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# APPLICATION OF MOLECULAR GENOMICS IN ADDRESSING QUESTIONS ON TERMITE BIOLOGY

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**Abstract** In termites, the mechanisms underlying caste differentiation and related physiological processes have remained elusive. To address this problem, we undertook a genomic research program that uses the common U.S. termite *Reticulitermes flavipes* Kollar as a model. These studies have allowed us to uncover a number of genes that are expressed during caste differentiation. Additionally, this genomic approach has also allowed us to identify genes that are differentially expressed among castes. To date, we have identified > 50 differentially expressed genes among workers, nymphs, presoldiers and soldiers that appear to have major impacts on either caste differentiation or caste-specific biology.

In this review, summaries are provided for termite genes that have been identified and sequenced, how these genes relate to termite biology, and results of functional studies connecting individual genes (and proteins) to specific biological/developmental processes. Through these studies has come an understanding of termite biology, biochemistry, physiology and caste differentiation that once was not approachable, even through decades of pre-genomic research.
**Key Words** Genomics, sociogenomics, Isoptera, hexamerin, RNA interference, juvenile hormone

## INTRODUCTION

Molecular biology is defined as the study of biology at the molecular level. It is an interdisciplinary approach that integrates biochemistry, physiology and genetics. Molecular biology emphasizes the Central Dogma, which is the relationship of DNA, RNA and protein: genetic information flows from DNA to RNA to protein, and thus, by studying DNA we can learn about proteins and basic biology. Genomics is a relatively new field of molecular biology that is defined as the comprehensive study of whole sets of genes and their interactions, rather than individual genes. Genomics enables the unambiguous identification of entire sets of genes associated with a specific biological process, or from entire organisms, as is the case with genome sequencing. With the advent of molecular biology and genomics has come an unprecedented ability to understand the biology of pest organisms. While it is basic in nature, the information provided by molecular biology and genomics has direct application towards the improvement of pest management tools and strategies.

Perhaps the best examples of insect genomics research are the *Drosophila melanogaster*, *Anopheles gambiae* and *Apis mellifera* genome sequencing projects. However, genome sequencing is an expensive endeavor reserved for only the most economically, medically and/or scientifically important insect species. Thankfully, more inexpensive approaches to insect genomics exist, particularly in the integrated use of cDNA libraries, robotic array printing and high throughput EST sequencing. With these combined approaches, large groups of genes that are associated with key biological, physiological, toxicological processes can be efficiently isolated and sequenced from cDNA libraries containing thousands of genes.

Here, we overview our progress in termite genomics research over the past 4-5 years. Our objectives are to provide examples of (1) our research approach, (2) termite genes that have been identified/sequenced, and (3) results of functional studies that show the importance of hexamerin genes and proteins to termite caste differentiation.

## MATERIALS AND METHODS

### Termites

*Reticulitermes flavipes* field colonies were collected in and around the Purdue University campus (West Lafayette, IN, USA), or in the University of Florida campus (Gainesville, FL, USA). Species identity was verified by a combination of soldier morphology (Nutting, 1990) and either mitochondrial DNA sequence (Ye et al., 2004) or restriction enzyme digestion patterns for polymerase chain reaction (PCR)-amplified 16S mitochondrial-ribosomal RNA (Szalanski et al., 2003). Termites were held in the laboratory for >4 months before inclusion in studies. Laboratory termite colonies were maintained in darkness within sealed plastic boxes (30 x 24 x 10 cm), at 26 ± 1ºC and 68 ± 2% RH. Termites were considered workers if they did not possess any sign of wing buds or distended abdomens, and had pronotal widths > mesonotal widths (Lainé and Wright, 2003). Other castes and phenotypes were classified according to Thorne (1996), Buchli (1958) or Lainé and Wright (2003).

