INTRODUCTION
Termites are social insects that live in colonies, which, in turn, function because of the complementary roles played by the different castes (Wilson, 1971). As with all social insects, termite castes are the result of an elaborate system of developmental polyphenism (Evans and Wheeler, 2001; West-Eberhard, 2003). Termite colonies produce worker, soldier and reproductive caste phenotypes that each display unique suites of physiological-behavioral characteristics and accomplish non-overlapping tasks. Specifically, workers assist reproductives, build tunnels and feed the colony; soldiers defend the colony; and reproductives pass genes to successive generations (via the production of offspring). With respect to lower termites, the worker caste is a temporally arrested immature form that retains the ability to differentiate into terminally-developed soldier- or reproductive-caste phenotypes (Buchli, 1958; Noirot, 1985; Noirot, 1990; Lainé and Wright, 2003).

While in their temporally arrested juvenile state, workers of the more primitive lower termites display altruistic helping behaviors that serve the colony and maximize its inclusive fitness (Myles and Nutting, 1988; Nalepa, 1994). Under specific circumstances, however, termite workers readily undergo caste differentiation. With respect to worker-to-soldier differentiation, elevated titers of the morphogenetic juvenile hormone (JH) drive this transition (Park and Raina, 2004; Mao et al., 2005). To counter the effects of JH, Reticulitermes flavipes workers produce hexamerin proteins that are part of a status quo regulatory mechanism that serves to block the irreversible worker-to-soldier transition (Zhou et al., 2006a; Zhou et al., 2006b). Therefore, the hexamerins have been, at least in part, co-opted through social evolution to meet the need for a numerically dominant worker force.

JH has been known to play a role in termite caste polyphenism for several decades (Lüschner, 1960; Lüschner, 1976; Noirot, 1969; Henderson, 1998). However, despite recent advances in the areas of termite endocrinology (Park and Raina, 2004; Mao et al., 2005) and gene-expression profiling (e.g. Miura et al., 1999; Scharf et al., 2003a; Scharf et al., 2005a; Miura, 2005; Koshikawa et al., 2005; Hojo et al., 2005; Cornette et al., 2006; Zhou et al., 2006c), our collective understanding of the molecular basis of termite caste regulation has remained limited. In this regard, the recent identification of the hexamerin caste-regulatory mechanism provided some of the first detailed molecular evidence of a caste-regulating mechanism from a termite (Zhou et al., 2006a).

The question of exactly how does the hexamerins function in termite caste regulation has not yet been answered. Hexamerins of solitary insects are normally involved in nutrient storage and nutritional signaling (Burmeister and Scheller, 1999), but have also been authenticated as bona fide JH-binding proteins (Braun and Wyatt, 1996; Gilbert et al., 2000; Tawfik et al., 2006). One hypothesis for termite hexamerins is that they serve as a signaling mechanism for nutritional status and that pre-soldier differentiation is suppressed when certain nutritional requirements are met (Zhou et al., 2006a). An alternative hypothesis is that the hexamerins are part of a mechanism that sequesters JH, thus preventing it from eliciting downstream effects on developmental gene expression (Zhou et al., 2006a). No evidence has yet been obtained that supports a role for the termite hexamerins in caste regulation via nutritional signaling. Evidence supporting the JH-sequestration hypothesis is the observed increase in JH-dependent caste differentiation after the
silencing of the *R. flavipes* hexamerins (Zhou et al., 2006a), as well as JH-induction (Scharf et al., 2005b) and recognition of the *R. flavipes* hexamerins by anti-JH antiserum (Zhou et al., 2006b). Termite JH titers increase in response to a release from colony conditions (Okot-Kotber et al., 1993; Mao et al., 2005). Thus, because the hexamerins are both JH-responsive and attenuate JH efficacy, they have apparently been selected during social evolution to function as a proximate socio-regulatory mechanism (Zhou et al., 2006a). The studies reported here were undertaken in an effort to identify members of a hexamerin-controlled, JH-dependent caste-regulatory gene network in developmentally plastic *R. flavipes* workers. These studies used a combination of RNA interference (RNAi), gene-expression profiling and regression analyses. The specific objectives of these studies were as follows: (I) to examine the downstream impacts of hexamerin gene silencing on a putative network of 20 genes; as well as (II) to attempt to correlate downstream impacts of hexamerin silencing with (i) colony-release effects, and (ii) effects of ectopic JH treatment. These findings revealed a significant correlation between hexamerin silencing and the downstream impacts of hexamerin silencing with (i) colony-release effects, and (ii) effects of ectopic JH treatment only, which provides additional evidence in support of earlier conclusions that the hexamerins function, at least in part, through the modulation of JH-mediated pleiotropy. These experiments also revealed members of a putative JH-responsive gene network, which, in addition to the hexamerins, includes transcription/translation factors, signal transducers, cuticle proteins and muscle proteins. Additionally, this research validates an experimental framework for dissecting caste-regulatory gene networks in social insects, and for investigating the ecological and evolutionary significance of environmentally responsive socio-regulatory mechanisms that exist specifically in termites.

**MATERIALS AND METHODS**

**Experimental animals**

Worker termites used in these experiments were from laboratory colonies held at a constant 22°C. All colonies were collected on the University of Florida campus (Gainesville, FL, USA). All caste phenotypes and developmental stages were readily observable in the colonies, including soldiers, nymphs, supplementary reproductives, larvae and eggs. Sampled workers were from later instars; they possessed sclerotized heads, rich symbiotic communities and prominent fat bodies.

