTCATACCTCTCTACTACTACAACAACCCCCGTTCTTCACAGCACCGAGTACGGCGTTCATTTC
AACCGTCGCGGTGAGCAGTTCTACTACAAAACCCAGCAGGTCCTAAGCC

>REGGIEIII-HEX2-R
CACGGCCAGTACTGCTCCGCGCGGTCGAAAGAACGCCGTACTCGGTGCTGTTGAAGAACGTGGGGTAG
TTGTAGTAGTAGTAGAGGTAGTACGTGCTGAGTCCAACGTCTTCCGT

>TR-HEX-2
CGGAACTAATACCTCTCTACTACTACAACTACCCCACGTTCTTCACAGCACCGAGTACGGCGTTCATTT
CGACCGTCGCGGTGAGCAGTTCTACTACAAAATCCAGCAGGTCCTAACCCGTGGGCCTGGA

>TR-HEX2-R
TTAGGCCAGTGCCTGCTCCGCGCGGTCGAAAGAACGCCGTACTCGGTGCTGTTGAAGAACGTGGGGTA
GTTGTAGTAGTAGTAGAGGTAGTACGTGCTGAGTCCAACGTCTTCCGTA

>Y-HEX-2
GANGACCTCATACTCTATACTACTACAACAACCCCCGTTCTTCACAGCACCGATTACGGCGTTAATTTC
AACCGTCGCGGTGAGCAGTTCTACTACAAAACCCAGCAGGTCCTAA

>Y-HEX2-R
CTGGGCCCAGTACTGCTCCGCGCGGTCGAAAGAACGCCGTACTCGGTGCTGTTGAAGAACGTGGGGTA
GTTGTAGTAGTAGTAGAGGTAGTACGTGCTGAGTCCAACGTCTTCCGTAACTT

SECTION 3- Sequences of qRT-PCR Products That Correspond to ESTs Identified by Tartar et al. (2009)

>CJUN1- ACCESSION NO. FL638224
CTCGCCTGGTGGATAGCCGATTGTAAAAGAACCAACTTCCTGTAATGTCGAGTGCATGACCGGCCCAG
CATACGGAAACGTTGGTGCCTGGTGCGAGGCTAGATAGGCAGCAACTGGCCTTGCCTTCACTTGCGTA
ATTCATCGCCACAACGTCATGTTGCGAACCGGATTAGAACTTTGTTGCCAGCCC

>CJUN-1-R
AACCGTTCGCATGAGTTGTGGCGATGAATTACGCAAGTGAAGGCAAGGCAGTTGCTGCCTATCTAGCC
TCGCACCAGGCACCAACGTTTCCGTATGCTGGGCCGGTCATGCACTCGACATTACAGTGAAGTTTGGTT
TCTTTTACAAATCTGGCTACTCCACACAGTTCAGTATTGAGCATGTCATGGACCCC

>CYP6-2- FL637360
CCCCCTAACGATATTACGACCTTCTTCCGCACGCTTGTGCACGAGACCGTCAAATTCAGGGAGCAACA
TTCCGTAACTCGCAACGACTTCTTGCAACTTCTGATCCGACTCAAGAACAAGGAAAGCCTTGAATCCG
ATACTTCAACCGAAGACTGCGATGGAAACTCTGCTGGTATGACAAGGGCCA

>CYP6-2-R

TTCATCCGAATTTTTGGTTGGAATTTGGGTTCCGGGTTTTCTTGGTTTTGAATCCGAAAAAAGTTGCAA
AAAGTCCTTGCCAATTACCGAATGGTGCCCCCTGGATTTGACGGGTTTCGGCCCCAACCGGGCGAAAA
AGGGCCAAATTTCCTTTGGGGGGGTCCCTTTTCCCAAAAACCTTGGGCCCACCC

>EPOX1- FL640608, FL636393, FL635113
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CTGCATCCTACCAGGATCTTTTGGAGGGTGAAGCGGCGTCAGACAGCTCCTGAGTGCCTCACTGACCC
TGCCTACGGGATCCATGGATATCTGCCAATTGAGGGAGTGA

>EPOX-1-R
TACAGGTCCTGTAGCAGGGTCGTGAGGCACTCAGGAGCTGTCTGACGCCGCTTCACCCTCCAAAAGAT
CCTGGTAGGATGCAGGAGGATGCTGAGACATAGCCGGCCGAAGGCTAGAGAAGCCACAAAGAAAGCT
AC

>FAMET1- FL638251, FL637991
TTCTCGTTGTGAGGGGATTTGAAGTCTATCTTACCAACAGTGGAATACCTACTCCTGTGGCTGACAACG
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TAATTTCCCTGCTGTATAAGTGGTCAAAAAAAT

>FAMET-1-R
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ATATGACTTTCAAATTCCCTCACATACACTACTCTGTGGTTTGCAAAAGTGG

>FAMET2- FL639748, FL638947
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GCTGGGAGAACACAGCATCTGTCATTCGCTACAACCGCCAGAAACCAGACAAGGTTCGGGCAGACAC
GCCTGGACTCTTGACCAACAGTGACTACAGTCGCTTCTTGATAGAGTGGCA

>FAMET-2-R
CAGTATCTGTTGGTAAGGTCCGGCGTGTCTGCCCGAACCTTGTCTGGTTTCTGGCGGTTGTAGCGAATG
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GTTAGAGCAATGTGTGCATTGGAGGGGGCACGAACCTCAATGTGCAGT

>FAMET3- FL636743
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GCCATGTGGCAGACTGCAAGTGAACCCACCTCTAATGCGACCCTGCTGCACTAGGCCGACGAAGGCTA
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>FAMET-3-R
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CGCCGTTGTTGGGAACTTTTTTGGCAGGTAAAATGTCAAACTCATAATACGCCCGTCCAATGTACAGG
GACCCTCCATTTTTGTCGTGTCCTCAAA

>JHEST1- FL636973, FL640151, FL638979- Carbx-1
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GATCTTCGGGCCCGCCCTCGTTCAGGAGGAGGAAAGGAAA

>JHEST1-R
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>JHEST2- FL638686- Carbx-2
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TGAAGAGACACAGCAAGACAAGGACA

