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Transcriptome profiling of female alates and egg-laying queens of the Formosan subterranean termite

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ABSTRACT

Termites are known to have an extraordinary reproductive plasticity and capacity, but the underlying genetic patterns of termite reproductive biology are relatively understudied. The goal of this study was to identify genes for which expression levels differ between dealated precopulatory females (virgins) and egg-laying queens of the Formosan subterranean termite, *Coptotermes formosanus* Shiraki. We constructed a normalized polyphenic expressed sequence tag (EST) library that represents genomic material from most of the castes and life stages of the Formosan subterranean termite. Microarrays were designed using probes from this EST library and public genomic resources. Virgin females and queens were competitively hybridized to these microarrays and differentially expressed candidate genes were identified. Differential expression of eight genes was subsequently confirmed via reverse transcriptase quantitative PCR (RT-QPCR). When compared to virgins, queens had higher expression of genes coding for proteins related to immunity (gram negative binding protein), nutrition (e.g., termite-derived endo-beta-1,4-glucanase), protein storage, regulation of caste differentiation and reproduction (hexamerin, juvenile hormone binding protein). Queens also had higher transcript levels for genes involved in metabolism of xenobiotics, fat, and juvenile hormone (glutathione-S-transferase-like proteins, and cytochrome P450), among others. In particular, hexamerin, juvenile hormone binding protein, and a cytochrome P450 from the 4C subfamily are likely to be involved in initiating the inactive period during the reproductive cycle of the queen. *Vice versa*, virgins had higher expression than queens of genes related to respiration, probably due to recent flight activity, and several genes of unknown function.

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1. Introduction

Termites (order Blattaria, formerly known as Isoptera, Inward et al., 2007) are social insects of immense ecological and economical importance. Gene expression in termites has received increasing interest in the last decade (Scharf et al., 2003, 2005a) mainly, because several termite species are important pests and the study of functional genomics identified promising leads for pest control (Zhou et al., 2008); furthermore, there are unique and interesting features of termite biology that are far from being understood.

The expression of genes involved in caste differentiation (Zhou et al., 2006a, 2007) and cellulose digestion (Watanabe and Tokuda, 2001; Scharf and Tartar, 2008) has received the most attention in the field of termite functional genomics. However, genes involved in reproduction are also of prime interest for basic scientific and applied reasons (Scharf et al., 2005a; Weil et al., 2007, 2009; Korb et al., 2009). The breeding system of many termite species, especially among the subterranean termites (Rhinotermitidae), shows remarkable plasticity (Thorne, 1997; Vargo and Husseneder, 2009). Colonies may be headed by a single pair or multiple reproductives with varying degrees of inbreeding (Husseneder et al., 1999; Thorne et al., 1999; reviewed in Vargo and Husseneder, 2009). Reproductives can be derived from alates (i.e., winged adult forms) or from nymphs and workers (i.e., inbreeding immature forms, Thorne, 1997). Some subterranean termite queens reproduce by parthenogenesis (Matsuura et al., 2009). Our understanding of the processes underlying termite reproductive biology, including sex differentiation, mate choice (Husseneder and Simms, 2008), and development into reproductive forms (Vargo and Husseneder, 2009), remains exceptionally limited.

Only a few studies (Scharf et al., 2003, 2005a; Raina et al., 2004, 2007; Brent et al., 2007; Elliott and Stay, 2007; Maekawa et al., 2010) have addressed the underlying molecular, cellular, and physiological processes that drive reproduction in termites. As a first step to describe gene expression involved in formation of reproductives in termite colonies, Scharf et al. (2005a) identified genes that were differentially expressed in nymphs, alates (future reproductives) and neotenic reproductives (inbreeding offspring of a colony) of

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Reticulitermes flavipes. Maekawa et al. (2010) subsequently described the reproductive cycle of *Reticulitermes speratus* in relation to changing juvenile hormone titers and vitellogenin gene expression. Only one other study investigated differential gene expression involved in division of reproductive labor in another lower termite (*Cryptotermes secundus*, Kalotermitidae): Weil et al. (2007) described five overexpressed genes in female neotenics in this species. Four of these genes putatively coded for secretory proteins implicated in pheromone and hormone processing. One gene that was expressed in all body parts of female neotenics and also in male reproductives coded for a vitellogenin (egg yolk and storage proteins).

We chose the Formosan subterranean termite, *Coptotermes formosanus* (Blattaria, Rhinotermitidae) as our model to study gene expression in reproductives. This invasive species is an important pest in Asia, the Pacific Rim and the United States (Su and Tamashiro, 1987) and increased knowledge of reproductive biology may lead to novel biotechnology-based termite treatments that disrupt reproduction and colony propagation. Colonies of the Formosan subterranean termite are founded by a single pair of reproductives, which mate for life and raise their offspring cooperatively (Vargo et al., 2003, 2006; Husseneder et al., 2005; Husseneder and Simms, 2008). The founding pair originates from alates, i.e., the winged adults, which are produced by mature colonies during swarming season (April through July in New Orleans, Louisiana, Henderson and Delaplane, 1994). After the flight, alates drop to the ground, shed their wings and form tandem pairs with potential partners (precopulatory pairs, Raina et al., 2003; Husseneder and Simms, 2008). As soon as a suitable nest site is found, tandem pairs become the founders of new colonies. Major morphological, physiological, and behavioral changes mark the transition from precopulatory adults to reproductive pairs. Mating occurs within 1 day in the laboratory and multiple matings, as often as three times per day, are essential to maximize reproduction (Raina et al., 2003). Egg-laying starts 2 days after mating and about 40 eggs are laid within the first reproductive cycle, i.e. 60–70 days after colony foundation (Raina et al., 2003). The reproductive pair cares for the first generation of eggs and larvae. Males and females increase in weight after colony foundation, with the queen becoming increasingly physogastric (Raina et al., 2003). Flight muscles degenerate, presumably to fuel egg production (Tian et al., 2004). Ovaries, spermathecae, and testes increase in size (Raina et al., 2003, 2007).

We conducted this seminal study of gene expression in female colony founders of the Formosan subterranean termite to describe the changes in transcription when virgin alates become egg-laying queens. This study adds critical knowledge about underlying molecular genetic patterns and processes that drive differentiation and breeding of reproductives to the previous data gained from the study of nymphs, alates and neotenics (Scharf et al., 2005a; Weil et al., 2007). We (1) constructed a normalized expressed sequence tag (EST) library of multiple castes and life-stages of Formosan subterranean termites, (2) designed microarrays using ESTs as probes to screen for differentially expressed candidate genes in virgins and queens, and (3) quantified differential expression of eight candidate genes via reverse transcriptase quantitative polymerase chain reaction (RT-QPCR).

2. Materials and methods

2.1. Tissue collection for the cDNA library

We collected different castes and developmental stages of Formosan subterranean termites from the New Orleans, Louisiana population. Eggs, multiple larva stages, workers, presoldiers, soldiers, male and female nymphs (immature reproductives) and neotenic queens were collected from a carton nest provided by the City of New Orleans Mosquito and Termite Control Board in spring 2005. Guts from workers were extirpated using sterile forceps during the collection process to reduce the abundance of symbiont material in the samples. The tissue samples were snap-frozen in liquid nitrogen and stored at −80 °C. Tandem-running males and females that had shed their wings were collected during an alate swarm in New Orleans (May 2005). Alate swarms contain a mixture of alates from different colonies (Husseneder et al., 2006; Husseneder and Guillot, 2010) from the New Orleans population. Usually the female leads the male in the tandem, but sex of the individuals was confirmed in addition by examining the terminal abdominal sterna using a stereomicroscope (Husseneder and Simms, 2008). Twenty pairs were immediately preserved in liquid nitrogen (precopulatory males and females, i.e., virgins), and the remaining pairs were used to establish 100 incipient laboratory colonies. The paired colony founders (kings and queens) were kept in 125 ml beakers containing moist pine shavings and vermiculite. Two months after formation of incipient laboratory colonies and while queens were laying eggs, queens were snap-frozen in liquid nitrogen and stored at −80 °C.

2.2. cDNA library construction

We submitted the samples on dry ice to Invitrogen (Carlsbad, CA) for RNA extraction and construction of a custom normalized cDNA library (Gubler and Hoffman, 1983). Invitrogen extracted total RNA from each sample and combined equal amounts of RNA of each of the different castes and developmental stages to a total amount of at least 1 mg of RNA. Messenger RNA (mRNA) was purified from total RNA and used to construct cDNA. The cDNA library was normalized using Invitrogen's subtraction technology to reduce the high variance in abundance of the same mRNA/cDNA type (Soares et al., 1994). The library was confirmed to have at least 3×10^6 clones with an average insert length of >1.4 kb; more than 50% of the clones had full length cDNA inserts.