**Genes**

Extensive preliminary studies led to the identification of >300 housekeeping, structural and candidate caste-regulatory genes from *R. flavipes*. These preliminary investigations used a combination of random library sequencing and cDNA arrays. Random sequencing allowed us to obtain housekeeping-gene sequences for use as references in qRT-PCR (Wu-Scharf et al., 2002). The arrays allowed us to identify >100 differentially-expressed genes among different caste phenotypes, including workers and soldiers (Scharf et al., 2003a), nymphs (Scharf et al., 2005a), and pre-soldiers (Scharf et al., 2005c; Zhou et al., 2006b) (X.Z. and M.E.S., unpublished). The 19 candidate genes chosen for evaluation here have significant homology (i.e. e<1×10⁻10) to well-defined developmental genes from *Drosophila* and other insects; in particular, broad, nanos, bicaudal, COP9 complex homolog subunit 5, *LIM* (Legs incomplete and malformed), troponin and *PIP* (phospho-inositol-phosphate) kinase. See Table 1 for identities and Genbank accession numbers for the 19 target and three control genes investigated here.

**RNA interference**

Silencing of hexamerin expression by RNAi was accomplished as described previously (Zhou et al., 2006a; Zhou et al., 2006b). Briefly, double-stranded (ds) short-interfering (si) RNA was synthesized using a commercially available kit (Silencer™, Ambion, Austin, TX). The dsRNA template from which the dsRNA was synthesized corresponded to a ~500 bp portion from

### Table 1. Gene identities, abbreviations, accession numbers and qRT-PCR primer sequences

<table>
<thead>
<tr>
<th>Gene identity (abbreviation)</th>
<th>Accession no.</th>
<th>Forward primer (5′-3′)</th>
<th>Reverse primer (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ligand-binding proteins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Hexamerin-1 (Hex-1)</td>
<td>AY572858</td>
<td>GATCCATCCCAAAGACAGG</td>
<td>ACATTTCACCGTGACTCC</td>
</tr>
<tr>
<td>2. Hexamerin-2 (Hex-2)</td>
<td>AY572859</td>
<td>ACGGAAAGACCTGGACTCAG</td>
<td>GAGGACTCTGGAGATCTTTG</td>
</tr>
<tr>
<td><strong>Cuticle and muscle proteins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Larval Cuticle Protein A3A-like (LCP)</td>
<td>DN792534</td>
<td>CGTCGACACCGCATCGACAG</td>
<td>GTCGACCGTGACTCCAG</td>
</tr>
<tr>
<td>4. Rf1 Troponin-1 (wup-like) (Trop-1)</td>
<td>CB518302</td>
<td>CGACCTAGAATACGAAGTGG</td>
<td>TCTTCTCTCCCTTCCCTCC</td>
</tr>
<tr>
<td>5. Rf2 Troponin-1 (wup-like) (Trop-2)</td>
<td>CB518303</td>
<td>GAAGATTTGGAAGAAGGACAGG</td>
<td>TTGTCCCACCGCCTTACG</td>
</tr>
<tr>
<td><strong>Transcription and translation factors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. nanos</td>
<td>BQ788190</td>
<td>CCACATGACTAAATGTAGG</td>
<td>TCAAGGCTCAAACACTCTT</td>
</tr>
<tr>
<td>7. COP9 Complex Homolog Subunit 5 (COP9)</td>
<td>DN792518</td>
<td>CTGACGAAAGGACATTCAC</td>
<td>GTGCTGCCTCCTGATG</td>
</tr>
<tr>
<td>8. BTB-POZ [broad-like]</td>
<td>AY258590</td>
<td>CGAGGCAAGACTATCATCTCTCTC</td>
<td>GGTGTGACCTGATTACAG</td>
</tr>
<tr>
<td>9. 18S rRNA-like (18S)</td>
<td>AY572860</td>
<td>TATCTGTCTCCCTGCTTGG</td>
<td>TCGGAAATGAGAAGGAGCC</td>
</tr>
<tr>
<td>10. 28S rRNA-like (28S)</td>
<td>CK906357</td>
<td>GGCAATGTGGAGGCGCTT</td>
<td>ACAGCGCAGTCCTGCTC</td>
</tr>
<tr>
<td>11. bicaudal (bic)</td>
<td>AY258589</td>
<td>GAGGCAAGATCAGGATTGAG</td>
<td>CTCTTCATGTGAAACACAG</td>
</tr>
<tr>
<td>12. LIM</td>
<td>CB518301</td>
<td>GTCGCTCACTGAGGTATG</td>
<td>GTCGACCTGAGAACACAG</td>
</tr>
<tr>
<td><strong>Signal transduction factors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13. Malonyl Co-A Decarboxylase (M-CoA)</td>
<td>AY572861</td>
<td>GCTAAGGGGAGCTTCTAC</td>
<td>GAGGACAGCTGATTCTCC</td>
</tr>
<tr>
<td>14. Phospho-Inositol Phosphate (PIP) Kinase</td>
<td>CK906365</td>
<td>AATTCTGTGGCTCCCTTGG</td>
<td>ACCTGCTGCGACTACATC</td>
</tr>
<tr>
<td>15. Apoptosis Inhibitor (Apop)</td>
<td>CK906364</td>
<td>CGTACTGTGGAGCGAGTT</td>
<td>AACTCACTGAGGGGACAG</td>
</tr>
<tr>
<td>16. AMP Deaminase (AMP-de)</td>
<td>CK906363</td>
<td>GTGACATGTGGAGCTTGG</td>
<td>ACCAGCTACCTTCTGTTG</td>
</tr>
<tr>
<td>17. GTPase Activating Protein (GAP)</td>
<td>BQ788178</td>
<td>TCCGAAACACGAGACACAGAC</td>
<td>TAAGTCCTGGAGGACGAC</td>
</tr>
<tr>
<td>18. ATPas</td>
<td>BQ788171</td>
<td>TCGAAGATCTGAGTATG</td>
<td>TAAGTCCTGGAGGACGAC</td>
</tr>
<tr>
<td>19. SH3 Domain Kinase (SH3)</td>
<td>CB518513</td>
<td>GAGGCTGGCCTGATG</td>
<td>ACAACATCTTCCGCGCTC</td>
</tr>
<tr>
<td><strong>Reference/control genes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20. β-actin</td>
<td>DQ206832</td>
<td>AGAGGAGAATCTGCTGCA</td>
<td>CAATAGTGTGACCTGCGCT</td>
</tr>
<tr>
<td>21. NADH-dh</td>
<td>BQ788175</td>
<td>GCTGGGGAAGTTTCCCTT</td>
<td>GCCATACACAAAGACCAAAA</td>
</tr>
<tr>
<td>22. HSP-70</td>
<td>BQ788164</td>
<td>AGAAGCAAGTGGCCATGA</td>
<td>CAACTGCTTACGTCCTCC</td>
</tr>
</tbody>
</table>
the open reading frame of the Hexamerin-2 (Hex-2) gene, which shares >50% identity with the Hexamerin-1 (Hex-1) gene across the same region. Thus, our approach allowed for the silencing of both hexamerin genes by a single dsRNA fragment (Zhou et al., 2006a). The template cDNAs were amplified by PCR primers that had T7 RNA polymerase sequences (5'-TAATACGACTCACTATAGGG-3') appended to their 5' ends. After synthesis and purification, the 500 bp dsRNAs were digested into siRNAs (15-25 bp) with RNase III. The siRNAs were diluted to 15 ng/μl in nuclease-free water and injected into the side of the thorax. Each termite received a 35-nl injection that contained 0.5 ng siRNA. For injection, a micro-injector that was fitted with custom-pulled borosilicate glass needles, and a custom vacuum manifold that facilitated termite immobilization, was used. After siRNA injection, groups of 15 workers were held for 24 hours, after which time they were destructively sampled for RNA isolation. Control workers received injections of nuclease-free water alone.