>JHEST2-R
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AGTTGACAGCAGCTTGAATTCTGGCA

>LPRS- FL635452,FL636727, FL636380, FL637727, FL636288
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GCAAGGCTCAGCCCTAATGAGACGTCACTGTGCTGTGTCAAGTGGGCAGACTTTAGAGCAAGTGTGCT
CAAGCCCTTTCTGCTGAGGTGCATCGCTCAAAGTGGTA

>LPRS-R
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CTGAGCCTTGCCTAGTTATACATAATCATTTGTTCCAGGACTTGTGAGTGGCTCCTGGGCCTCCTCTCC
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>MEVASE-1- FL639092
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GCAAGGTCATTCTTCACGGTGAGCATTCAGTGGTTCAACC

>MEVASE1-R
TCGTCATCCTTGCCGGTGCTGAACTCTGAGGCTAGAGGATGTTTATGTGGTACATCATGACTGAATACT
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CTGCTTATGTGTACTGTATAGGAACCGTGACCTCAGA

>NADH-DH-F- BQ788175
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TCTCATAACCCCCAAAAAGAGACCCCACACCTCTAGGTATGAATGCCCACCCCAAGCAAC

>NADH-DH-R
CGACTAAAAAGAGAGTAAAAGGCATATAAACAGATAAACCCCCCATAAAACGGATATCCTGAGAATC
TCCTATGAAAGAGAAACACCCCCCCCACACCCCGCTTCTTCTACTGCGCCCACCGAGCCGAAAATCAC
CAGCGCGCCGCTCCCACGGTCGAGAAAACCGATATGCACACCGGCGGCATTCAACG

>STERO-1- FL639110, FL636382, FL635522
CCGTGACCGGGGCCCCACAGCCTGGTAAAGCTGACTGTACACTAACACTTGCTGATGAGGACATGGTA
CAAATTGGATCAGGAAAGTTGAATCGACAGGCAGCATTCATACAGGGCAAGTTGAAGGTGGCAGGCA
ACATTATGCTGACACAGAAATTGTCTGCGTTGCTAAGGGA

>STERO-1-R
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ATGCAATTTGTACCATGTCCTCATCAGCAAGTGTTAGTGTACAGTCAGCTTTACCAGTCTGTGGTGGTC
CCTGGTACACACTAGGCCTCTTAAGGTCCACAGTCCAAA

SECTION 4- Sequences from Library Clones Identified from Tartar et al. (2009)

>AACTIN- SP6
TAGCATTTAGGTGAACTATAGAATACTCAAGCTATGCATCCAACGCGTTGGGAGCTCTCCCATATGGT
CGACCTGCAGGCGGCCGCGAATTCACTAGTGATTCGCTCAGTCAGGATCTTCATCAGGTAGTCGGTCA
AGTCACGGCCAGCCAAGTCCAGACGCAGGATGGCATGGGGCAGAGCGTAACCTTCATAGATGGGGAC

GGTGTGGGAGACACCATCACCTGAGTCCAGCACGATACCAGTGGTACGACCGGAAGCGTACAGGGAC
AGGACAGCCTGGATTGCGACATACATGGAATCGAATTCCCGCGGCCGCCATGGCGGCCGGGAGCATG
CGACGTCGGGCCCAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTACAACGTCG
TGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCG
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GTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATTC
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CGGTATTTCACACCCGCATCAGGTGGCACTTTTCGGGGAAATGTGCGCGGACCCTATTTGTTTATTTTT
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>BTUBE-SP6- CB518304
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ATGGCGGCCGGGAGCATGCGACGTCGGGCCCAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGG
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CGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACG
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CGCAATTTTGGCGCT

>CEL2-R24- DQ014511
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>HEX-1-R24- AY572858
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>HMG_COA-M13R- FL638074, FL637763, FL638896, FL640394, FL638646
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>MYOSIN- SP6- CB518305
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CTTCA

>NADH-R-24- BQ788175
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>P450-CYP2V1-R24-DQ279463
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SECTION 5- Sequences from Library Clones Identified from Tartar et al. (2009)

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SECTION 6- High Throughput Array Genes Sequences

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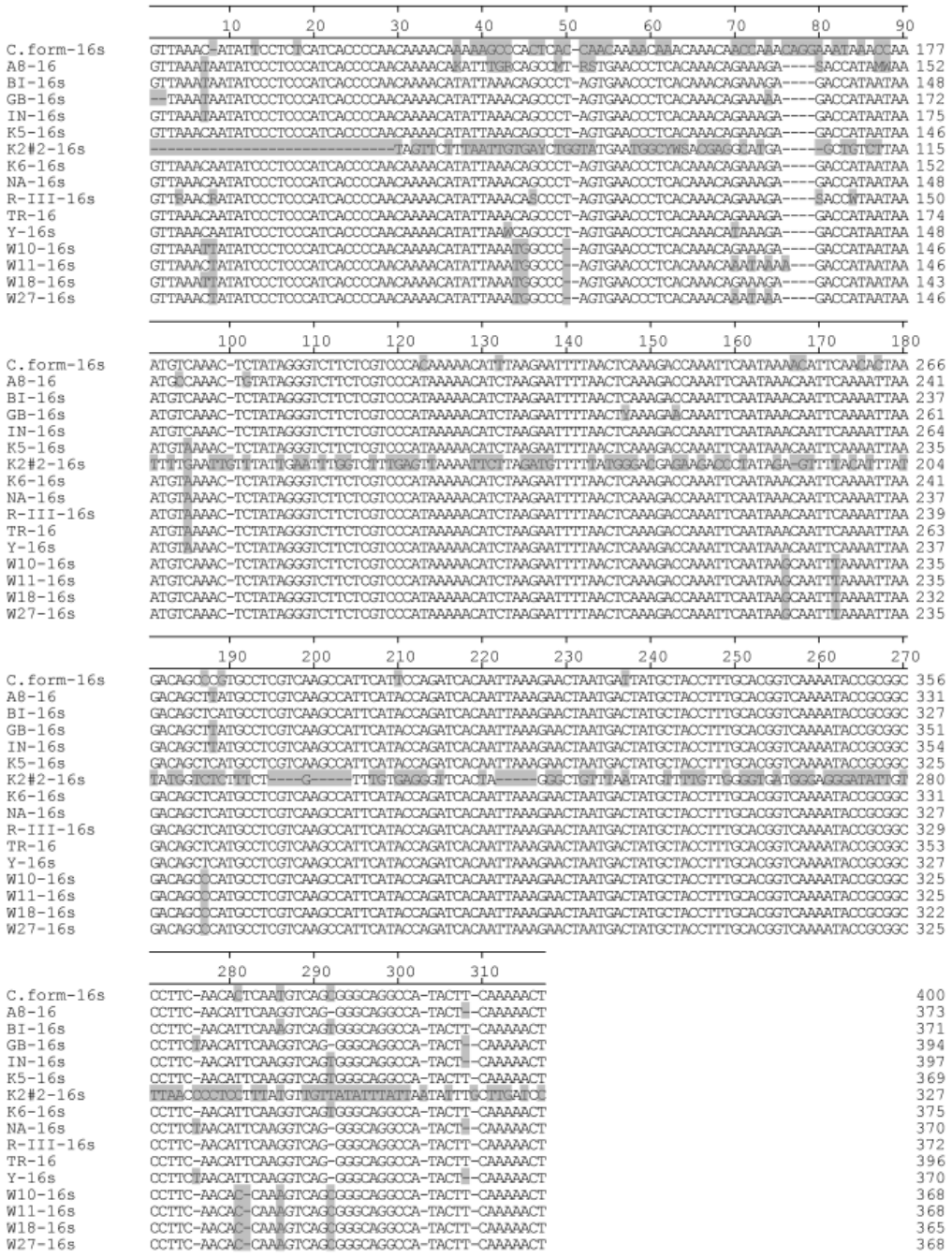