### Genomic Techniques

A "polyphenic" cDNA library was synthesized as described previously (Wu-Scharf et al., 2003; Scharf et al., 2003a). Primary array results for workers, soldiers and nymphs were reported previously (Scharf et al., 2003a; 2005a). Presoldier array results are reported here for the first time. Expression levels were initially compared between array autoradiograms by manual alignment. For secondary verification, dot blots were performed using plasmid DNA (corresponding to specific array positions) that was affixed to nitrocellulose membranes (e.g., Scharf et al., 2003a). Array and dot blot-positive clones were sequenced from their 5' ends (T3 promoter) using a high throughput EST sequencing procedure. All high throughput sequencing was performed by the Purdue University Genomics Core Facility. When >1 EST was found with a given identity, sequences were aligned and contiguous sequences assembled by the CAP3 sequence assembly program. Full-length gene sequences were obtained by aligning contiguous EST sequences, primer walking, and 5' Rapid Amplification of cDNA ends (RACE).

### Model JH Assays

Model JH assays were conducted as described previously (Scharf et al., 2003b), with minor modification (Scharf et al., 2005b). Paired laboratory paper towel sandwiches (3.5 cm diam) were treated with 300 µl reagent-grade acetone containing 150 µg JH III (75% purity; Sigma, St. Louis, MO, USA). The filter paper served as both a substrate for JH treatment, as well as a food source. Controls were treated with acetone alone. After acetone had evaporated, the paper sandwiches were wet with 100 ml distilled water and placed in 5 cm plastic Petri plates with 30 worker termites ($4^{th}$ – $5^{th}$ instar) and a cotton-plugged Eppendorf tube (0.5 ml size) containing ca. 0.3 ml water. Plates were held in complete darkness at room temperature (25-27 ºC), within sealed plastic containers.

### Hemolymph Protein Studies

Hemolymph proteins were isolated from groups of 25-50 termites per caste or treatment by the following procedure. Under a viewing scope, termites were immobilized and secured dorsal-side-down on a piece of adhesive tape. Next, either their legs or abdominal cuticle were carefully clipped with micro-dissecting scissors; termite hemolymph readily bled from the wounds. Approximately 1-3-µl of hemolymph was collected quickly into a 10-µl capillary tube, then transferred to an Eppendorf tube containing 50-µl phosphate-buffered saline solution (PBS; pH 7.6). The hemolymph of 25-50 termites per treatment was placed into a single tube of PBS. Proteins were quantified with a commercially available Bradford assay (Bio-Rad) against a standard curve of bovine serum albumin. All electrophoresis was conducted on PAGE resolving gels containing 8% acrylamide and 10% SDS. Stacking gels contained a lesser quantity of acrylamide (4%) and the same amount of SDS. A dis-continuous Tris-Glycine buffering system was used, and protein sample buffer contained dithiothreitol (0.15 M) in place of ß-mercaptoethanol. Molecular weight markers were prestained broad-range markers (Bio-Rad, Hercules, CA, USA). N-terminal protein sequencing was performed using the Edmann Degradation on proteins transferred to PVDF membrane, by the University of Florida Protein Core Facility.

**Quantification of Gene Expression**

Quantitative real-time PCR (qRT-PCR) was performed to precisely quantify hexamerin gene expression. cDNA templates for quantitative PCR were synthesized from DNase-treated total RNA of whole termites. Total RNA isolation and DNase treatment were performed with commercially available protocols (Qiagen; Valencia, CA, USA) (Ambion; Austin, TX, USA). The quantity and quality of RNA were assessed before and after DNase treatment by spectrophotometry and formaldehyde-agarose electrophoresis (Sambrook et al., 1989). cDNA was synthesized from 5 ìg total RNA with an oligo-$dT_{15}$ primer and 50 U Superscript II reverse transcriptase (Invitrogen; Carlsbad, CA, USA) at 42ºC for 50 min. Quantitative PCR was performed as described previously (Scharf et al. 2003b; 2005a; 2005b) with â-actin as a control gene (Giulietti et al., 2001). Primer sequences and annealing temperatures ($T_m$) for the respective genes were as follows: *Hexamerin I* (Forward = GATCCATTCCACAAGCACG; Reverse = ACATTCTCCACCGTCACTCC; $T_m$ = 60.0ºC), *Hexamerin II* (Forward = ACGGAAGACGTTGGACTCAG; Reverse = GAGGACCTGCTGGATCTTGT; $T_m$ = 59.0ºC), and *â-actin* (Forward = AGAGGGAAATCGTGCGTGAC; Reverse = CAATAGTGATGACCTGGCCGT; Tm = 60.0ºC). qRT-PCR was performed under the following temperature cycles: 1 cycle of 94ºC (3 min) followed by 45 cycles of 94ºC (30 sec), $T_m$ (30 sec) and 72ºC (20 sec), a final extension of 72ºC (10 min), then a "melting curve" for verification of single PCR products.