**Bioassays**

Replicated groups of 15 workers were held on one of two treatments that included either 150 μg JH III (Sigma-Aldrich, Milwaukee, WI, USA) or analytical-grade acetone (the solvent carrier for JH). Termites were held within 5 cm diameter plastic Petri dishes on moistened filter papers that were pre-treated with either JH dissolved in acetone or acetone alone. These treatments allowed for the determination of JH-responsive gene expression and the effects of colony-release on gene expression, respectively. Additionally, four holding times were examined in the experiment (0, 5, 10 and 15 days); each was replicated three times with 15 workers. Using this bioassay format, pre-soldier differentiation is typically first observed on day 15 of JH exposure, whereas pre-soldier differentiation is absent in control treatments (Scharf et al., 2005b; Scharf et al., 2005c; Zhou et al., 2006a). At each time point, workers were destructively sampled for RNA isolation and cDNA synthesis (see below).

**RNA isolation, cDNA synthesis and quantitative real-time PCR**

All quantitative real-time PCR (qRT-PCR) primer sequences and Genbank accession numbers are presented in Table 1. qRT-PCR was performed using an iCycler qPCR real-time PCR detection system with iQ™ SYBR™ Green Supermix (Bio-Rad, Hercules, CA, USA). cDNA, which served as the template for qRT-PCR, was synthesized independently from total RNA of 15 individuals. Total RNA and cDNA were obtained using the SV total RNA Isolation System (Promega, Madison, WI, USA) and the iScript™ cDNA Synthesis Kit (Bio-Rad), respectively, following manufacturer protocols. The suitability of the three reference/control genes β-actin, 70 kDa cognate heat shock protein (HSP-70) and nicotinamide adenine dinucleotide-dehydrogenase (NADH-dh) were evaluated using the two software packages Bestkeeper (Pfaffl et al., 2004) and NormFinder (Andersen et al., 2004) as described previously (Zhou et al., 2006a). From these analyses, β-actin was determined to be the most reliable reference gene. Relative gene expression was determined using the method of Livak and Schmittgen (Livak and Schmittgen, 2001), as described in the next section.