2.3. EST sequencing, contig assembly and annotation

We transformed *Escherichia coli* colonies with pCMV.SPRT6.1 vectors containing the cDNA inserts, according to Invitrogen's recommended methods. The resulting plasmids were sequenced using pTV-poly T anchored primers by the IIGB-Core Facility, University of California at Riverside and the DNA Sequencing Core Facility, University of Florida. Clones were selected at random, sequenced, trimmed, and submitted to dbEST (Boguski et al., 1993) (http://www.ncbi.nlm.nih.gov/Genbank/index.html).

We formed contiguous sequences from the ESTs using the the SeqMan Pro Assembler software package (DNASTAR® Lasergene®, Version 7.2; DNASTAR Inc., Madison, WI). We used the parameters: Match Size 25; Minimum Match Percentage 80; Match Spacing 150; Minimum Sequence Length 100; Maximum Mismatch end bases 15. Additionally, we scanned for and eliminated vector sequence during assembly. We then selected the longest, high-quality sequences for use as microarray probes (described in Section 2.4).

We annotated a subset of the ESTs that represented the longest unique (non-redundant) sequences using the BLAST2GO software suite (Conesa et al., 2005). First, the BLASTX algorithm (Altschul et al., 1997) was used to query the non-redundant protein database at the National Center for Biotechnology Information; we used a minimum E-value level of 1.0E-5 and a high-scoring pair length cutoff of 33 bp. Next, we used the “mapping” function to obtain gene ontology (GO) information for each of the sequences (Ashburner et al., 2000). We then used the ANNEX (Annotation Augmentation) function to verify and remove low-level GO terms. Annotation, protein domain, and KEGG enzyme searches were then performed using the “annotation”, “Interpro search”, and “GO-enzyme code” search functions, respectively, for which we used the default parameters. Finally, we merged the Interpro search results with the annotation results,

and removed sequences that originated from the same contig or Genbank accession number.

2.4. Microarray design

We submitted 9190 EST sequences to the Agilent online eArray software suite for use as probe templates. Default settings were used for probe design. There were 7801 probes printed to the array, of which 4154 originated from the *C. formosanus* EST library, 1404 from dampwood termites (*Hodotermopsis sjoestedti*) and 1102 from other subterranean termites (*R. flavipes*), 10 from honey bees (*Apis mellifera*), 781 from gut protozoa of *C. formosanus* and *R. speratus* and 385 from cockroaches (*Blattella germanica* and *Periplaneta americana*) to fill the remaining spots on the array. Ten probes for *Arabidopsis thaliana* were included as negative controls. The subarray of probes was printed eight times on the slide for eight biological replicates (competitive hybridizations of individual queens and virgins). Each probe was printed twice in each subarray at a non-adjacent site, except those of cockroaches and *A. thaliana* (negative control), which were only printed once, and Agilent added its Spike-In controls to reach a total of 15,744 features on the array.

2.5. Microarray protocols

Total RNA was extracted from whole bodies of precopulatory females (virgins) from eight tandem-running pairs and eight egg-laying queens from the laboratory colonies using the Qiagen Plant RNAeasy system (Qiagen), and stored at −80 °C until use in microarray-related reactions. We synthesized Cy3- and Cy5-labeled complementary RNAs (cRNA) from mRNAs using the Agilent Quick-Amp Labeling kit, according to the manufacturer's instructions. Eight separate mixes (biological replicates) of Cy3- and Cy5-labeled cRNAs (each representing one virgin and one queen, 500 ng per sample, 1 μg total) were hybridized to eight individual subarrays. Four of the mixtures were prepared with virgins labeled with Cy3 and queens labeled with Cy5, and four of the mixtures were prepared as a dye-swap to account for dye incorporation effects (Zakharkin et al., 2005). We included the recommended elongation factor 1-alpha controls for use in quality control and discarding spots (described in Section 2.6). We hybridized 1 of 8 separate mixes (biological replicates) of Cy3- and Cy5-labeled cRNAs (representing one virgin and one queen) to each individual subarray, and covered them using a compartmentalized slide. The microarray with hybridized samples was incubated for 16 h at 65 °C, washed the next morning using the Agilent recommended buffers, and scanned immediately thereafter. We scanned the slide using a GenePix Personal 4100A scanner (Molecular Devices, Sunnyvale, CA, USA) and spots were quantified using the GenePix Pro 6 platform (Molecular Devices).

2.6. Microarray data analysis

Fluorescence data was exported into Microsoft Excel (Microsoft Inc., Redmond, WA, USA) for processing. For quality control, we discarded all spots for which intensity was below that of the lowest spike-in control (~4475 pixels) which represented the lowest intensity at which spots could be visually inspected using the software interface. Raw intensity values were converted to a log2 scale, and normalized using the "efficiency common array dye-swap" method as implemented in the Bioconductor suite for the R software environment (Gentleman et al., 2004). We averaged the two replicate spots after eCADS normalization but before statistical analysis. We used a mixed analysis of variance to compare normalized log2 signal between virgins and queens using the SAS system (PROC MIXED, SAS/STAT Software, SAS Institute Inc., 1999), using a model statement that accounted for the random effect of individuals nested within dyes. We calculated Q-values for each EST using the software Q-value (Storey and Tibshirani, 2003). The use of Q-values is based on false discovery rate approaches (Benjamini and Hochberg, 1995) that avoid problems with multiple testing in large microarray datasets. We adopted a false discovery rate of 5%; effects were considered to be significant when $Q<0.05$.

2.7. Validation of microarray results using reverse transcriptase quantitative PCR (RT-QPCR)

We performed technical validation of the microarray data for eight genes for which transcript concentration was different between virgins and queens. Complementary DNA (cDNA) was reverse-transcribed from the original total RNA samples using the Invitrogen Superscript II kit with oligo d(T) primers. We designed primers for ESTs that originated from *C. formosanus* sequences using the PRIMER-BLAST software suite located at the NCBI website. Amplicons were designed to be less than 100 bp to ensure efficient amplification. For ESTs that originated from other termite species, we used the assay design service of Primer Design Ltd, Southampton, UK (www.primerdesign.co.uk) (Table 1). RT-QPCR was performed using the iCycler IQ real-time PCR detection system with SYBR-green product tagging (BioRad, Hercules, CA, USA). Annealing temperature of 55 °C was used for all primers. Template was amplified in reactions that each contained 10 ng of cDNA (as measured by total RNA input), 12.5 μL of SYBR-green master mix (BioRad), a concentration of 150 nmol forward and reverse primer, and water for a total

Table 1
Primer sequences and melting temperatures (T) for RT-QPCR amplification of eight ESTs to quantify differential expression between queens and virgins.

Putative gene function	Species of origin	Acc #	Primer sequences	T [°C]
GNBP 2	*Drepanotermes rubriceps*	DQ058934	CTTCCCAGTGTCGAAAGCTC	60.0
			TGGGTACCCCATAGGAGATG	59.6
NADH–ubiquinone oxidoreductase Fe-S protein 7	*Hodotermopsis sjoestedti*	DC236251	CTCAACCGCGCTCACCAT	60.1
			GTCTTCCGATCCGCTAGTGG	60.4
Endo-beta-1,4-glucanase	*Coptotermes formosanus*	FK835315	GCGAATATCACCAGACCCCA	60.1
			TCCAATGGCGTACACAGCC	60.1
JHBP	*Reticulitermes speratus*	BJ979031	TGGATACACGCCCCTACAAT	60.2
			ACCTGAAGCACCCTGAAAGA	59.8
Hexamerin 1	*Coptotermes formosanus*	FK833856	AAGTCCTTGCTGCGGATCTG	59.9
			TGACAAGCCACTGGGATTCC	59.7
CYP4C9	*Coptotermes acinaciformis*	AF046008	TCTTTCAGGGTGCTTCAGGT	59.8
			CTGTGGAGCTTTCGACACTG	59.6
GST	*Hodotermopsis sjoestedti*	DC239424	GTTTCAAGCCTCGCTTTGAC	60.0
			CGTCACTGTGAGACTGCCAT	59.9
Fibrillin/von Willebrand factor domain	*Coptotermes formosanus*	FK831422	CCGGATCCAGCAGTAGGGTT	59.9
			CGAAGCACTTGAGGACATCG	60.2

volume of 25 μL. Signal intensity after each cycle and raw fluorescence data were captured using the included BioRad software and exported into Microsoft Excel for further analysis.

Gene expression was calculated by the relative concentration (RC) of a target gene transcript (*target*) compared to an endogenous control transcript of a reference gene (*ref*) using the formula [RC = $(E_r^{cT\,(target)}/E_r^{cT(ref)})$], where E_r is the reaction-specific amplification efficiency estimated using LinRegPCR software (Ramakers et al., 2003) and cT is the fractional cycle at which amplification reached a detection threshold. Putative proteasome subunit beta 7 (Genbank accession no. DC236606 from *H. sjoestedti*) was chosen as the reference gene because it varied the least among the microarray samples, and furthermore because cT did not differ between virgins and queens (ANOVA; $P = 0.33$). Average RC was compared between virgins and queens using a mixed model analysis of variance using the SAS system (PROC MIXED, SAS/STAT Software, SAS Institute Inc., 1999), using a model statement that accounted for the random effect of individuals. Differences were considered to be significant when $P < 0.05$.