Figure C-1- Clustal W alignment of nucleotide sequences for *R. flavipes 16s* mitochondrial gene. Shaded residues indicate differences to *C. formosanus* (AY558910).

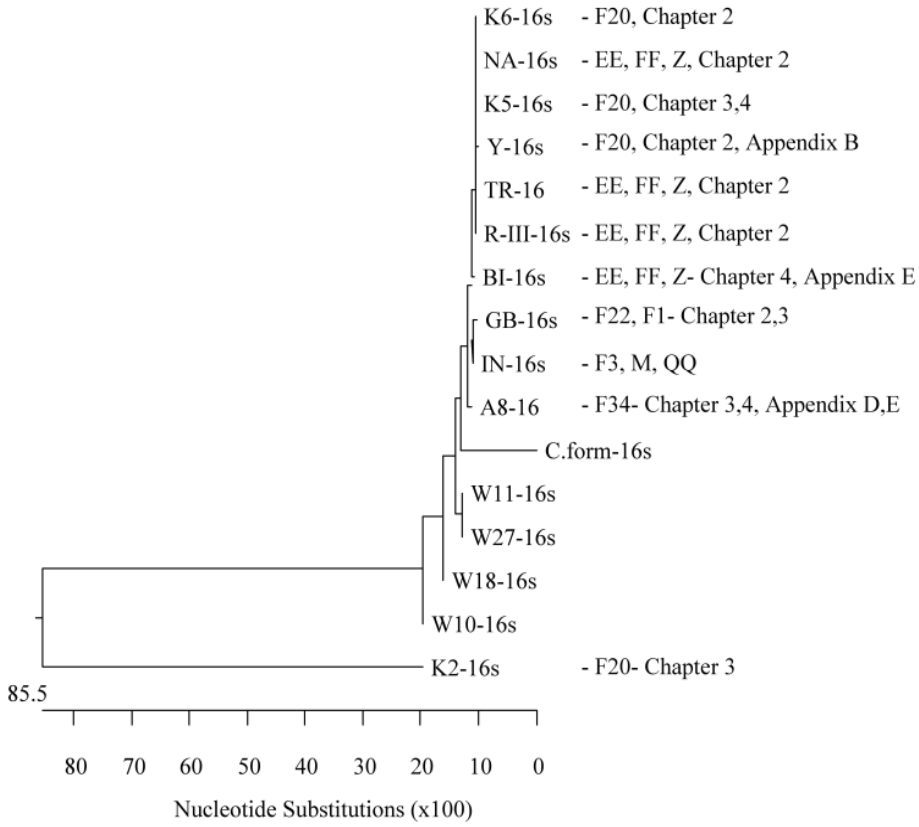

Figure C-2- Clustal W phylogenetic tree of nucleotide sequences for *R. flavipes 16s* mitochondrial gene. Next to the colony name is the top Blast haplotype and the chapter where the colony was used.