**RNA Interference**

RNA interference (RNAi) techniques followed those for the silencing of honey bee vitellogenin, as reported by Amdam et al. (2003). Short-interfering RNA (siRNA) was synthesized using a commercially available kit (Silencer$^{TM}$, Ambion, Austin, TX, USA). siRNA templates corresponded to 500 bp portions from the center of the *hexamerin I* and *II* genes; they were amplified with PCR primers that had T7 RNA polymerase sequences appended to their 5' ends. The siRNA-template PCR primers were as follows: *hexamerin I* (Forward = TAATACGACTCACTATAGGG-ctggtccacaggattcatca, Reverse = TAATACGACTCACTATAGGG-CCTTGCTCTTCATGGTGTGA); *hexamerin II* (Forward = TAATACGACTCACTATAGGG-ATACGCCAATGGACAGGAAG, Reverse = TAATACGACTCACTATAGGG-GCGCTTGAGGATTTGGTAGT). After synthesis, siRNAs were diluted in nuclease-free water to ca. 15 pg/nl for injection into individual worker termites. siRNAs (0.5 ng) were injected into the side of the thorax in 32 nl volumes, using a Nanoliter 2000$^{TM}$ injector (World Precision Instruments, Sarasota, FL, USA) fitted with a custom-pulled borosilicate glass needle. Sham injection treatments received nuclease-free water alone. After injection, replicated groups of 15 termites were subjected to the model JH assay described above.

**Data Analysis**

Genbank (http://www.ncbi.nlm.nih.gov/BLAST/) database searches were performed using both blastx and blastn under default settings, with *E*-values for significance of identity being $1x10^{-10}$ and smaller (Altschul et al., 1990). Gene ontology and functional assignments were characterized based on significant homology to database sequences catalogued at either Genbank or Flybase. Quantitative PCR was performed in three replicates per caste or phenotype; relative expression levels were calculated using REST software (Pfaffl et al., 2002). Mean separation tests on quantitative PCR data were made by the least significant difference *t*-test ($P<0.05$) after ANOVA. RNAi experiments were conducted in triplicate, with each of the three replicates being performed on successive days. Average caste differentiation proportions were compared among treatments using the least significant difference *t*-test ($P<0.05$) after ANOVA.

## RESULTS AND DISCUSSION

**Gene Discovery**

To facilitate rapid and cost-effective discovery of major-impact genes, we used cDNA macro-arrays printed from a polyphenic cDNA library representing all castes and phenotypes except primary reproductives (Wu-Scharf et al., 2003). These arrays facilitated the comparison of gene expression between castes and developmental stages via the separate probing of arrays with labeled mRNAs from various castes and stages. Using this approach, vast differences occurred in the sequence composition of array-positive (up-regulated) clones between workers and soldiers (Scharf et al., 2003a), nymphs (Scharf et al., 2005a) and presoldiers (X.G.Z. *unpublished*) (Figure 1). To date, > 50 genes have been identified that represent solid candidates for further functional characterization studies. Four of these genes encode hemolymph proteins with potential roles as juvenile hormone (JH) binding proteins in insects (vitellogenins and hexamerins). Two of the genes encode the hemolymph hexamerin proteins that were our first targets for functional characterization studies (see below).