Since fertile queens were only available from incipient laboratory colonies, whereas virgins could only be collected from the field, we performed an additional experiment to increase our confidence that the differential gene expression between queens and virgins was not a lab artifact. We compared gene expression of four genes between freshly-collected workers or soldiers, and workers or soldiers that had been kept in the laboratory for two months from the same colony (n = 4 for each category). RNA collection, cDNA synthesis, and RT-QPCR protocols were the same as described above.

We tested for differences in gene expression between fresh-collected and lab-held individuals (rearing condition) and between soldiers and workers (caste) and for the interaction of rearing condition and caste using a two-way ANOVA (PROC MIXED, SAS/STAT Software, SAS Institute Inc., 1999). Differences were considered to be significant when $P < 0.05$. Additionally, we used the “SLICE” function to test for differences within the levels of rearing condition and caste.

3. Results and discussion

3.1. EST generation

A total of 14,600 randomly picked clones were sequenced, which resulted in 7663 high-quality sequences that were trimmed and submitted to dbEST (Boguski et al., 1993). The ESTs were awarded identifiers 59261965 to 59269627 and GenBank accession numbers FK829415 to FK837077 (http://www.ncbi.nlm.nih.gov/Genbank/index.html). A total of 4726 contiguous sequences were assembled from the 7663 sequenced clones, which were submitted to eArray for microarray probe design.

3.2. Putative identities for Formosan subterranean termite ESTs

After annotation using BLAST2GO and removal of sequences which originated from the same contig or accession number, there were 3436 unique EST sequences without redundancy to other ESTs. Of these, 1062 had significant (E < 10-5) matches to the nr protein database, although not all of the matches had known identities (e.g., hypothetical proteins). Forty-six of these (4%) had an E-value of 1e-100 or less and are therefore considered to have highly significant homology, 582 (55%) had significant homology (1e-20 and 1e-99) and are moderately similar, and 434 (41%) had weak homology (1e-05 and 1e-19) and are thus weakly similar (Habermann et al., 2004; Coblentz et al., 2006, Fig. 1). Among the top BLAST hits from ESTs that had significant matches in Genbank, 472 (44%) top BLAST hits were to insect ESTs [the body louse (*Pediculus humanus corporis*), the flour beetle (*Tribolium castaneum*), the honey bee (*A. mellifera*), a parasitoid wasp (*Nasonia vitripennis*), vinegar flies (*Drosophila sp.*), the pea aphid

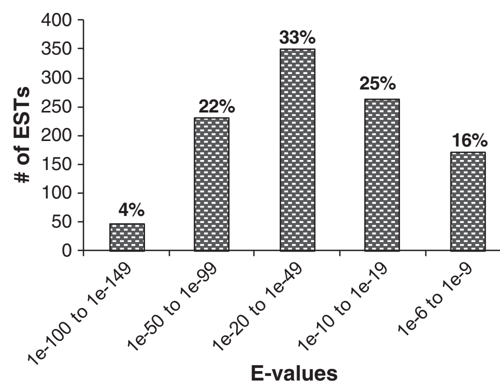


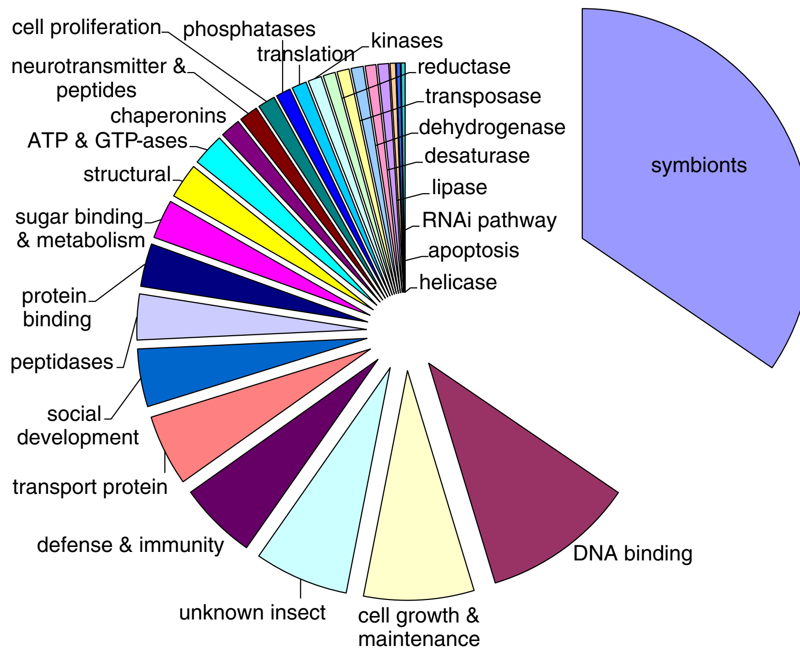
Fig. 1. E-value distributions of EST matches.

(*Acyrthosiphon pisum*) and the mosquito *Aedes aegypti*], 444 (42%) were to bacterial or protist ESTs, and the remaining 146 (14%) were to other ESTs (vertebrates, plants, and fungi). Nearly a third (305) of these BLAST hits originated from the pathogenic flagellate *Trichomonas vaginalis*. Fig. 2 shows the proportional representation of putative gene function categories based on GO terms and literature searches.

The purpose of this study was to use the EST library and existing gene resources as platforms to design microarrays to identify genes important in termite reproduction. Therefore, we only present a preliminary annotation of the EST library; more candidate genes of interest may be revealed when searches for targeted pathways are completed. Since it was not our goal to use the EST library as a standalone screening method, but as a platform for designing microarrays to specifically investigate differential gene expression among virgins and queens, we only briefly present the library results with emphasis on genes that reflect special characters of termite biology.

A third of the ESTs in the library were of bacterial and protozoan origin, underscoring the importance of symbiosis in subterranean termite biology (reviewed in Husseneder, 2010). Although we removed guts from the workers, which are responsible for food digestion and contain the bulk of the protozoan (Lai et al., 1983) and bacterial symbionts (Shinzato et al., 2005; Husseneder et al., 2010), symbionts in the guts of larvae, soldiers, nymphs and alates likely ‘contaminated’ the library.

Besides protein families involved in regular cellular processes, symbiont genes of interest encode putative membrane proteins (bacterial and protozoan ankyrins, e.g., FK830769, FK835454) and surface antigens (bacterial and protozoan BspA-like proteins, e.g., FK835561, FK836786), which are possibly involved in colonization of the host and aggregation (Sharma et al., 1998), and proteins for protozoan motility (e.g., dynein (FK833686, FK834676, FK836946), Satir, 2003). The library also contained genes that are important in symbiont metabolism and energy production, e.g., genes similar to those coding for alcohol dehydrogenase (FK830138, FK836942), malate-dehydrogenase (FK836591), pyruvate:ferredoxin oxidoreductases (FK836517, FK836935, Upcroft and Upcroft, 1999), iron hydrogenases (FK831040, FK833833, FK834619, Inoue et al., 2007; Cao et al., 2010), and metallopeptidases (FK829586 FK837021, FK834724), among others. Carbohydrate metabolism enzymes of the symbionts received previously the most attention, since termite survival is dependent on symbionts aiding in cellulose digestion and the termite gut is considered a reservoir of enzymes with potential application for industrial biomass degradation (Sun and Scharf, 2010). Our library contains symbiont genes coding for enzymes in gluconeogenesis (FK834487, FK836790, FK836793) and glycolysis (e.g. Enolases, FK833849, FK834626, FK834808) as well as various cellulases and hemicellulases. Interestingly, we found cellulases and hemicellulases of putative bacterial origin, e.g., several candidate beta-glucosidases (glycoside



Proportions are given from the largest (symbionts origin) to the smallest category (helicase).

Fig. 2. Annotation of the EST library.

hydrolase family GHF3, FK834553, FK835592, FK834973) and beta-galactosidases (FK834973, FK835502) from different bacteria species, but the usually abundant and well described protozoan cellulases from over a dozen glycoside hydrolase families (Nakashima et al., 2002a; Watanabe et al., 2002; Inoue et al., 2005; Todaka et al., 2007; Tartar et al., 2009) were missing from this library (probably due the removal of the worker guts prior to library construction).