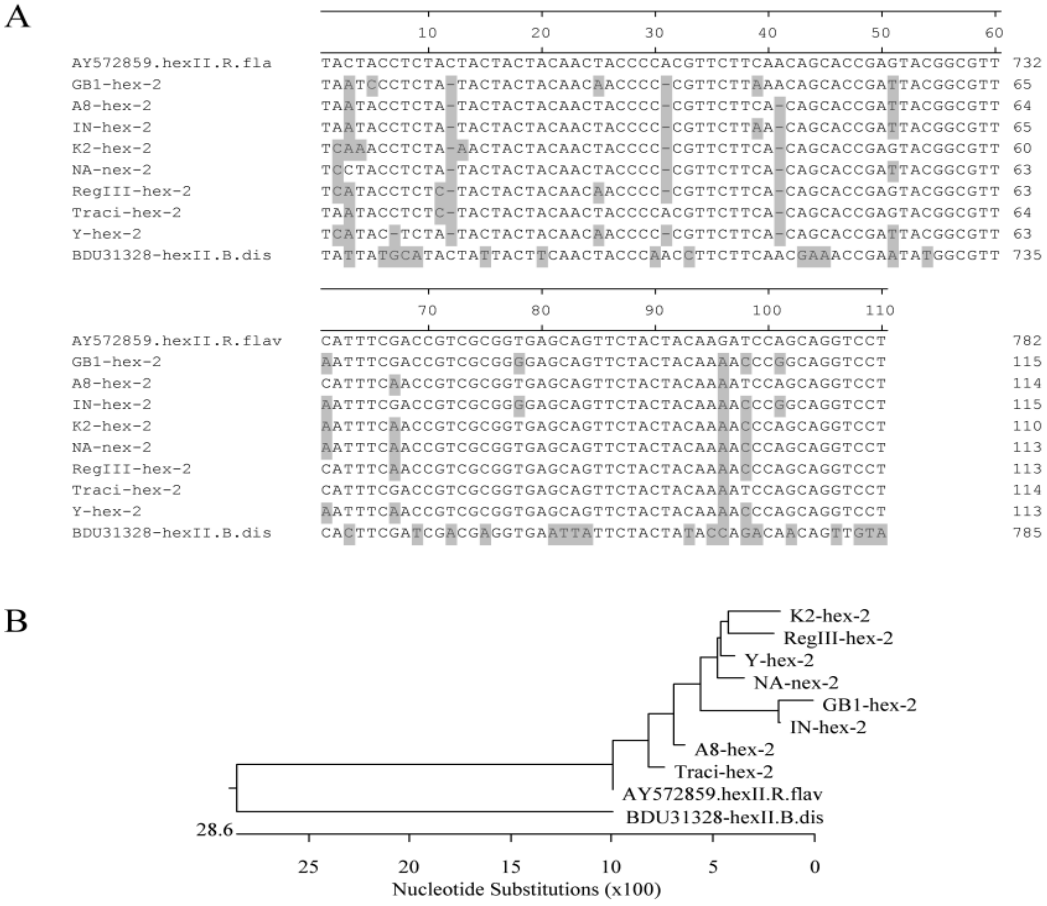

Figure C-3- Clustal W alignment of *Hex-2* sequences (A) and phylogenetic tree (B) of nucleotide sequences for *R. flavipes Hexamerin-2* gene. *B.discoidalis Hexamerin-2* gene (BDU31328) was used as an out-group.

APPENDIX D
SOLDIER INFLUENCE ON WORKER CASTE DIFFERENTIATION

Introduction

Past research in other termite species has shown that live soldiers inhibit the formation of additional soldiers in laboratory settings (Henderson, 1998; Mao et al., 2005). However, research completed in Chapter 1 found that extracts from soldier's heads synergistically increased worker to soldier morphogenesis when applied in combination with JH III. Additional experiments as described here were conducted to investigate if live soldiers from Florida *Reticulitermes flavipes* colonies can suppress soldier differentiation in worker termites in the absence of ectopic JH.

Methods

Two treatments were examined to assess the influence of live solders on soldier formation in larger experimental groups than tested in other chapters (n=100). Control treatments (100 workers) and soldier treatments (90 workers and 10 soldiers) from one colony were placed into large Petri dishes (diameter 9cm). Caste composition and survival were monitored every ten days for a total of 40 days. Each treatment was replicated five times on a single colony (A8).

Results

Results from the gene expression experiments (Chapter 3) suggested that live soldiers are capable of impacting expression of genes important in caste differentiation, thereby potentially inhibiting workers from becoming soldiers. Throughout this dissertation research I observed only a single incidence of presoldier formation in untreated controls (Appendix A). One possibility for this is that the bioassay utilizes a small number of workers (n=15) and is only 25 days long. Therefore, to test if live soldiers can indeed inhibit nestmate soldier formation, we tested the effect that live soldiers have on a larger number of workers (n=100) in a longer assay. Forty days after treatment no additional soldiers had formed in the bioassays that had included 10% soldiers

(Figure D-1a). However, in the treatments with only workers, soldier differentiation did occur and reached a level of 0.6% by day 40 (Figure D-1a). Survival rates between each treatment were similar with each treatment having around 83% survival after 40 days (Figure D-1b). These results verify that soldier caste members inhibit the formation of new soldiers in *R. flavipes*.