**Experimental design and data analysis**

Downstream effects of hexamerin silencing were determined independently from five individuals showing maximal silencing effects at 24-36 hours after siRNA injection. The hexamerin silencing in these five individuals averaged >85% relative to water-injected controls (Fig. 1A). In bioassay experiments comparing colony-release and ectopic JH impacts on gene expression, two analysis procedures were used. In the first procedure, gene expression on assay days 5, 10 and 15 was determined relative to day-0 colony workers (see arrows at the top of Fig. 2A,B). In the second procedure, relative gene-expression levels in JH-treated individuals on each assay day were determined relative to colony-release controls on the same day (see diagram at the right in Fig. 2C). All relative expression levels were calculated by the 2-ΔΔCT normalization algorithm (Livak and Schmittgen, 2001). Mean and standard error were determined by averaging relative expression levels across three independent replicates, each determined in triplicate. Mean expression levels were compared statistically by pairwise Kruskal-Wallis tests in SAS (P<0.05) (SAS Institute, Cary, NC, USA). Linear regressions (shown in Fig. 3) were conducted using the PROC REG procedure in SAS.

**RESULTS**

**Apparent downstream members of a caste-regulatory gene network**

In our first experiment, we investigated the impacts of RNAi-based hexamerin silencing on the expression of well-characterized regulatory and structural genes with homologs that are linked to a number of insect developmental processes. Following RNAi, the two hexamerin genes were silenced 85-95%, whereas the control genes cellulase-1, β-actin, NADH-dh and HSP-70 were unaffected (for details, see Zhou et al., 2006a). In the same individuals, impacts of hexamerin silencing on 15 out of 17 suspected downstream genes were also observed (Fig. 1). Specifically, hexamerin silencing impacts an apparent network of downstream genes/proteins that include signal transduction factors, transcription/translation factors, cuticle proteins and muscle proteins. The downstream genes that

![Fig. 1. Impacts of hexamerin silencing on downstream gene expression. Downstream effects of hexamerin silencing (A) on the expression of 17 putative downstream genes, and the three reference/control genes β-actin, NADH-dh and HSP-70 (B). Results were determined from quantitative real-time PCR data. See Table 1 for gene abbreviations. The y-axis represents the percentage of gene expression relative to water-injected controls 24 hours after treatment (bars represent the mean of five individuals, each determined in triplicate). Bars with asterisks represent significant differences from water-injected controls at P<0.01 (**), P<0.05 (**) or P<0.1 (*), obtained by pairwise Kruskal-Wallis tests. Error bars represent standard error of the mean (s.e.m.).](image-url)
were most highly significantly impacted were a larval cuticle protein (LCP) and the translation factor nanos. Additionally, 13 other genes were impacted significantly. These genes encode four transcription/translation factors (BTB/POZ, 18-S, 28-S, bicaudal), seven signal transducers (PIP kinase, COP9, malonyl Co-A decarboxylase, apoptosis inhibitor, AMP deaminase, GTPase activating protein, ATPase), and two muscle troponin isoforms (Trop-1 and Trop-2). In addition to the control genes noted above, the two genes LIM and SH3 kinase were unaffected by hexamerin silencing.

After observing these effects, we re-verified that no sequence homology exists between the hexamerins and the members of the apparent gene network that showed reduced expression. Thus, we conclude that the observed downstream impacts are not the result of off-target RNAi impacts. Rather, these results suggested that the hexamerins function by regulating the expression of JH-responsive genes, such as the 15 significantly-impacted genes described above. Based on our observation of JH affinity by Hex-1 (Zhou et al., 2006b), we hypothesized that, by binding JH and restricting its availability, the hexamerins apparently influence JH-dependent caste differentiation. To further investigate this possibility, we designed and conducted experiments that tested the impacts of: (i) colony-release and (ii) ectopically-applied JH on a potential genomic network that includes the 15 significantly impacted genes.

**Release from colony conditions impacts expression of hexamerin-responsive genes**

The first step towards testing the hypothesis that the hexamerins regulate JH-responsive gene networks was to determine the effects of colony-release on worker gene expression. These experiments did not involve RNAi, but simply examined gene expression in wild-type termites held away from the colony. The impetus for pursuing this experiment is based on prior evidence showing that, in *R. flavipes*, colony-release significantly influences the expression of several cytochrome P450 genes (Zhou et al., 2006c), as well as that of hexamerin and vitellogenin genes (Scharf et al., 2005c). These experiments were conducted following the bioassay approach presented by Scharf et al. (Scharf et al., 2003b; Scharf et al., 2005b; Scharf et al., 2005c), in which workers are isolated in small groups away from the colony. However, in the current experiments, we evaluated the expression of the 17 genes noted above, as well as *Hex-1* and *Hex-2* and the control genes β-actin, NADH-dh and HSP-70.
The isolation of workers away from colony conditions has pronounced impacts on the expression of the majority of genes at 5, 10 and 15 days of isolation, relative to day 0 (Fig. 2A). For the sake of brevity, we only focus on day 5 results here. In total, seven genes were significantly up-regulated on day 5 of colony-release. These up-regulated genes include Hex-2 (1.86×), nanos (6.38×), COP9 (2.22×), BTB/POZ (18.67×), bicaudal (5.79×), apoptosis inhibitor (2.15×) and GTPase-activating protein (3.90×). A total of four genes were significantly downregulated on day 5 of colony-release. These downregulated genes include larval cuticle protein (0.46×), 28S rRNA (0.55×), AMP deaminase (0.16×), and SH3 kinase (0.13×). Finally, seven genes showed no significant changes by day 5 of colony-release. These findings illustrate the strong influence of the colony on worker gene expression, and emphasize the importance of controlling for colony-release effects when investigating the impacts of experimental treatments on gene expression.