These hexamerin genes, named *hexamerin (hex) I* and *II*, were identified from nymph arrays (predominantly *hex I*) and presoldier arrays (predominantly *hex II*). Full-length sequences for these two genes were obtained by aligning contiguous EST sequences, primer walking, and 5' RACE.

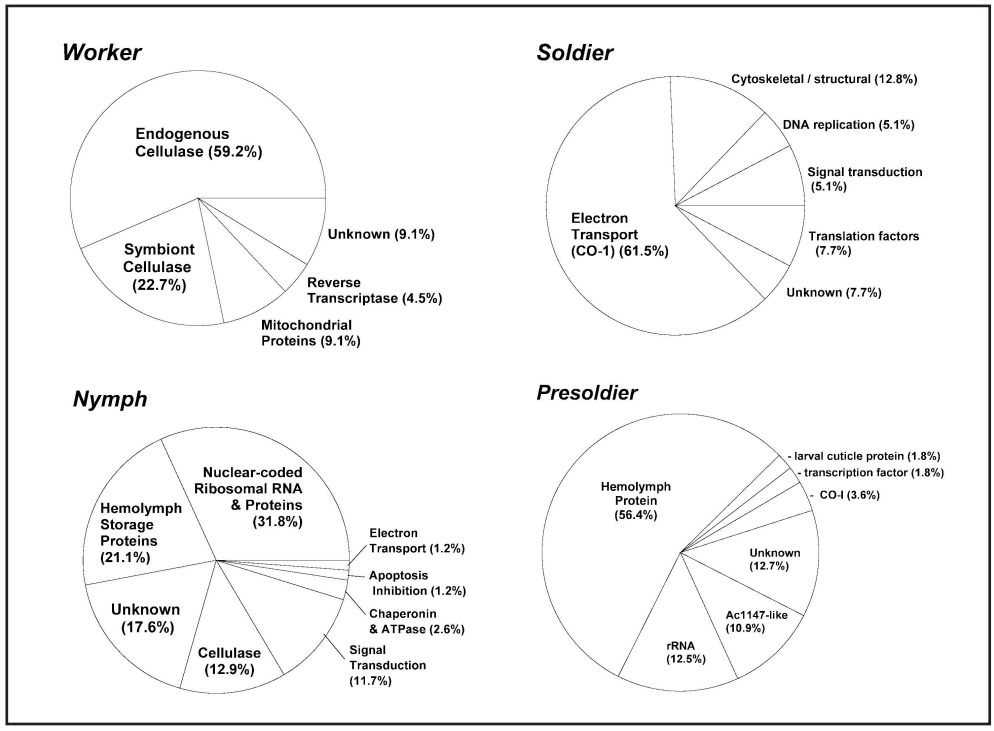


**Figure 1.** Comparison of *Reticulitermes flavipes* EST sequences from directed array screens showing sequence compositions for differentially expressed genes among castes and developmental stages. Shown are categories of EST sequences identified from workers (Scharf et al., 2003a), soldiers (Scharf et al., 2003a), nymphs (Scharf et al., 2005a) and presoldiers (X.G.Z. unpublished).

## JH Model Assays to Induce Caste Differentiation

Because of the apparent importance of JH to termite caste differentiation (Lüscher, 1960; Henderson, 1998), we also conducted parallel investigations that focused on JH and its effects on presoldier induction. Our first step was to optimize model JH assays originally described by Okot-Kotber et al. (1991) for induction of presoldier differentiation (Scharf et al., 2003b). We found that a "dish" assay format was most applicable for forcing workers to begin differentiating along the soldier developmental pathway. Following placement of established laboratory colony workers on JH III-treated substrates, presoldier differentiation typically begins on day 14, progresses linearly through day 25, and generally reaches a plateau at around day 25 (Figure 2). In these assays, mortality is typically less than 4%. We also found that workers from established laboratory colonies show higher levels of presoldier induction than workers collected directly from the field (see Figure 2 for a comparison of the same colony before and after two months in the laboratory). Based on these findings, laboratory colony workers were used in all subsequent studies so that more uniform responses could be induced for protein, gene expression and RNAi studies.