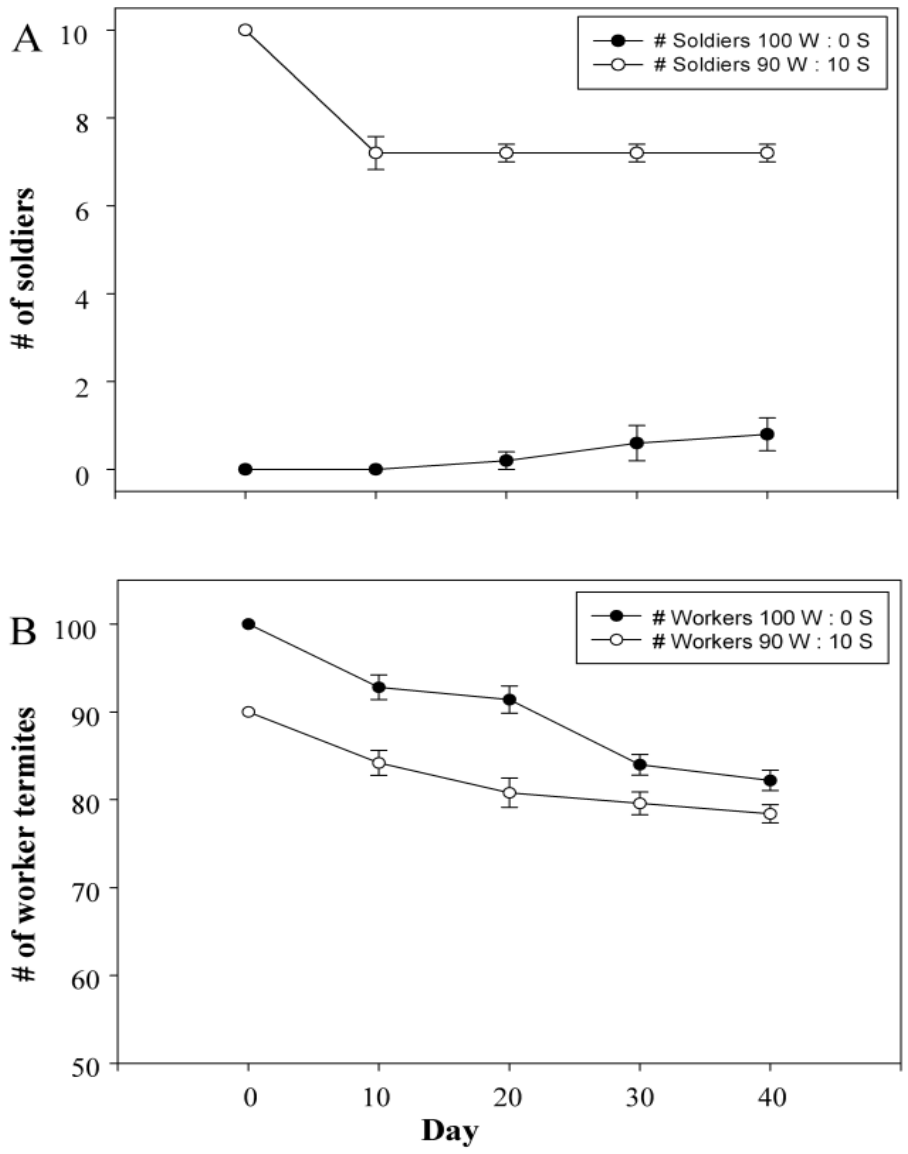

Figure D-1.Influence of live soldiers on worker nestmate caste differentiation. A) The Y-axis represents soldier numbers in bioassays. B) The number of worker termites was monitored 0-40 days. Lines with white and black circles, respectively, represent bioassays, that began with 10% and 0% soldiers (90% and 100% workers).

APPENDIX E
GENE SILENCING THROUGH RNAI

Introduction

The goal of these studies was to attempt to functionally characterize responsive genes identified in Chapter 3 through RNAi-based transcriptional silencing. The hypothesis was that, if worker to presoldier differentiation is affected by target gene silencing, then the target gene must play a role in caste differentiation. However, if RNAi does not affect caste differentiation, then 1) the target gene does not play a role in caste differentiation, 2) my method was not able to adequately silence the transcript, or 3) the bioassay strategies or detection methods are not able to detect effects.

RNAi Materials and Methods

Termites

Termites were collected form colonies around the Gainesville, FL area. Colonies were maintained in sealed plastic boxes for alteast one month before assay data. Colonies were identified as *R. flavipes* through *16s* DNA sequencing (Szalanski et al., 2003). Only worker termites were used in bioassays.

dsRNA/ siRNA Synthesis

dsRNA was synthesized using a commercially available kit (Silencer Ambion, Austin, TX) and eluted with H_20. dsRNA was then quantified using a spectrophotometer (A260 method). For RNAi experiments #7,10, and 13 dsRNA was RNAse III-digested using the Ambion Silencer kit. RNAi template primers were T7-appended 41 mers. Primers were: 1) RfCyp15F1-F-TTTCTCTGATGGCCCGTACT, RfCyp15F1-R-TTACAAGGCAATAATCCGGC, 2) RfCyp15A1-F-ATGGATGCCAGTGGTAGGAA, RfCyp15A1-R-CACTGAATGACAAACGCCTG, 3)

RfFaMet2-F- ACTGCCATCAGACGAGACC, RfFaMet2-R-AGTCGCTTCTTGATAGAGTGG, 4) RfVit-1-F-ACTGCCGTCAACGTATCCAT, RfVit-1-R-ATGAGTTGCCAAGTGGAGCTG, 5)RfVit-2-F- GGGTGAAATGGAACAAAGC, RfVit-2-F TACCCTATGGACCTTGGCAA, 6)RfLacF1-GCTCCCGGACATCAACTACT, RfLacR1-TTACTGGTTCCCTCACTGCC siRNA for experiment RNAi#15 was purchased directly from Invitrogen, "Stealth siRNA". RfEst1- stealth_550 GCAGAUGUCGUUGUAGUCACUUUAA. A pre-designed siRNA control was a scrambled Stealth RNAi siRNA duplex (48% GC content)-negative Control-STEALTH RNAI NEG CTRL MED GC#2 Cat no. 12935112.

dsRNA Feeding Assays

dsRNA feeding assays were performed as described by Zhou et al. (2008) with slight modifications. Termites were immediately removed from colonies and placed into 35-mm assay dishes that contained an 18-mm paper disk. Paper disks received dsRNA, JHIII or control treatments. dsRNA disks received 20 µg per disk. Disks treated with JHIII received 56µg of JHIII in acetone. Papers were treated only once at the beginning of assays. These procedures differ from Zhou et al. (2008) in that Zhou et al. pre-starved termites 24 hours before assays, and they also replaced treated papers every 8 days in bioassays.

Treatments

For experiment RNAi#7, six different genes were tested; *GFP*, *Famet-2*, *Cyp15F1*, *Cyp15A1*, *Vit-1*, and *Vit-2*. Each was tested with and without JHIII. An untreated control was also included. Four biological replicates were used for each treatment.

For experiment RNAi#11, termites were feed with nothing as a control or *Laccase* dsRNA and collected at 1,2,4, and 8 days after treatment. Termites guts were dissected into PBS and immediately frozen at -80 ^{o}C. RNA was extracted, normalized, transcribed into cDNA, and analyzed by qRT-PCR as described in full in Chapter 3. *β-actin* was used as a control gene. qRT-

PCR primers are Laccase-F1-AATCAAACTGGGTGCTTTGG, Laccase-R1-GGCTACGCGATCATCAAGTT

For experiment RNAi#13, seven treatments were used 1) Control –Day 0, 2) control- day-2, 3) *GFP*, 4) *Cyp15F1*, 5) *Cyp15A1*, 6) *RfEst1*, and 7) H_20. All treatments were injected with 500 pg siRNA per termite (15 ng/µl), except for controls. Injections were conducted exactly as described in Zhou et al. (2006). Treatments 2-7 were destructively sampled two days after treatment and frozen at -80 ^oC. RNA was extracted, normalized, transcribed into cDNA, and analyzed by qRT-PCR as described in full in Chapter 3. *Stero-1* was used as a control gene and *Cyp15F1*, *Cyp15A1* and *RfEst1* were target genes. Primer sequences are listed in Chapter 3, Table 3-1.