**Ectopic JH treatment also impacts the expression of hexamerin-responsive genes**

The next step towards testing the hypothesis that the hexamerins regulate JH-responsive gene expression was to compare the effects of ectopic JH treatment to the effects of colony-release on gene expression. In this experiment, wild-type (non-RNAi treated) workers were isolated in groups as in the previous experiment; however, filter paper substrates were treated with a concentration of ectopic JH that is capable of inducing pre-soldier differentiation in the majority of individuals (150 μg) (Scharf et al., 2005b). This combination of worker isolation plus JH treatment resulted in more pronounced changes in gene expression relative to worker isolation (Fig. 2B). With JH treatment, the majority of evaluated genes had significantly increased expression on days 5 and 10 of isolation, relative to day 0. Of these, the most significantly upregulated genes on day 5 were Hexamerin-1 and Hexamerin-2 (6.06× and 12.93×, respectively), BTB/POZ (50.32×), bicaudal (15.27×), apoptosis inhibitor (22.36×), GTPase A.P. (11.74×) and both troponin isoforms (Trop-1, 4.87×; and Trop-2, 8.24×). The expression of other genes, such as LCP, AMP-deaminase, ATPase and SH3 kinase, either changed insignificantly or were slightly downregulated.

When observing JH impacts in this manner, however, we suspected that gene expression would probably be exaggerated because of the dual effects of colony-release and JH-induction. In an effort to control for colony-release effects, JH-impacted gene expression (see Fig. 2B) was normalized within day, to colony-release-associated gene expression from Fig. 2A (see Fig. 2C). These results revealed more modest expression changes. For example, expression of Hexamerin-1 and Hexamerin-2 were still significantly induced, but at reduced levels of 2.69- to 2.75-fold greater than at day 0. Other genes, such as nanos and COP9 – which showed pronounced JH-induction relative to colony workers, reversed from significant induction to significant repression when normalizing to colony-release controls. Additionally, other highly JH-induced genes, such as BTB/POZ, bicaudal, apoptosis inhibitor and both troponins, showed reduced induction levels when normalizing to colony-release controls. Thus, by controlling for colony-release effects on gene expression, the impacts of excess JH on gene expression can be more realistically estimated.
Downstream impacts of hexamerin silencing correlate significantly with JH-dependent changes in gene expression

Regression analyses were conducted to specifically test the hypothesis that the hexamerins regulate JH-dependent gene expression (Fig. 3). These analyses tested for correlations between gene expression after RNAi-based hexamerin silencing versus (i) baseline colony-release effects, and (ii) JH-dependent effects. These regressions included only the 17 cytoskeletal, transcription/translation and signal transduction genes compared in Fig. 1. The three reference genes and the two hexamerins were not included in the regression analyses. In the first regression, which compared downstream effects after hexamerin silencing to colony-release effects at days 5, 10 and 15 of isolation, no correlations were significant (Fig. 3, top row). Although not significant, the colony-release regressions showed apparently important outlier trends for the BTB/POZ and larval cuticle protein genes, respectively, on days 5-10 and 15. Specifically, these results for BTB/POZ and LCP suggest that these genes may play roles in suppressing the expression of other network members in response to the release from colony-based suppression.

Regressions comparing gene expression after hexamerin silencing to day-0-normalized JH-associated gene expression (Fig. 2B) showed no significant correlations (graphs not shown). Here, correlation coefficients ($r^2$) for days 5, 10 and 15 were 0.009, 0.049 and 0.022, respectively. In regressions comparing downstream effects after hexamerin silencing with true JH-dependent gene expression on assay days 5, 10 and 15 (Fig. 2C), significant correlations were observed for all days (Fig. 3, bottom row). Furthermore, when apparent outlier genes were removed from the analysis (day 5: apoptosis inhibitor and ATPase; day 10: larval cuticle protein and nanos; day 15: SH3 kinase), the regressions improved considerably (see reduced models in bottom row of Fig. 3). These latter results are highly interesting, in that they suggest temporal changes in the JH-responsive gene network throughout pre-soldier development.

DISCUSSION

This study revealed members of a putative genomic network of JH-responsive genes with links to termite soldier-caste differentiation. The majority of these network members share significant homology to well-characterized developmental genes of both holo- and hemi-metabolous insects. Although other network members probably await discovery, the current list of JH-responsive genes includes 13 genes from the signal transduction, transcription/translation, integumental and cytoskeletal/structural ontogeny categories. These findings also provide novel lines of evidence supporting the idea that the hexamerins modulate JH availability, and therefore regulate JH-mediated phenotypic plasticity.

Although JH signaling is a process that remains poorly understood in insects, JH is widely recognized as the pleiotropic master regulator of insect development and metamorphosis (Truman and Riddiford, 1999; Gilbert et al., 2000; Flatt et al., 2005). In higher holometabolous insects, such as M. sexta, JH functions in suppressing adult-tissue differentiation by inhibiting intrinsic signaling independently of nutritional state and ecdysteroids (Truman et al., 2006). In this respect, JH has been proposed to function as a lipid-signaling system parallel to retinoic acid of mammals and protein prenylation in yeast and fungi (Wheeler and Nijhout, 2003). In termites, which are hemimetabolous insects that display complex and highly derived developmental plasticity, JH has adopted broader functions (Henderson, 1998). Two of the most well-defined of these functions are soldier differentiation at high JH titers and status quo worker-to-worker molts at lower JH titers (Park and Raina, 2004; Mao et al., 2005).