About 2/3 of the EST's were of putative insect origin. Termite-derived genes involved in sugar binding and metabolism include cellulases and hemicellulases, which are known to complement those of the symbionts to form the dual lignocellulose digesting system of the termite gut (Nakashima et al., 2002b), as well as genes involved in glycolysis and glycogen metabolism. As expected, our library contained many endoglucanases from the most prominent glycosyl hydrolase family GHF 9 (endo-beta-1,4-glucanases, Nakashima et al., 2002b, FK835315, FK832326, FK834375, FK834385, FK836530, FK836533, FK836535, FK835602, FK833777, FK833778, FK833780, FK833782), which comprise the majority of termite endoglucanases that were likely inherited from a wood-feeding roach ancestor (Lo et al., 2000). In addition, genes from GHF 5 (a fungal exo-1,3-beta glucanase) and GHF 35 (beta-galactosidase, FK834977) were detected. The GHF 1 genes found in the library (beta-glucosidase, FK835429, FK834671) were similar to the genes overexpressed in female nymphs of several Cryptotermes species (Weil et al., 2009).

Tartar et al. (2009) found 25 genes encoding for termite-derived candidate genes involved in lignin digestion in their EST library of *R. flavipes* guts. In concordance with their study, we found genes encoding for several cytochrome P450 families known from other insects (FK834421, FK834422, FK834859, FK835350, FK835449, FK833807, FK833823, FK834655), which are likely playing a role in lignin side-chain oxidation (depolymerization) and demethylation and detoxification in general (Geib et al., 2008) as well as caste regulation (see below). Genes with predicted carboxylesterase function (FK834562) may also aid in the solubilization and depolymerization of lignin and hemicelluloses by hydrolyzing carboxylester bonds (Scharf and Tartar, 2008). Insect multicopper oxidases (FK835519) could function as laccases, which are known to degrade lignin (Scharf and Boucias, 2010).

Genes in our EST library putatively related to termite caste development include those coding for various putative enzymes and carrier proteins involved in synthesis, breakdown, binding, and transport of juvenile hormone (JH). Juvenile hormone, in general, regulates embryogenesis, larval development, and stimulates reproductive maturation in insects (Kolodziejczyk et al., 2003). In termites, specifically, it is known to regulate caste differentiation (Lüscher, 1960; Henderson, 1998; Scharf et al., 2005b; Elliott and Stay, 2008). Whereas high JH titers stimulate soldier differentiation, low JH titers are required for nymphal and alate differentiation (Nijhout and Wheeler, 1982; Cornette et al., 2008) and excess JH may block egg-production (Sutherland et al., 2000). Therefore, JH titers need to be tightly regulated. Our EST library contained genes for putative hormone and pheromone receptors, juvenile hormone binding proteins (JHBP-like, FK833677, FK835518, FK835549, FK834677, FK834866) for transport of JH and juvenile hormone esterases (FK830195) catabolizing JH (Henderson, 1998). Also likely to be involved in caste regulation are genes encoding for hexamerins (FK836845, FK833856), which are highly similar to the hexamerins 1 and 2 described in detail from *R. flavipes* (Scharf et al., 2005b; Zhou et al., 2006b). Both hexamerins are inducible by JH (Scharf et al., 2005b) and bind JH (Braun and Wyatt, 1996; Zhou et al., 2006b) and are thus involved in modulation of JH availability (similar to JHBP above) and regulation of hormone-dependent gene networks (Zhou et al., 2007). Cytochrome P450 genes (see above), are also implicated in the synthesis and metabolism of pheromones and hormones (Feyereisen, 1999; Zhou et al., 2006c), such as JH.

Termites are at increased risk for exposure to potentially pathogenic microorganisms because they nest and forage in high densities with genetically similar nestmates in an environment with high microbial density (decaying wood and soil) and a microclimate that is conducive to the growth of microorganisms (Rosengaus et al., 2003). Therefore, termites are considered to be valuable model systems for studying insect immunity. The library contained genes from several main pathways of innate immune defense (Schmid-Hempel, 2005),

e.g., a putative gene encoding for a TATA-binding protein-associated helicase that activates antimicrobial immune genes in *Drosophila* (Yagi and Ip, 2005, FK835420), genes that express antibacterial peptides (defensin-like, FK829620, FK829867, FK830579, FK830747, FK830879 FK831100 FK833556), lectin-like peptides for binding galactose and lipopolysaccharides (possibly for recognition of bacterial infection, FK835521 FK835392 FK834461), peptides from the immunoglobulin domain cell adhesion molecules subfamily (FK829690, FK833816, FK833819, FK833857, FK835285, FK835436), and putative chitin-binding proteins (FK829545, FK831407, FK832557, FK832766). We also found genes coding for serine proteases that would possibly activate enzymes of immune response pathways (Gorman and Paskewitz, 2001, FK831407, FK835442, FK835448). Gram-negative binding proteins (GNBPs), which are pattern recognition receptors and antimicrobial effectors in several termite species (Bulmer et al., 2009), were not detected in the EST library; however, they were found to be expressed in Formosan subterranean termites via microarrays (see Section 3.3).

Furthermore, the EST library contained genes from cytochrome P450 families (see above), which are necessary for detoxification of xenobiotics encountered in diet and soil during foraging and have been described in termites (Haritos et al., 1994; Zhou et al., 2006c and references therein). Genes from the glutathione-S-transferase (GST) family were not found in the library, however, GST-like genes similar to those of cockroaches were found to be expressed in termites via microarray. The putative multi antimicrobial extrusion protein (MatE 1), an antiporter integral membrane protein, may also be involved in detoxification (Kaatz et al., 2005, FK830598, FK831154, FK833694). Variable activity of detoxification enzymes may be the cause for the observed range of susceptibility of subterranean termite colonies against commonly used termiticides (Osbrink et al., 2001; Valles and Woodson, 2002; Gatti et al., 2002).

A longevity associated gene similar to those from the *A. mellifera* genome was found in the library (FK835433). Members of the longevity assurance protein family are involved in determining life span, which is particularly interesting in social insects, since queens (and kings in case of termites) have unusually high life expectations among insects (10 yrs and longer), although they are genetically identical with the shorter lived sterile castes and lifespan differences must therefore be determined by gene expression differences. The molecular mechanisms by which longevity is determined are unclear, although some evidence suggests a participation in ceramide synthesis (Obeid and Hannun, 2003). Future studies will compare expression of these genes in different castes and developmental stages of the Formosan subterranean termite.

Considering the focus of this study, we were especially interested in gene ontologies of reproduction or oogenesis. The library contained genes responsible for egg yolk production and nutrient storage (vitellogenin, Scharf et al., 2003, 2005a, FK834516), chorion hardening in eggs (chorion peroxidase, Han et al., 2000, FK834667), juvenile hormone binding/storage proteins (e.g., JHBP and hexamerins, Scharf et al., 2003, 2005a, see above), and many genes that may be involved in general with morphological and physiological changes during queen development, such as genes for protein translation and folding, fatty acid biosynthesis, sugar-, ion- and lipid-transport, among others. Other genes related to reproduction were found in other termite and cockroach species, e.g., ovary maturation (Brent et al., 2007) and included on the microarray.

3.3. Differential gene expression

We spotted probes designed from our EST library and from genomic resources from other species found in public databases on microarrays to get a first insight into reproductive biology of subterranean termites by investigating differential gene expression between virgin females and reproductive queens. Of the 7801 probes printed on the array, 922 showed cross-hybridization (signal) with Formosan subterranean termite virgins and queens. A total of 512 probes (6.6% of the total array) met the standard for minimum signal intensity. At the time the microarrays were designed, the genomic resources for Formosan subterranean termites were limited. Therefore, we included probes from other species. Most of these probes were from closely related termites and cockroaches (which were recently combined into the same insect order, Inward et al., 2007). Nevertheless, cDNA from Formosan subterranean termites only cross-hybridized to a fraction of the probes from other species. Forty-four percent of the included probes were from the Formosan subterranean termite and its symbionts, followed by the dampwood termite *H. sjoestedti* (38%); the remaining organisms/probesets each contributed less than 10% (Table 2). Transcript concentration differed between virgins and queens for 94 probes (18% of included probes), of which 38 (40% of the probes detecting differential expression) were from the *H. sjoestedti* probeset, 36 (38%) were from the *C. formosanus* probeset and the remaining probes (22%) were from other termite species and cockroaches (Table 3).

There were 81 (86%) probes for which transcript concentration was higher in queens (Table 4) and 13 (14%) probes for which

Table 2
Species origin of the 512 ESTs represented by probes on the microarray that met the minimum criteria for inclusion in analysis of variance.