For experiment RNAi#15 termites were injected with either negative control siRNA or *RfEst1* at a rate of 37 ng (identically to Korb et al., 2009b) and collected at 2,4, and 6 days after treatment and stored at -80 ^oC. RNA was extracted, normalized, transcribed into cDNA, and analyzed by qRT-PCR as described in full in Chapter 3. *Stero-1* was used as a control gene and *RfEst1* was the target gene. Expression of the target gene was normalized to the control gene in both negative-control-injected and target-gene-injected treatments and plotted over the course of 6 days.

For each experiment fifteen termites were used for each replicate. If needed, termites were counted every five days and scored for mortality and caste differentiation. Water was added *ad libitum*. Termites were kept at 27 °C.

Statistical Analysis

ANOVAs with mean separations by Tukey's HSD were used to determine significant differences on percent presoldier formation for phenotypic experiments, and delta CT for transcript level.

Results and Discussion

Feeding

Prior studies conducted in the Scharf laboratory documented transcript knockdown and phenotypic impact by dsRNA feeding in *R. flavipes* (Zhou et al., 2008). A number of dsRNA feeding experiments were performed but I am only presenting a few representative experiments. For RNAi experiment #7 termites were fed dsRNA and held both with and without JH. Results show that termites treated with JH+GFP and JH+Vit-1 had an increase in mortality but there was no significant difference between treatments through 25 days (Figure E-1a).

No presoldiers formed in dsRNA-alone treatments. There was a slight increase in presoldier production in *Famet-2* and *Cyp15A1*-alone treatments; however, it was not significantly different from the control treatments (Figure E-1b). Of most interest, in termites treated with dsRNA+JH, there was on average a 58% increase in presoldier production with JH. Termites treated with JH+ *Cyp15F1*, *Cyp15A1*, *Vit-1*, and *Vit-2* dsRNA showed a non-significant decrease in presoldier formation when compared to the JH alone positive control treatment (df 13,55; $F=7.6136$; $p<0.0001$) (Figure E-1b).

Grouping of individuals could also impact gene silencing detection. For knockdown quantification studies, because the groups of termites were initially separated into groups of 15 per biological rep, there was a possibility I was not able to detect an expression knockdown because of baseline expression variability, or non-uniform silencing among individuals. As a result, I next sought to measure transcript level in individual termites. Target gene expression was compared in individual termites fed siRNA for a digestive Laccase relative to untreated controls. Results indicate that there was a large amount of variability between individual termites, and there was no trend in transcript knockdown over the four days tested (1,2,4,8). Days 1 and 4 are shown as an example (Figure E-2).

Injection

As shown above, feeding termites dsRNA did not reduce the target gene transcript level or reveal any repeatable phenotypic differences. Therefore, I proceeded to inject termites with siRNA, similar to Zhou et al. (2006a). A number of dsRNA/siRNA injection experiments were performed but only a representative few are shown.

In RNAi experiment #14, transcript levels were measured for the target gene in termites that were injected with nothing, H_20, *GFP* siRNA, or the target siRNA. Three different target genes were tested, *Cyp15F1*, *Cyp15A1*, and *RfEst1*. No significant reductions in transcript levels were identified one day after injection for the three target genes *Cyp15F1*, *Cyp15A1*, and *RfEst1* when compared to the *GFP* siRNA and H_20 controls (Figure E-3a,b,c).

To monitor transcript levels over a number of days, siRNA control and *RfEst1* siRNA were injected and termites were destructively sampled over a six day time course (RNAi#15). There was no significant reduction in transcript level after injection of Stealth *RfEst1* siRNA compared to the siRNA control over six days (Figure E-4). However, as shown in Chapter 4, there was a significant reduction in an esterase protein isoform 5 days after injection with *RfEst1* siRNA.