The removal of worker termites from the colony leads to increases in JH titers (Okot-Kotber et al., 1993; Mao et al., 2005), which can result in soldier-caste differentiation in Coptotermes termites (Mao et al., 2005). In Reticulitermes, however, the hexamerins have apparently been co-opted to counter the effects of JH and retain a status quo work force (Zhou et al., 2006a). This also explains why, in Reticulitermes, worker removal from colonies does not lead to pre-soldier differentiation (Okot-Kotber et al., 1993; Scharf et al., 2005b). To overcome the effects of the hexamerins in blocking colony-release-associated caste differentiation, we adopted the model system employed in the current study (Scharf et al., 2003b; Scharf et al., 2005b). Our model system relies on ectopic JH exposure to overwhelm the attenuating effects of the hexamerins and synchronize worker differentiation. Thus, whereas colony-release effects are modest and do not normally permit soldier-caste differentiation in a short time frame, ectopic JH exposure is an effective alternative to induce high levels of soldier-caste differentiation (Scharf et al., 2005b). In this respect, results of the current study provide further evidence supporting the involvement of termite hexamerins in blocking JH-dependent gene expression and morphogenesis.
JH-responsive genes and putative gene networks

Fig. 4A illustrates how members of a JH-responsive genomic network may be interconnected, as inferred from findings of the present study, gene identities, sequence features and homology to other genes with defined functions. At the top of the network are the two hexamerin genes, which are known to respond to JH (present study) (Scharf et al., 2005b; Zhou et al., 2006b) and environmental conditions (M.E.S. et al., unpublished). Immediately downstream, in a manner similar to that proposed by Wheeler and Nijhout (Wheeler and Nijhout, 2003), are downstream network genes that mediate JH signaling. These genes include five signal transducers, seven transcription/translation factors, one cuticle protein and two muscle proteins. At the present time, we propose that these genes are arranged in simple cascades, whereby the genes are sequentially connected in a simple hierarchical fashion (Tapscott, 2005). However, other configurations are certainly possible, such as feed-forward signaling, in which early genes differentially influence multiple later genes, or a single-input cascade, where JH directly influences each network member independently through pleiotropy (Tapscott, 2005). Additionally, at the present time, we have no basis for knowing if responsive genes are members of single or multiple gene networks, or if there is inter- or cross-talk between levels, such as between transcriptional and translational factors. Studies to define network hierarchy are in progress. Details relating to what is known regarding the function of some of the JH-dependent genes, as well as their suspected interconnections, are provided in the following paragraphs.

Signal transducers

Signal transduction is the process by which extracellular signals are transmitted through the cytoplasm, both to and from the nucleus. In the JH-responsive network, our findings suggest that these factors may be acting both upstream and downstream of the transcription and translation factors, but upstream of cuticle and muscle proteins. As proposed by Wheeler and Nijhout (Wheeler and Nijhout, 2003), the possibility should also be considered that some or all of these gene products may directly participate in JH signaling.

The signal transducers malonyl-CoA, PIP kinase, apoptosis inhibitor, AMP-deaminase and ATPase were identified from previous nymphal arrays (Scharf et al., 2005a), whereas the GTPase activating protein (GAP) was identified from soldier arrays (Scharf et al., 2003a). The COP9 gene was identified from pre-soldier arrays (Genbank Accession No. DN792518). Of these signal-transduction factors, most have multiple functions that can vary widely between organisms. Thus, we do not elaborate on their potential roles in termite caste regulation/differentiation. However, the numerous kinase-associated factors apparently play very interconnected roles in myogenesis and cytoskeletal assembly. In particular, the central role of the COP9 gene in these processes is discussed below.

Of the signal transducers investigated here, the COP9 complex homolog subunit 5 gene appears to be very important. Specifically, COP9 expression increases 10- to 20-fold on days 5 and 10 of worker isolation, but remains unchanged with ectopic JH treatment. These findings suggest an important role for COP9 in the suppression of other developmental genes in response to natural, low-level increases in JH titer. The COP9 complex homolog is a complex of eight subunits (termed CSN1-CSN8) that play key roles in development (Harari-Steinberg and Chamovitz, 2004). The termite COP9 subunit investigated here shares significant homology with the Drosophila subunit 5 (blastx e-value: 4×10^{-137}), which plays key roles in Drosophila development by participating in hormonal signaling and by regulating the degradation of gene products that control tissue differentiation and body plan (Freilich et al., 1999; Oron et al., 2002; Björklund et al., 2006). The COP9 complex homolog is a central component of kinase-mediated signal-transduction pathways, with direct impacts on transcriptional regulation (Harari-Steinberg and Chamovitz, 2004; Björklund et al., 2006). The recognized link between the COP9 complex homolog, kinases and transcriptional regulation is very much in-line with our identification of multiple genes related to kinase-signaling and transcriptional regulation. These commonalities, coupled with the highly phosphorylated nature of the hexamerin proteins and their function in endocytosis-based transport, suggest that COP9 and the other signal transducers play highly interconnected developmental roles.