Species	No. ESTs	Average length	% of total	% with identity	% insect	% bacteria, protozoa	% other
Coptotermes formosanus	225	752	43.9	65.3	25.9	51	23.1
Hodotermopsis sjoestedti	192	601	37.5	84.4	87.7	5.6	6.8
Reticulitermes speratus	47	464	9.2	83	28.2	53.2	18.6
Blattella germanica	23	655	4.5	73.9	82.3	0	17.6
Holomastigotoides mirabile	5	1191	1	100	0	100	0
Pseudotrichonympha grassii	4	1299	0.8	100	0	100	0
Reticulitermes flavipes	4	947	0.8	100	100	0	0
Coptotermes acinaciformis	2	1088	<0.01	100	100	0	0
Coptotermes lacteus symbiont	1	1085	<0.01	100	0	100	0
Drepanotermes rubriceps	1	1123	<0.01	100	100	0	0
Mastotermes darwiniensis	1	1347	<0.01	100	100	0	0
Nasutitermes comatus	1	442	<0.01	100	100	0	0
Nasutitermes dixoni	1	1140	<0.01	100	100	0	0
Nasutitermes pluvialis	1	1122	<0.01	100	100	0	0
Nasutitermes triodiae	1	1122	<0.01	100	100	0	0
Neotermes koshunensis	1	1358	<0.01	100	100	0	0
Odontotermes formosanus	1	1444	<0.01	100	100	0	0
Sinocapritermes mushae	1	1259	<0.01	100	100	0	0

Table 3
Species origin of the 94 probes on the microarray for which transcript concentration differed between virgins and queens.

Species	No. ESTs	Average length	% of total	% with identity	% insect	% bacteria, protozoa	% other
Hodotermopsis sjoestedti	38	632	40.4	84.2	90.6	0	9.4
Coptotermes formosanus	36	747	38.3	47.2	76.5	17.6	11.8
Blattella germanica	5	600	5.3	80	75	0	25
Reticulitermes speratus	5	492	5.3	80	33	66	0
Reticulitermes flavipes	2	930	2.1	100	100	0	0
Coptotermes acinaciformis	1	400	1.1	100	100	0	0
Drepanotermes rubriceps	1	1123	1.1	100	100	0	0
Mastotermes darwiniensis	1	1347	1.1	100	100	0	0
Nasutitermes pluvialis	1	1122	1.1	100	100	0	0
Nasutitermes triodiae	1	1122	1.1	100	100	0	0
Neotermes koshunensis	1	1358	1.1	100	100	0	0
Odontotermes formosanus	1	1444	1.1	100	100	0	0
Sinocapritermes mushae	1	1259	1.1	100	100	0	0

transcript concentration was higher in virgins (Table 5). We performed technical validation via RT-QPCR for eight of these ESTs (endo-beta-1,4-glucanase, gram negative binding protein (GNBP 2), Bulmer et al., 2009), a cytochrome P450 of the invertebrate specific subfamily 4C (CYP4C9), glutathione-S-transferase (GST)-like protein, hexamerin 1 (Scharf et al., 2005b; Zhou et al., 2006b), a putative juvenile hormone binding protein (JHBP), NADH–ubiquinone oxidoreductase Fe-S protein 7 and fibrillin (Table 1). For all these genes, statistical tests for RT-QPCR data supported the differential expression indicated by the microarray data (Fig. 3).

3.4. Gene expression in freshly collected vs. lab-reared workers and soldiers

Virgins were freshly collected from the field and egg-laying queens were obtained from two month old laboratory colonies. To test for the influence of laboratory rearing on gene expression, we selected four ESTs (endo-beta-1,4-glucanase, GNBP 2, CYP4C9, and GST-like protein) out of the list of eight above and compared transcript levels of freshly-collected workers and soldiers to those of workers and soldiers that had been kept in the laboratory for 2 months. When gene expression levels of field collected workers or soldiers were compared to lab reared individuals of the same caste, no significant differences in the expression of the four tested genes were detected ($P>0.05$) (Fig. 4). Since there was no effect of rearing condition on gene expression levels, we feel confident that the difference in gene expression between virgins (field collected) and queens (lab reared) is not solely due to lab artifacts.

However, there were instances of significant differences in transcript concentrations between workers and soldiers (Fig. 4) within the same "rearing condition" (freshly collected or lab reared). Within freshly-collected termites, workers had significantly higher transcript concentrations than soldiers ($P<0.05$) for endo-beta-1,4-glucanase (Fig. 4A), GNBP 2 (Fig. 4C), and CYP4C9 (Fig. 4D). Within lab-reared termites, workers retained significantly higher transcript concentrations than soldiers ($P<0.05$) only for endo-beta-1,4-glucanase (Fig. 4A). Since workers are the caste responsible for foraging, food digestion, feeding and grooming colony members, and nest building, it is not surprising that workers collected from the field had higher expression levels of cellulases, such as endo-beta-1,4-glucanase, and of immune related genes, such as the gene expressing GNBP 2. The GNBP 2 belongs to a class of conserved receptors critical for recognizing bacterial infection and triggering immune response via inducible beta-1,3-glucanase activity in termites (Bulmer et al., 2009). Active GNBP 2 was found in body tissues, salivary gland secretions and cuticular washes of workers and soldiers of *Nasutitermes corniger* and is also present in nest material (Bulmer et al., 2009). Also, workers from field colonies had higher transcript levels of CYP4C9 than soldiers. In general, gene products from the cytochrome P450 family play broad roles ranging from the breakdown of xenobiotics (insecticides and toxic compounds produced by plants) and the synthesis and metabolism of pheromones, ecdysteroids (hormones involved in molting and reproduction) and juvenile hormone (JH) in insects (Feyereisen, 1999, 2005). Higher transcript levels of cytochrome P450 genes in workers than soldiers would explain previous observations in subterranean termites, which showed that workers are more resistant to a wide variety of insecticides than soldiers (Osbrink et al., 2001; Gatti et al., 2002) presumably due to higher levels of enzymes that metabolize/detoxify pesticide components. Soldiers show only a fraction of detoxification enzyme activity of workers (Valles and Woodson, 2002). The specific functions of enzymes from the subfamily CYP4C are not known, except for CYP4C7, which is believed to metabolize JH in roaches (Sutherland et al., 2000) and could thus be involved in caste regulation, i.e., keeping the JH titers in workers low until differentiation to presoldiers and soldiers is needed (Lüscher, 1960). The transcript levels of the GST-like protein, which is also involved in clearance of xenobiotics, were not significantly different between the sterile castes (Fig. 4B).

Lab reared workers expressed higher levels of cellulases than soldiers, but not of GNBP 2 and CYP4C9. When feeding on filter paper (>98% alpha cellulose content) in the laboratory, workers need to continue cellulose digestion and endo-beta-1,4-glucanase is critical for the first step in cellulose digestion, i.e., the hydrolysis of internal glycosidic bonds within the cellulose polymers (Watanabe and Tokuda, 2001). However, lab-reared workers are isolated from their natural environment and their colony, i.e., they have no contact with soil, limited social interactions, and no need for nest maintenance. Thus, there is reduced need for the production of costly detoxification and immune enzymes. Zhou et al. (2006c) reported a reduction in CYP4C9 expression of *R. flavipes* workers that were isolated from their colonies for at least 15 days. Our study showed a tentative but not significant reduction in the expression of the CYP4C9 in lab-reared workers after two months (Fig. 4D), which supports previous findings of environmental and/or colony factors influencing termite gene expression (Zhou et al., 2006c).

3.5. Overexpression in queens

Of the genes upregulated in queens, the one encoding the muscle-contraction protein troponin C showed the largest difference in transcript concentration between queens and virgins on the microarray (Table 4). Troponin C plays a fundamental role in the regulation of vertebrate and invertebrate skeletal muscle contraction, for example, of flight muscles in insects (Qiu et al., 2003). We were surprised to find overexpression in egg-laying queens and not in virgins. Although the young queens were only in the beginning stages of becoming physogastric and were therefore more mobile than mature queens, we would have expected that virgin alates that just had completed

Table 4
Differentially expressed genes with higher transcript concentration in queens. Difference in expression (DIFF) is expressed as the difference in log2 signals between queens and virgins. Note that probesets from two ESTs of *C. formosanus* for *endo-beta-1,4-glucanase* (FK834385 and FK836533) originated from the same gene (BAB40697).