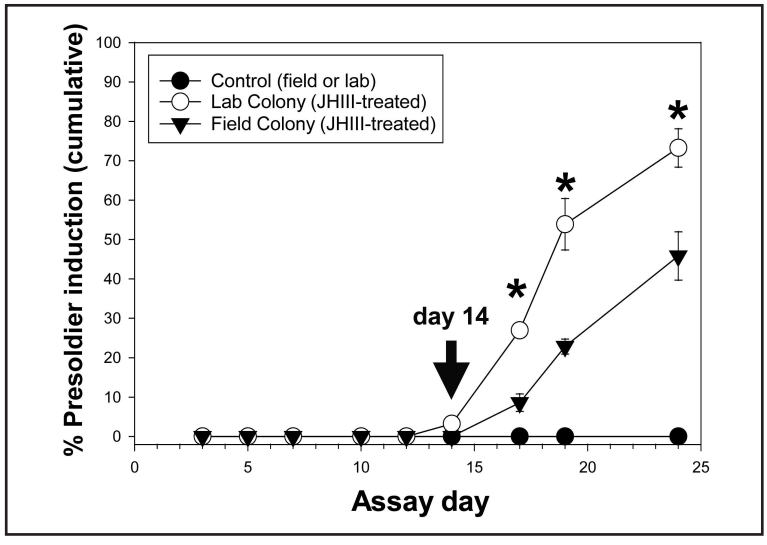


**Figure 2.** Presoldier induction by JH III in laboratory versus field-collected *Reticulitermes flavipes* workers. Data shown represents cumulative percentage presoldier induction in an established laboratory colony, over a 25-day assay. Assays were initiated by placing 25 worker termites on sandwiches of laboratory paper towel treated with 150 µg of JH III in 300 µl acetone. Control assay dishes received acetone alone. All data points in the same day with an asterisk (*) are significantly different by the LSD t-test (P<0.01, n=5).

## Hemolymph Protein Studies

Five major hemolymph proteins are resolved by SDS polyacrylamide gel electrophoresis (PAGE) with Coomassie staining in workers, presoldiers and soldiers; with only three major proteins in nymphs (Figure 3A). Hemolymph protein composition changes significantly in non-differentiated workers exposed to JH III in model assays for 16-24 d (Figure 3B) (Scharf et al., 2005b). These changes include an increase in band 3, and a decrease in band 5. N-terminal protein sequencing studies (X.G.Z. unpublished) have revealed that the two ~85 kDa bands which increase in abundance during JH-induced presoldier differentiation are *hex-2* (band 3) and *hex-1* (band 4). The identity of the JH-suppressible band with a molecular weight around 60 kDa (band 5) remains to be determined.