Transcription and translation factors

The translation factors bicaudal and nanos, and the transcription factor BTB/POZ were identified from previous soldier array studies (Scharf et al., 2003a), whereas the translation factor-like 18S- and 28S-rRNA genes were identified from arrays investigating gene expression in immature reproductives (i.e. ‘nymphs’) (Scharf et al., 2005a). Based on their well-defined functions in other organisms, these genes most probably play roles in the transcription and/or translation of downstream structural genes; but also possibly of signal-transduction factors and other transcription/translation factors (Fig. 4A). At the present time, it is not clear if the 18S- and 28S-like genes are true ribosomal RNAs, or if they are pseudo-rRNAs buried in the coding region other genes that participate in ribosomal filtering (Mauro and Edelman, 1997; Mauro and Edelman, 2002; Chappell et al., 2006). The fact that the rRNA-like sequences contain poly-A tails supports the latter possibility (Scharf et al., 2005a).

Although the nanos and bicaudal genes are highly interesting, BTB/POZ-like genes have a far-more conserved importance in insect development (Riddiford et al., 2003; Erezylmaz et al., 2006). The termite BTB/POZ gene is similar to transcriptional regulators, which contain conserved zinc-finger motifs that participate in DNA binding. BTB/POZ is so named because it contains a conserved BTB/POZ domain in its N-terminal region (Scharf et al., 2003a). The termite BTB/POZ gene product shares significant homology with the broad gene identified from numerous insects; for example, the hemimetabolous insect Oncopeltus fasciatus (blastx e-value: 1.0×10^{-33}). Broad, which participates in ecdysone signaling, is a transcription factor from the BTB/POZ family that is characterized by an N-terminal BTB domain and a C-terminal pair of zinc-finger motifs (Zhou and Riddiford, 2002; Riddiford et al., 2003). In Oncopeltus, RNAi-based silencing of broad results in pronounced pigmentation and wing malformations, but has no effect on anisometric growth or molting (Erezylmaz et al., 2006). Interestingly, the Oncopeltus deformities resulting from the silencing of broad are highly consistent with regressive molts that occur in nymphal Reticulitermes (Buchli, 1958; Lainé and Wright, 2003), as well as with aberrations to adult imago termites noted after ectopic ecdysone treatment during immature nymphal instars (Lüscher, 1960).

Cuticle and muscle proteins

Consistent with impacts on cytoskeletal assembly and myogenesis noted above for the signal transducers, we previously identified a larval cuticle protein (Genbank Accession No. DN792534) and two troponin isoforms (Scharf et al., 2003a). These three genes are apparently the furthest downstream members of the putative JH-responsive gene network (Fig. 4A). Cuticle proteins occur in differentiating tissues that are extremely sensitive to JH and ecdysone (e.g. imaginal disks); they are involved in processes related
to chitin binding and cuticle hardening, among others (Willis, 1999; Takeda et al., 2001). Because of the high degree of sclerotization of the termite soldier head, and because soldier differentiation is induced by JH, it is logical to suspect that cuticle proteins would be JH-responsive. In this regard, the R. flavipes cuticle protein gene was only significantly induced on day 10 of JH exposure, and was markedly down regulated on days 5 and 15. This cuticle protein gene was also significantly upregulated on day 15 of colony-release; possibly reflecting a slower rise in JH titers after colony-release and/or other undefined primer phenome effects. Here, it is noteworthy that four similar cuticle proteins were identified during juvenoid-induced soldier differentiation in the termite Hodotermposis sjostedti (Koshikawa et al., 2005). The translated cuticle protein sequence of R. flavipes is most similar to the H. sjostedti cuticle protein HsjCP1 (86% identity; e-value: 4×10^{-17}). Most interesting here is that three of the four H. sjostedti cuticle proteins showed identical induction trends to that observed for the R. flavipes cuticle protein (see Fig. 2C).

JH-induced differentiation of the soldier caste is associated with a large increase in body mass and musculature, especially in the head where a large muscle mass is required to drive the enlarged soldier mandibles. The two troponin I isoforms that were evaluated here share similarities with heldup-mutant alleles that occur in Drosophila; specifically wings-up and wings-apart (Beall and Fryberg, 1991). Our findings showed consistent JH-induction of both forms of troponin across all 15 bioassay days. Originally, it was proposed that the expression of these troponin isoforms in soldiers might be associated with flight-muscle degeneration, as occurs in heldup mutants of Drosophila (Scharf et al., 2003a). Although the results of the present study do verify that both troponin forms are members of a putative JH-responsive gene network, their true functions remain unknown. Nonetheless, it is logical to deduce that troponin muscle proteins, as well as other muscle proteins not investigated here (Scharf et al., 2003a), are probably the most downstream members of the genomic network. Future studies that work to dissect this apparent gene network will investigate a number of cytoskeletal/muscle protein-encoding genes not included in the present analysis.