EST acc. #	EST species origin	DIFF	SD	Q	Description of closest match	Match acc. #	E-value	Sim.%
DC239220.1	*Hodotermopsis sjoestedti* whole body	5.07	2.66	0.000	Troponin c, *Blattella germanica*	ABB89298	1.2E-73	99
DQ058931.1	*Nasutitermes triodiae*	3.90	2.08	0.001	Gram negative bacteria binding protein 2, *N. triodiae*	AAZ08501	0	100
AB118795.1	*Hodotermopsis sjoestedti*	3.66	1.95	0.001	Endo-beta-1,4-glucanase, *H. sjoestedti*	BAD12003	0	100
AB058669.1	*Coptotermes formosanus*	3.44	1.82	0.001	Endo-beta-1,4-glucanase, *C. formosanus*	BAB40695	0	100
AB118802.1	*Odontotermes formosanus*	3.28	1.74	0.001	Endo-beta-1,4-glucanase, *O. formosanus*	BAD12010	0	100
AY572862.2	*Reticulitermes flavipes*	3.06	1.66	0.004	Endogenous cellulase, *R. flavipes*	AAU20853	0	100
FK835315	*Coptotermes formosanus*	2.82	1.61	0.009	Endo-beta-1,4-glucanase, *C. formosanus*	BAB40697	4.6E-38	96
DC239152.1	*Hodotermopsis sjoestedti* whole body	2.80	1.54	0.006	Beta-1,3(4)-glucanase, *Periplaneta americana*	ABR28480	1.6E-104	89
DC235716.1	*Hodotermopsis sjoestedti* salivary gland	2.55	1.45	0.011	Endo-beta-1,4-glucanase, *H. sjoestedti*	BAD12004	5.3E-75	83
DC239449.1	*Hodotermopsis sjoestedti* whole body	2.25	1.31	0.012	Alcohol dehydrogenase, *Gryllotalpa orientalis*	AAR84629	1.1E-71	83
DC239424.1	*Hodotermopsis sjoestedti* whole body	2.17	1.20	0.001	Glutathione S-transferase, *Blattella germanica*	CAO85744	2.5E-65	76
AB118806.1	*Sinocapritermes mushae*	2.11	1.21	0.016	Endo-beta-1,4-glucanase, *S. mushae*	BAD12014	0	95
AB118798.1	*Neotermes koshunensis*	2.05	1.21	0.021	Endo-beta-1,4-glucanase; *N. koshunensis*	BAD12006	0	100
DQ058934.1	*Drepanotermes rubriceps*	1.97	1.14	0.014	Gram negative bacteria binding protein 2, *D. rubriceps*	AAZ08504	0	100
DQ058930.1	*Nasutitermes pluvialis*	1.94	1.15	0.028	Gram negative bacteria binding protein 2, *N. pluvialis*	AAZ08500	0	100
AJ511343.1	*Mastotermes darwiniensis*	1.80	1.05	0.018	Endo-beta-1,4-glucanase, *M. darwiniensis*	CAD54730	0	100
DC238044.1	*Hodotermopsis sjoestedti* hindgut	1.61	0.87	0.001	Aldo-keto reductase, *Tribolium castaneum*	XP_969456	7.0E-74	87
DC237453.1	*Hodotermopsis sjoestedti* midgut	1.58	0.86	0.005	Chymotrypsin, *Teleogryllus emma*	ABV32556	1.6E-29	65
AF046008.1	*Coptotermes acinaciformis*	1.56	0.86	0.007	CYP4C9/family 4 cytochrome p450, *C. acinaciformis*	AC03109	6.8E-74	100
FG127280.1	*Blattella germanica*	1.33	0.75	0.010	Myosin heavy chain, *Acyrthosiphon pisum*	XP_001952092	8.0E-61	97
FK834385	*Coptotermes formosanus*	1.29	0.70	0.000	endo-beta-1,4-glucanase, *C. formosanus*	BAB40697	7.0E-134	100
DC238772.1	*Hodotermopsis sjoestedti* whole body	1.18	0.68	0.007	N/A			
FK833771	*Coptotermes formosanus*	1.16	0.65	0.009	Vacuolar sorting protein 4A, *Pediculus humanus corporis*	EEB18887	6.7E-36	61
DC238679.1	*Hodotermopsis sjoestedti* whole body	1.15	0.66	0.007	Myosin heavy chain, *Acyrthosiphon pisum*	XP_001952213	2.7E-08	90
DC239126.1	*Hodotermopsis sjoestedti* whole body	1.06	0.61	0.009	Paramyosin, long form, *P. humanus corporis*	EEB19617	4.0E-50	99
FK833856	*Coptotermes formosanus*	0.99	0.60	0.014	Hexamerin 2 beta, *P. americana*	AAB09632	5.4E-33	90
FK836533	*Coptotermes formosanus*	0.97	0.54	0.001	Endo-beta-1,4-glucanase, *C. formosanus*	BAB40697	1.5E-153	100
DC236056.1	*Hodotermopsis sjoestedti* foregut	0.92	0.54	0.011	Hypothetical protein, *Branchiostoma floridae*, ligand of numb-protein X1	XP_002219274	1.9E-20	62
FG126824.1	*Blattella germanica*	0.89	0.54	0.023	N/A			
FK830854	*Coptotermes formosanus*	0.84	0.50	0.012	N/A			
FK836845	*Coptotermes formosanus*	0.83	0.50	0.012	Hexamerin 1, *R. speratus*	BAG48838	6.8E-82	89
FK833714	*Coptotermes formosanus*	0.83	0.45	0.001	Muscle myosin heavy chain, *Papilio xuthus*	BAG30740	1.3E-75	85
FK833034	*Coptotermes formosanus*	0.75	0.50	0.023	N/A			
FK830787	*Coptotermes formosanus*	0.69	0.39	0.006	N/A			
DC236948.1	*Hodotermopsis sjoestedti* midgut	0.69	0.41	0.028	Similar to alcohol dehydrogenase, *Nasonia vitripennis*	XP_001603755	1.8E-52	76
DQ279466.1	*Reticulitermes flavipes*	0.68	0.41	0.028	CYPU3/family 4 cytochrome p450, *R. flavipes*	ABB86767	2.1E-67	100
FK832358	*Coptotermes formosanus*	0.66	0.47	0.039	Gamma 1 hypothetical protein, *Brugia malayi*	XP_001893676	1.9E-30	90
FK832530	*Coptotermes formosanus*	0.65	0.38	0.012	Similar to gustatory receptor, *Apis mellifera*	XP_001603636	1.1E-06	72
DC238066.1	*Hodotermopsis sjoestedti* hindgut	0.65	0.38	0.026	Tubulin beta-2 chain, *P. humanus corporis*	EEB15535	3.6E-127	97
FK830784	*Coptotermes formosanus*	0.62	0.36	0.012	N/A			
FK833539	*Coptotermes formosanus*	0.61	0.35	0.013	N/A			
FK829716	*Coptotermes formosanus*	0.60	0.36	0.007	N/A			
DC238804.1	*Hodotermopsis sjoestedti* whole body	0.60	0.39	0.046	Similar to ribosomal protein S15A, *N. vitripennis*	XP_001607263	1.7E-67	98
DC239360.1	*Hodotermopsis sjoestedti* whole body	0.59	0.38	0.044	Hypothetical protein, *Triatoma infestans*	ABR27858	2.9E-49	76
FK830784	*Coptotermes formosanus*	0.62	0.36	0.012	N/A			
FK833539	*Coptotermes formosanus*	0.61	0.35	0.013	N/A			
FK829716	*Coptotermes formosanus*	0.60	0.36	0.007	N/A			
DC238804.1	*Hodotermopsis sjoestedti* whole body	0.60	0.39	0.046	Similar to ribosomal protein S15A, *N. vitripennis*	XP_001607263	1.7E-67	98
DC239360.1	*Hodotermopsis sjoestedti* whole body	0.59	0.38	0.044	Hypothetical protein, *Triatoma infestans*	ABR27858	2.9E-49	76
BJ979043.1	*Reticulitermes speratus* gut	0.58	0.34	0.012	Polyubiquitin, *A. pisum*	XP_001950434	1.2E-79	100
BJ979031.1	*Reticulitermes speratus* gut	0.57	0.33	0.012	N/A			
DC238524.1	*Hodotermopsis sjoestedti* hindgut	0.56	0.37	0.029	Similar to S14e ribosomal protein, *T. castaneum*	XP_970498	1.9E-55	99
FK830720	*Coptotermes formosanus*	0.53	0.33	0.012	N/A			

(continued on next page)

Table 4 (*continued*)