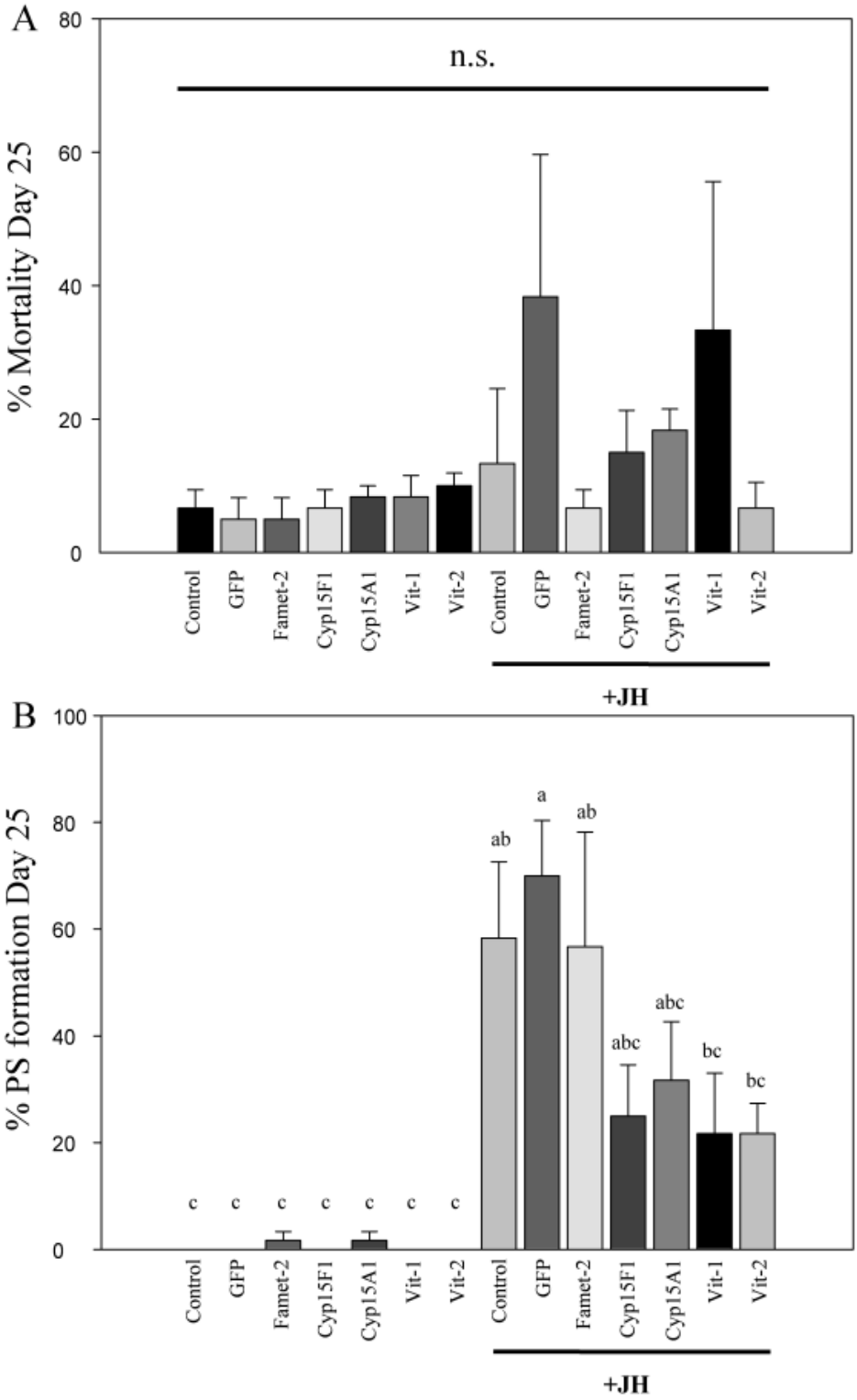

Figure E-1. dsRNA feeding assay. A) Percent mortality at Day 25 of termites feed dsRNA of six target genes with and without JHIII. B) Percent cumulative presoldier formation at Day 25 of termites feed dsRNA of six target genes with and without JHIII. Bars with different letters are significantly different ($p<0.05$).

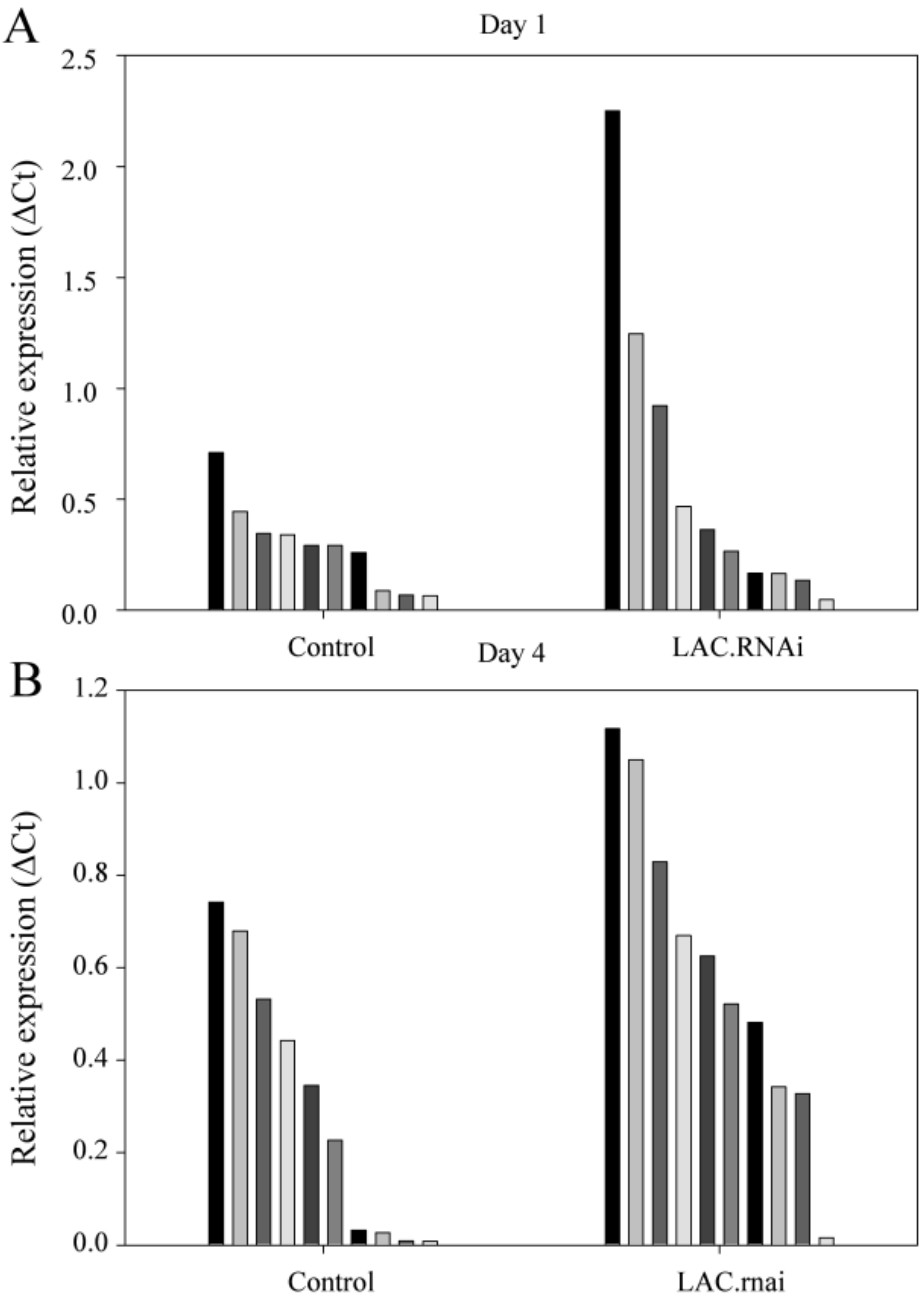

Figure E-2.Relative expression of the Laccase gene of termites fed control vs. Laccase dsRNA. Each bar represents an individual termite. Data from days 1 and 4 after treatment are shown.