**Termite hexamerins and impending issues**

Evidence from anti-JH blotting studies suggests that the hemolymph-soluble Hexamerin-1 protein is capable of covalent JH binding. Alternatively, the hemolymph-soluble Hexamerin-2 protein exhibits no such JH affinity, but it does have membrane-binding characteristics much like the well-studied hexamerin receptors of higher solitary insects (Zhou et al., 2006b). The R. flavipes hexamerins, which constitute a majority percentage of total worker protein, also have highly unique sequence features relative to most other known hexamerins. For example, Hex-1 has a completely unique hydrophobic tail and prenylation motif that is similar to non-insect sesquiterpene-binding motifs (Zhou et al., 2006b). Furthermore, Hex-2 has a long hydrophilic insertion plus several protease-cleavage-like sites that are consistent with hexamerin receptors (Zhou et al., 2006b). Thus, when taking these characteristics into consideration, along with previous RNAi results (Zhou et al., 2006a) and JH-binding functions known for other insect hexamerins (Braun and Wyatt, 1996; Tawfik et al., 2006), it is not unreasonable to hypothesize that these two proteins cooperate to sequester JH and modulate its efficacy. In a broader context, these unique proximate characteristics support the idea that hexamerins have been co-opted in termites to ultimately inhibit irreversible morphogenesis towards the soldier caste.

The hexamerins apparently modulate JH-dependent gene expression both by being JH-inducible and by sequestering JH (Fig. 4B). Thus, when hexamerin titers are high, JH availability (not necessarily JH titer) is presumably low and pre-soldier differentiation is attenuated. Evidence in support of the JH sequestration hypothesis came from previous RNAi studies in which it was observed that, when worker hexamerin levels are attenuated by RNAi, JH-dependent pre-soldier differentiation is significantly elevated (Zhou et al., 2006a). As shown in the present study, colony-release also has pronounced impacts on hexamerin and affiliated downstream gene expression, apparently as a result of rising JH titers and/or a release from other primer phenome influences that result from a removal of colony-based suppression (Okot-Kotber et al., 1993). To conclusively test the hypothesis that the hexamerins are capable of JH-sequestration, JH-binding studies will be necessary. Other studies, such as those measuring JH titers after hexamerin silencing, might also be informative, but this might also yield confusing results because of competing colony-release-dependent increases in JH titer. Regardless, despite a lack of conclusive JH-binding data, evidence obtained to date shows a clear responsiveness by the hexamerins to JH treatment as well as attenuation of JH-dependent caste differentiation.

More importantly, the growing body of evidence discussed above points towards an important question: what are the socio-regulatory factors that impact relative titers of both JH and hexamerins? (Fig. 4B). Because solitary-insect hexamerins play a very well-documented role in nutrient storage (Burmester and Scheller, 1999), one possibility is that food quality/quantity and nutritional status are important extrinsic and intrinsic factors that impact hexamerin titers (Nalepa, 1994). Colony conditions, such as caste composition (reviewed in Wilson, 1971), auditory stimuli (Evans et al., 2005) and primer pheromones (Wilson and Bossert, 1963; Lefevue and Bordereau, 1984; Vander Meer et al., 1998; Korb et al., 2003), may also have either direct or indirect impacts on hexamerin titers. With respect to environmental influences on JH, conditions such as season, temperature and moisture must also certainly play a role (Huang and Robinson, 1995; Liu et al., 2005a; Liu et al., 2005b; Suzuki and Nijhout, 2006). Finally, other intrinsic factors, such as sex, developmental instar and the JH-modulating allatostatin peptides (Yagi et al., 2005), are also likely to play roles in influencing hexamerin titers via the modulation of JH synthesis. To characterize this system more fully, future research should be aimed towards more than hexamerin-JH interactions alone; such studies should also be aimed towards understanding the elaborate interactions of these potential intrinsic and extrinsic socio-regulatory factors. As discussed below, the current study provides important insights into how to proceed in addressing these questions.

**Future research considerations and conclusions**

In addition to elucidating a mechanism responsible for regulating the JH-dependent expression of developmental genes, the findings of this research have refined questions and approaches for future investigations into eusocial polyphenism. Most notably, we observed significant effects of colony-release on gene expression. This suggests that colony-release effects must be taken into account when conducting any experiments that involve the isolation of colony members away from colony influences. For this purpose, we found that normalization of gene expression for treated individuals to colony-release-control individuals provides for a more realistic assessment of gene expression. Also, in a broader context, our observation of significant colony-release effects suggests interesting and readily approachable ecological-developmental research.
questions (Gilbert, 2001): specifically, how is gene expression influenced under variable extrinsic conditions such as caste composition, food quality and seasonality? Finally, as also shown here, the use of RNAi to quantify downstream effects by upstream regulatory genes is a useful approach for delineation of caste-regulatory gene networks. This approach is being applied further in ongoing investigations that will define the hierarchy of JH-responsive networks and how their members are interconnected.

In conclusion, we have shown here that, in R. flaveipes workers, targeted hexamerin silencing results in extensive downstream impacts on an apparent genomic network of developmental and structural genes. Of the findings reported here, the most important is that downstream impacts of hexamerin silencing correlate significantly with JH-dependent changes in gene expression. This determination provides strong evidence to support the idea that the hexamerins modulate JH availability, thereby attenuating true JH-responsive networks and how their members are interconnected.

We thank Daniel Hahn for providing a critical reading and valuable comments to the revised manuscript draft. This work was supported by the Florida Agricultural Experiment Station, by a gift from Procter and Gamble Inc., and with start-up funds provided to M.E.S. by the Institute of Food and Agricultural Sciences of the University of Florida.

References