EST acc. #	EST species origin	DIFF	SD	Q	Description of closest match	Match acc. #	E-value	Sim.%
DC238683.1	*Hodotermopsis sjoestedti* whole body	0.52	0.34	0.028	N/A			
FG125869.1	*Blattella germanica*	0.51	0.33	0.038	Similar to adenine nucleotide translocase, *T. castaneum*	XP_968561	1.3E-92	93
BJ978699.1	*Reticulitermes speratus* gut	0.51	0.31	0.017	Polyubiquitin, *Monocercomonoides* sp.	AAW22168	1.6E-85	97
FK830216	*Coptotermes formosanus*	0.50	0.32	0.026	N/A			
DC237553.1	*Hodotermopsis sjoestedti* midgut	0.50	0.31	0.044	Similar to S7e ribosomal protein, *T. castaneum*	XP_968147	1.5E-54	96
FK834948	*Coptotermes formosanus*	0.49	0.28	0.015	Hypothetical protein, *Trichomonas vaginalis*	XP_001328699	2.6E-13	60
FK833954	*Coptotermes formosanus*	0.47	0.32	0.022	Actin, *Artemia* sp.	P18601	1.3E-92	100
DC236742.1	*Hodotermopsis sjoestedti* foregut	0.46	0.30	0.020	similar to CG8067-PA, *N. vitripennis*	XP_001601244	1.0E-26	83
FK833997	*Coptotermes formosanus*	0.45	0.30	0.014	Actin, *A. pisum*	XP_001943291 3.5E-93	100	
DC239071.1	*Hodotermopsis sjoestedti* whole body	0.45	0.33	0.023	Mitochondrial malate dehydrogenase, *N. vitripennis*	XP_001600547	3.0E-49	94
DC237551.1	*Hodotermopsis sjoestedti* midgut	0.44	0.29	0.044	Peroxiredoxin-like protein, *Phlebotomus papatasi*	ABV44727	3.0E-65	85
FK834754	*Coptotermes formosanus*	0.44	0.26	0.012	N/A			
FK830418	*Coptotermes formosanus*	0.43	0.28	0.010	N/A			
DC238757.1	*Hodotermopsis sjoestedti* whole body	0.42	0.28	0.022	N/A			
FK829638	*Coptotermes formosanus*	0.42	0.28	0.012	N/A			
FK830146	*Coptotermes formosanus*	0.41	0.26	0.043	N/A			
FK834744	*Coptotermes formosanus*	0.40	0.26	0.015	N/A			
FK833966	*Coptotermes formosanus*	0.39	0.29	0.034	Muscle actin, *Bombyx mori*	NP_001119725	2.1E-81	90
DC238237.1	*Hodotermopsis sjoestedti* hindgut	0.39	0.26	0.011	Nascent polypeptide associated complex protein alpha subunit, *A. mellifera*	XP_623555	3.8E-70	87
FK832671	*Coptotermes formosanus*	0.39	0.23	0.009	N/A			
DC236429.1	*Hodotermopsis sjoestedti* foregut	0.38	0.22	0.011	Keratinocyte associated protein 2, *T. castaneum*	XP_973093	8.9E-14	88
DC238779.1	*Hodotermopsis sjoestedti* whole body	0.37	0.22	0.012	Ring finger protein 7, *A. mellifera*	XP_001119874	5.5E-41	89
DC238314.1	*Hodotermopsis sjoestedti* hindgut	0.33	0.21	0.044	N/A			
DC236743.1	*Hodotermopsis sjoestedti* foregut	0.32	0.19	0.022	N/A			
FK830260	*Coptotermes formosanus*	0.31	0.21	0.033	Neprilysin-like protein, *N. vitripennis*	XP_001599086	1.3E-13	82
DC239211.1	*Hodotermopsis sjoestedti* whole body	0.31	0.21	0.016	Transmembrane emp24 domain-containing protein 10, *P. humanus corporis*	EEB13384	1.4E-49	88
FK830752	*Coptotermes formosanus*	0.30	0.21	0.039	Predicted protein, *Nematostella vectensis*	XP_001627135	1.5E-06	71
DC236687.1	*Hodotermopsis sjoestedti* foregut	0.29	0.21	0.013	Proteasome subunit beta type-6 precursor, *T. castaneum*	XP_973571	4.1E-74	90
FK829943	*Coptotermes formosanus*	0.28	0.18	0.046	N/A			
FG127938.1	*Blattella germanica*	0.28	0.19	0.023	Ubiquitin, *Equus caballus*	XP_001487876	3.3E-118	100
FG126489.1	*Blattella germanica*	0.26	0.16	0.040	protein tyrosine phosphatase type IVA, *P. humanus corporis*	EEB20445	7.4E-08	83
DC237613.1	*Hodotermopsis sjoestedti* midgut	0.26	0.17	0.033	Nucleotide binding protein 2, *A. mellifera*	XP_393995	1.1E-80	92
DC238816.1	*Hodotermopsis sjoestedti* whole body	0.23	0.14	0.022	Similar to ADP-ribosylation factor-like 2, *Ciona intestinalis*	XP_002120798	1.4E-57	93

their mating flight would have had higher expression in muscle contraction related proteins than queens. A recent study by Obinata et al. (2010) showed the involvement of the troponin complex in the ovulatory contractions and the expulsion of mature oocytes in *Caenorhabditis elegans*. Defective troponin C results in ovulation failure and accumulation of oocytes in the ovary. The overexpression

Table 5
Differentially expressed genes with higher transcript concentration in virgins. Note that probesets from two ESTs of *H. sjoestedti* (DC236443.1 and DC236251.1) were similar to the same sequence from *A. mellifera* (XP_392437).

EST acc. #	EST species origin	DIFF	SD	Q	Description of closest match	Match acc. #	E-Value	Sim.%
DC236443.1	*Hodotermopsis sjoestedti* foregut	−0.33	0.25	0.023	NADH–ubiquinone oxidoreductase FE-S protein 7, *A. mellifera*	XP_392437	8.7E-73	83
DC236697.1	*Hodotermopsis sjoestedti* foregut	−0.39	0.33	0.020	Cleft lip and palate transmembrane protein 1, *Drosophila yakuba*	XP_002087904	2.2E-22	85
DC236441.1	*Hodotermopsis sjoestedti* foregut	−0.45	0.30	0.048	N/A			
BJ978728.1	*Reticulitermes speratus* gut	−0.73	0.44	0.011	Alpha peptide from pUC9, *Cryptosporidium hominis*, similar to catalase	XP_664835	6.5E-12	100
DC237742.1	*Hodotermopsis sjoestedti* midgut	−0.78	0.50	0.012	Unknown, *Picea sitchensis*	ABK26259	3.2E-06	82
BJ979011.1	*Reticulitermes speratus* gut	−0.79	0.45	0.011	N/A			
FK829565	*Coptotermes formosanus*	−0.87	0.49	0.010	N/A			
FK832779	*Coptotermes formosanus*	−0.88	0.51	0.011	N/A			
DC236251.1	*Hodotermopsis sjoestedti* foregut	−0.95	0.57	0.010	NADH–ubiquinone oxidoreductase FE-S protein 7, *A. mellifera*	XP_392437	8.2E-32	74
FK832073	*Coptotermes formosanus*	−1.19	0.70	0.009	N/A			
DC238532.1	*Hodotermopsis sjoestedti* hindgut	−1.20	0.66	0.009	Conserved hypothetical protein, *Aedes aegypti*	XP_001659087	5.5E-51	59
FK831422	*Coptotermes formosanus*	−1.70q	0.91	0.001	Hypothetical protein, *B. floridae*	XP_002226217	7.9E-17	45
FK830009	*Coptotermes formosanus*	−2.88	1.64	0.011	Conserved hypothetical protein, *Yersinia pseudotuberculosis*	YP_071169	9.8E-07	60