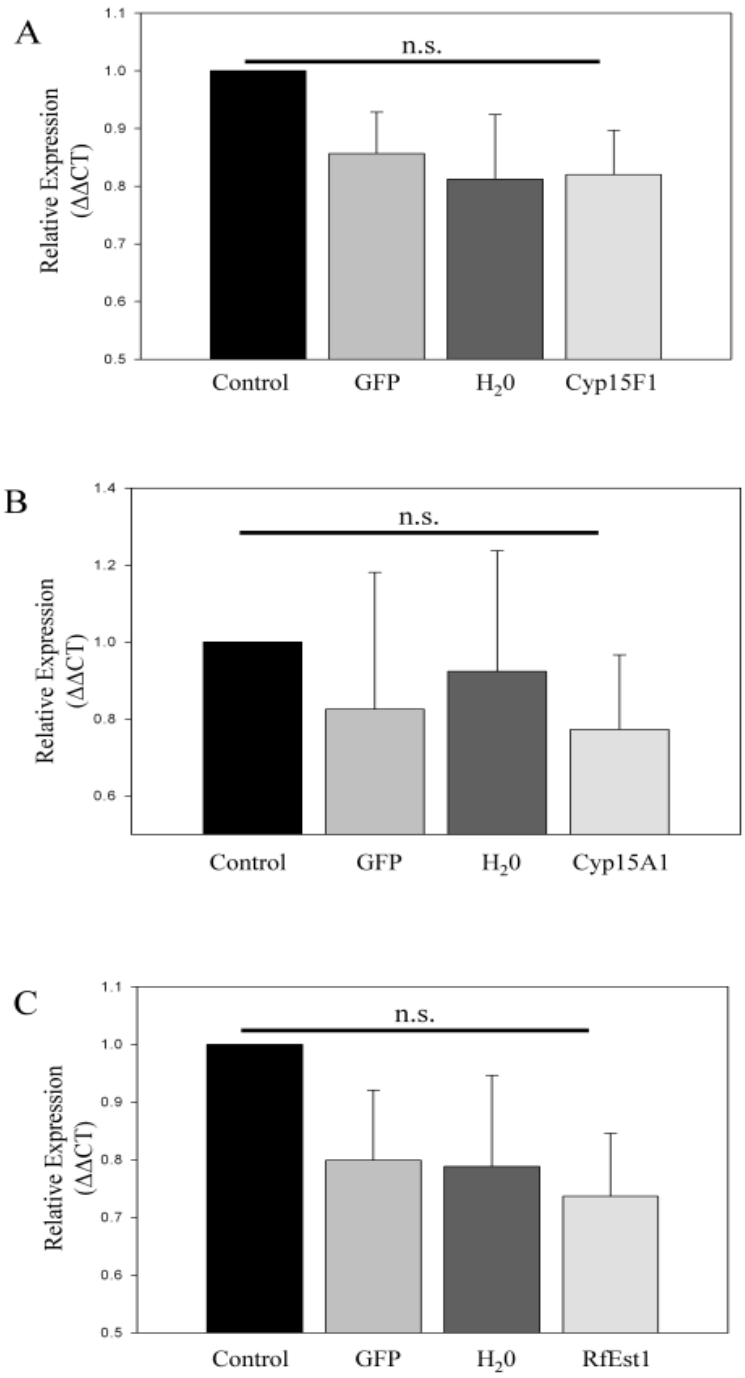

Figure E-3. Relative expression of siRNAi injected genes A) *Cyp15F1*, B) *Cyp15A1*, and C) *Rfest1* from termites injected with either; 1) nothing (Control), 2) GFP, 3) H_20, or 4) target gene (A) *Cyp15F1*, (B) *Cyp15A1*, (C) *Rfest1*. Bars with different are significantly different ($p<0.05$).

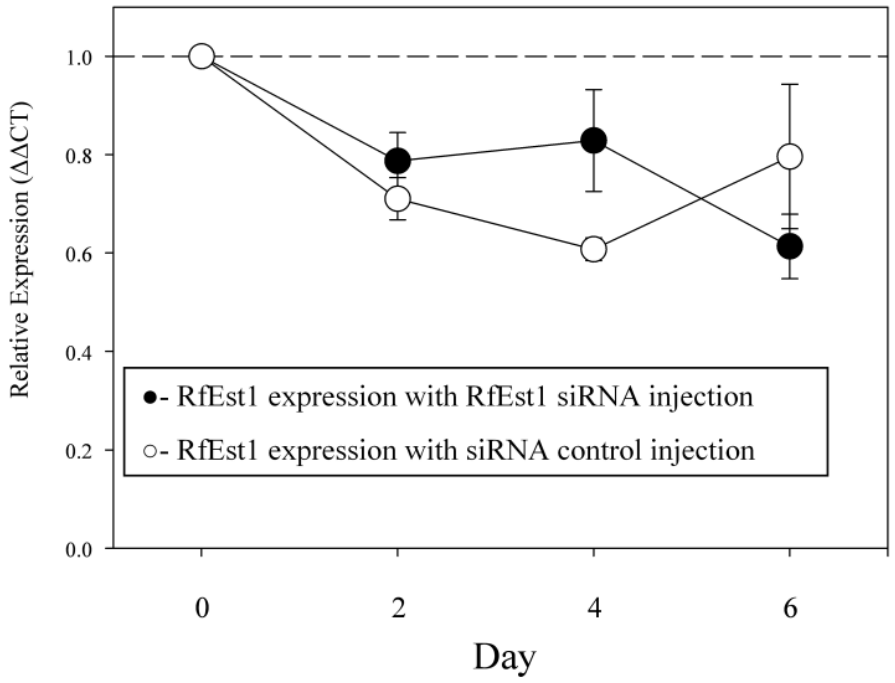

Figure E-4. Relative expression of the *RfEst1* gene in termites injected with siRNA negative control vs. *RfEst1* siRNA over six days.

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BIOGRAPHICAL SKETCH

Matthew R. Tarver grew up in Indiana and attended Purdue University for both his bachelor's and master's degree in entomology. Then he moved down to Florida to attend school at the University of Florida to study entomology. While in Gainesville he met his wonderful wife Megumi. During his stay in Gainesville, he has met many wonderful people who have made his life better in everyway. Thanks!!