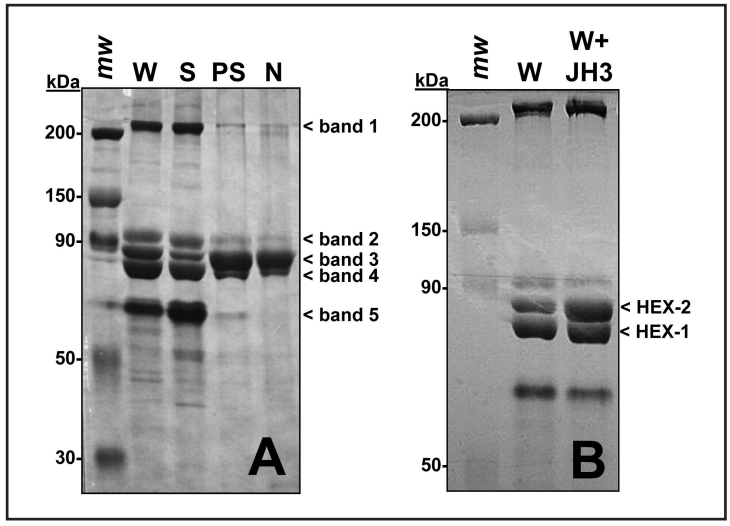


**Figure 3.** SDS-PAGE analysis of *Reticulitermes flavipes* hemolymph proteins. In A, hemolymph proteins were isolated from: colony workers (W), colony soldiers (S), colony presoldiers (PS) and 3rd-5th instar nymphs (N). In B, hemolymph proteins were isolated from colony workers (W) and non-differentiated workers exposed to JH III for 16-24 d (W+JH3). Protein bands labeled as "Hex-2" and "Hex-1" (bands 3 and 4) in B share 100% N-terminal protein identity with the N-terminal cDNA sequences identified from cDNA arrays. Numbers at left correspond to molecular weight markers (mw) in kilodaltons (kDa). All protein loadings were 20 g per lane.

**Caste and Development-Associated Gene Expression**

Quantitative PCR results show clear differences in hexamerin gene expression among castes, as well as during JH-induced presoldier differentiation in workers (Figure 4). More detailed studies of JH-induced hexamerin expression are reported in Scharf et al., (2005b). For *hex-1*, highest expression occurs in the nymph developmental stage, followed by female supplementary reproductives, then JH-treated workers. For *hex-2*, the highest overall expression levels occurred in association with JH treatment, during JH-induced presoldier differentiation. These results suggest that *hex-1* plays a role in reproductive differentiation, while *hex-2* apparently plays a role in either worker retention or presoldier differentiation.

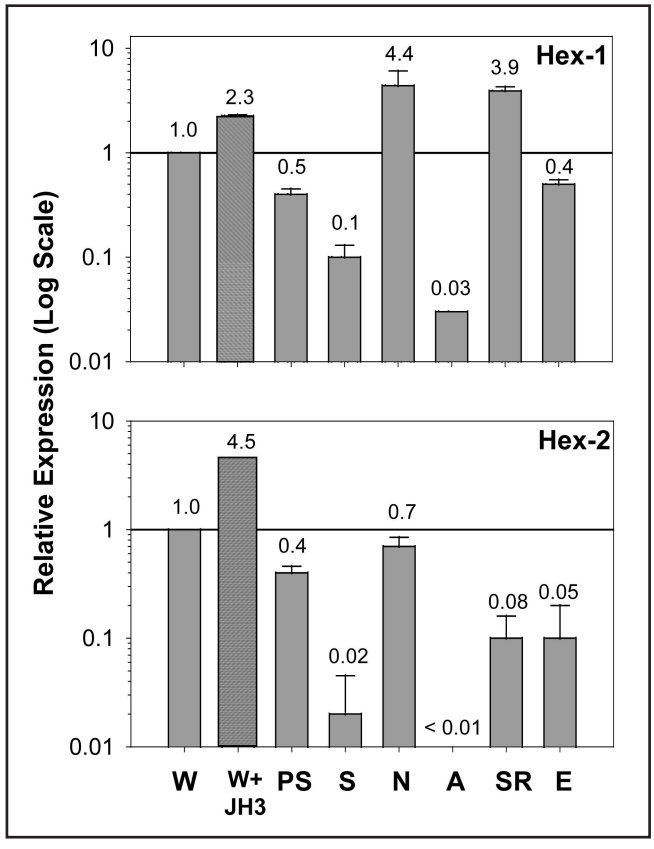


**Figure 4.** Relative expression levels for the Hex-1 and Hex-2 genes among *Reticulitermes flavipes* castes and developmental stages, and after JH III treatment. Expression levels were determined by quantitative real-time PCR relative to the control gene -actin. Hex-1 is most highly expressed in nymphs and supplementary reproductives, while Hex-2 is most highly expressed in workers that are undergoing presoldier differentiation in response to JH III treatment. Abbreviations:W = worker; W+JH3 = JH III- treated workers; PS = presoldier; S = soldier; N = nymph; A = alate; SR = supplementary reproductive; E = egg / embryo.

**RNA Interference Studies of Hexamerin Function**

Our next logical step in moving toward understanding the effects of the two hexamerins on soldier differentiation was to silence hexamerin expression via RNAi. The objectives in taking this step were to (i) definitively determine hexamerin's role in caste differentiation, and (ii) to develop RNAi protocols for termite research. RNAi is emerging as a powerful tool for functional characterization of genes identified in genomics research (Dykxhoorn et al., 2003). This technique has now been successfully executed on a number of arthropods (Gatehouse et al., 2004; Buchner and Klingle, 2004; Karim et al., 2004; Uhlirova et al., 2003). Most notably, with respect to our interests, injection of double-stranded short-interfering RNA into honeybee larvae had proved highly successful for silencing vitellogenin, a hemolymph storage protein (Amdam et al., 2003).

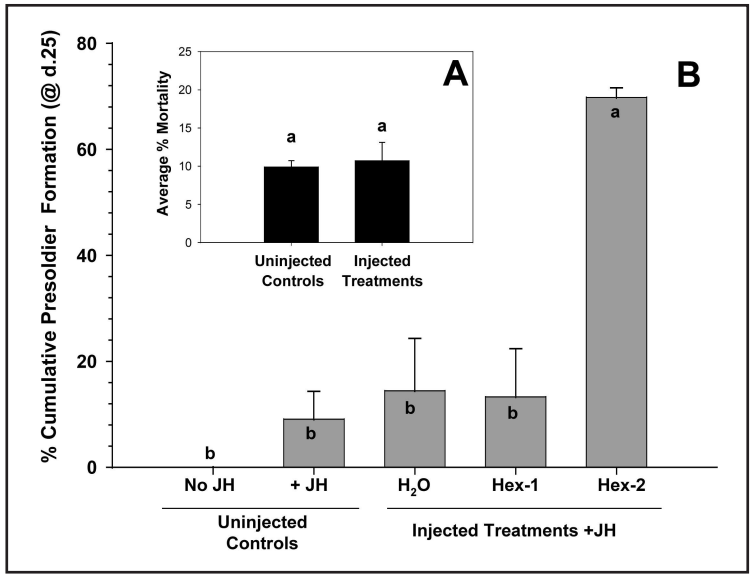


**Figure 5.** RNAi effects on presoldier induction, showing the apparent importance of Hex-2 as a caste regulatory protein. Individual worker termites ($4^{th}$-$5^{th}$ instar) were injected with 0.5 ng short-interfering RNAs, then subjected to JH model assays as described above. Treatments consisted of uninjected controls (No JH or JH+) and injected treatments ($H_2O$ = water injected; Hex-1 = Hexamerin-1 siRNA injected; Hex-2 = Hexamerin-2 siRNA injected). Shown in A (inset) are pooled mortality levels for uninjected controls and injected treatments. Bars in B with the same letter are not significantly different by the LSD t-test ($P<0.01$, n=3) (Note: A and B were analyzed separately).

Our approach consisted of injecting short interfering RNA fragments corresponding to portions of the hexamerin genes into the worker termite hemocoel, followed by subjecting the termites to JH model assays as described above. Since RNAi had never (to our knowledge) been attempted in termites, we had three alternate hypotheses: (1) termites would not survive injections and/or no effects would be observable, (2) hexamerin silencing would lead to a blockage of presoldier differentiation, or (3), hexamerin silencing would lead to enhanced presoldier differentiation.

The termites proved to be surprisingly robust to withstand the RNA injection process. In this regard, there were no significant differences in mortality between injected and uninjected treatments (pooled averages are shown in inset of Figure 5). After day 25 of model JH assays, there were significant increases (7-9-fold) in presoldier formation as a result of silencing the *hex-2* gene ($P < 0.001$); this was relative to the other treatments as well as all the controls in terms of presoldier induction (Figure 5). In addition, there were no significant differences in presoldier induction among the *hex-1*, water, and uninjected JH-treated groups relative to the acetone-treated uninjected control; however, clear numerical differences exist between these treatments and controls. In companion studies that examined protein expression after RNAi treatments, there are clear reductions in protein expression relative to uninjected and water-injected controls (X.G.Z. in preparation).

To our knowledge, these results are the first to demonstrate the use of RNAi techniques in termites. These findings suggest that *hex-2* plays a role in the suppression of soldier caste differentiation, while *hex-1* plays a less important role in this process. Because JH induces both presoldier differentiation and *hex-2* at the gene and protein levels, it appears that *hex-2* may be involved in JH binding. In this regard, hexamerins have been identified as JH binding proteins in other insects (Braun and Wyatt, 1996; Gilbert et al., 2000). Specifically, our findings support the hypothesis that *hex-2* sequesters JH, thus preventing it from interacting with extracellular receptors that would mediate JH-dependent developmental processes. In support of this hypothesis, western blotting studies that compared RNAi vs. non-RNAi termites have shown that JH binding is significantly reduced for hemolymph proteins in the vicinity of the *hex-2* protein, in only *hex-2* silenced treatments (X.G.Z. in preparation). However, since hexamerins also have well-established roles in nutrition and nutritional signaling (Burmester and Scheller, 1999), it can not presently be ruled out that *hex-2* regulates caste differentiation by non-hormonal means; or whether interactions between hormonal and nutritional cues serve to regulate presoldier differentiation. Nonetheless, based on our collective findings, it appears logical to conclude that *hex-2* is a caste regulatory protein that apparently serves to maintain a *status quo* work force (workers) in termite colonies. Therefore, *hex-2* and its homologs in other termites may be centrally important to Isopteran eusociality. Studies

are currently in progress to further investigate the influences of colony composition on *hex-2* expression, as well as JH binding properties for the *hex-2* protein.

**Conclusions and Future Directions**

As outlined here, the approach we undertook began with the establishment of a non-normalized "polyphenic" cDNA library. After validation of the integrity of the library (Wu-Scharf et al., 2003), several duplicate "filter" or "macro" arrays were printed that each represented 18,000 cDNAs from the library. The arrays were subsequently probed with labeled RNAs from different termite castes or developmental stages that included workers, soldiers, nymphs and presoldiers (Scharf et al., 2003a; 2005a; unpublished). These array studies revealed > 50 differentially expressed genes, of which 35 (to date) have been shown conclusively by quantitative real-time PCR to have varying expression levels among castes and developmental stages. In order to validate the use of our emerging termite model system, we concurrently focused on model JH assays for inducing caste differentiation (Scharf et al., 2003b), as well as hemolymph proteins and their changes during JH-induced caste differentiation (Scharf et al., 2005b). Finally, as reported here for the first time, we have developed RNAi protocols for use within our termite model system. We have shown that (1) RNAi is possible in Isoptera and (2) that one of two hexamerin genes/proteins identified (*hex-2*) is apparently a major caste regulatory factor.

Here we have overviewed our termite genomics research over the past 4-5 years. The approach outlined here, which involved gene discovery combined with functional studies of hexamerin genes, was largely driven by the need to gain information on the basic biology underlying termite caste differentiation, as well as to make the maximum progress on a modest research budget. For this reason, our main focus on genes for functional characterization has been on highly expressed hemolymph proteins with apparent roles in JH binding and caste regulation. The emerging hexamerin story is but one example of potentially dozens of interesting stories that may be told by the multitude of genes identified from our cDNA array studies.

To date, in functional characterization studies we have been working on "downstream" genes that regulate developmental hormones, caste-related physiology and nutrition. Relative to nutrition, we have identified a host of endogenous and symbiotic cellulases. Currently, we are investigating the complementary roles of these diverse cellulases in termite nutrition. Our future research will focus on the several dozen *Reticulitermes flavipes* genes that show differential expression between castes, as well as during caste differentiation and development. In this regard, some highly interesting genes identified to date fall into a more "upstream" regulatory category, such as signal transducers, transcriptional and translational regulators, and homeotic genes. These functional genomic investigations will most certainly continue to advance our understanding of many of the long-standing basic questions surrounding termite biology, while at the same time, contributing significantly to applied goals that relate to improved termite management.

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