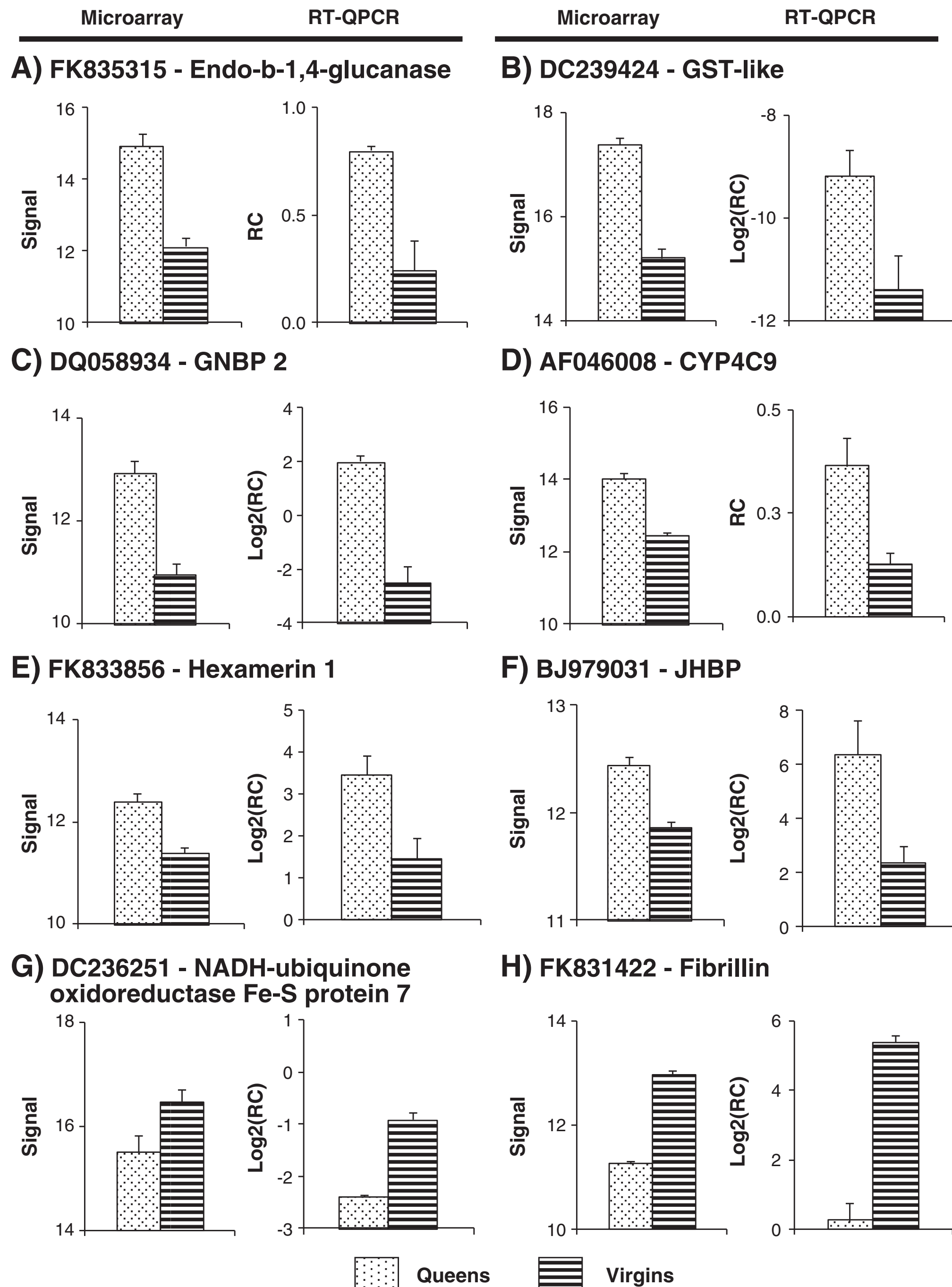


Fig. 3. Differential gene expression of egg-laying queens versus virgins of the Formosan subterranean termite measured as log2 signal intensity on the microarray (signal) and relative concentration (RC) by RT-QPCR.

of troponin C in egg-laying termite queens suggests that the troponin system is also essential for ovulation in insects.

Termite gram negative binding protein (GNBP 2, Bulmer and Crozier, 2006; Bulmer et al., 2009) also was overexpressed in queens. The signal strength of queen transcripts on the microarray as well as their relative concentration determined by RT-QPCR exceeds that of virgins considerably (Table 4, Fig. 4). Since queens are frequently groomed and fed by workers and mate repeatedly (Raina et al., 2003), they are constantly involved in social interactions and thus need protection from pathogen exposure to maintain their own health and that of their brood.

Similar to genes involved in immune response, gene expression of two enzymes involved in response to exposure to xenobiotics and detoxification also were overexpressed in queens, namely a glutathione-S-transferase (GST)-like gene and two genes from the cytochrome P450 family 4 (Table 4). Differential gene expression was confirmed with RT-QPCR for the GST-like protein gene and CYP4C9 (Fig. 4). We would not have expected upregulation of detoxification genes in queens, since queens are not exposed to the environment to the same degree as alates. Alates have to leave the parental colony, participate in swarming, find mates, and dig into the ground to found colonies, while queens are sheltered within the colony. However, like

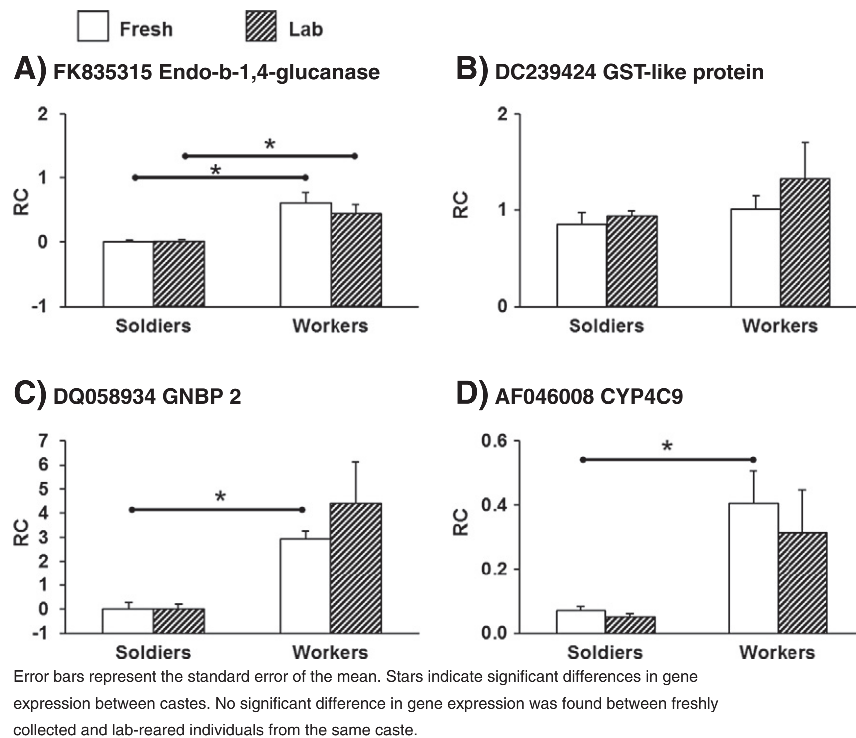


Fig. 4. Relative transcript concentration (RC) of freshly collected versus laboratory-reared workers and soldiers of the Formosan subterranean termite measured by RT-QPCR.

immune-related genes, the upregulation of detoxification genes in the initial workerless stages of incipient colonies may be important since queens still have to feed themselves and their first brood. Enzymes of the CYP4 family are involved in fat metabolism and are upregulated under conditions of starvation in insects (Grönke et al., 2005). Since the incipient termite colony in its initial stages is sustained solely by the fat reserves of the parents until the first generation of workers emerges to provide the reproductive and the brood with nutrition, the upregulation of cytochrome genes may signify increase utilization of lipids.

Transcript concentrations of a number of cellulases measured by microarrays were higher in queens than in virgins (Table 4) and differential expression of endo-beta-1,4-glucanase was confirmed by RT-QPCR (Fig. 4). In mature colonies workers and their gut symbionts both produce the majority of cellulases to achieve efficient wood digestion and to feed their colony mates, including the queen and developing alates. Reproductives in incipient colonies, however, lack workers and have to feed themselves and their offspring (Shellman-Reeve, 1990; Machida et al., 2001). Xylophagous termites and cockroaches who feed themselves have high expression levels of endogenous endo-beta-1,4-glucanase (Scharf et al., 2005a; Fujita and Miura, 2008; Shimada and Maekawa, 2008). Similar to our study, Shimada and Maekawa (2010) showed that endo-beta-1,4-glucanase expression is lower in alates than in queens heading incipient colonies in their early stages (up to 400 days after colony foundation). These authors concluded that “wood digestion ability is needed for parental feeding of young offspring and to obtain sufficient nourishment for reproduction” (Shimada and Maekawa, 2010). As soon as worker numbers increased after 400 days post colony foundation, the expression levels in queens dropped, most likely because workers gradually took over feeding brood and parents, and queens could devote their resources primarily to reproduction.

Also, the expression of hexamerins 1 (FK836845) and 2 (FK833856) from the Formosan subterranean termite library was also upregulated in queens compared to virgins (Table 4, Fig. 4). Hexamerins are known to be able to traverse membranes via receptor-mediated endocytosis and are generally involved in nutrient storage (and possibly egg provisioning in termites, Johnston and Wheeler, 2007) and signaling of nutritional status (Burmester and Scheller, 1999). Queens are being fed by their workers and signaling factors may thus be involved in maintaining queen nutrition. Furthermore, it has been shown that both hexamerins in termites are inducible by JH (Scharf et al., 2005b) and bind JH (Braun and Wyatt, 1996; Zhou et al., 2006b) and are thus involved in modulation of JH availability (similar to JHBP above) and maintenance of the optimal titers required for reproductive functions. In subterranean termite reproductives (*R. speratus*, Maekawa et al., 2010) and dampwood termites (*Zootermopsis angusticollis*, Brent et al., 2005), JH production increases as alates mature into queens and is correlated to an increase in the number of vitellogenic oocytes. Excess of JH, however, may interfere with oviposition; thus, JH titers need to be tightly regulated during the reproductive cycle (Sutherland et al., 1998, 2000).

In connection to JH regulation, queens also showed higher expression of a gene from the *R. speratus* gut (BJ979031.1, Table 4) with a predicted translation product that contains a conserved domain from the juvenile hormone binding protein (JHBP) superfamily. JHBP is a carrier protein only found in insects (Whitmore and Gilbert, 1972). It is putatively synthesized in the fat body and secreted into the hemolymph, where it binds small lipophilic molecules, such as JH, and transports them from the site of synthesis to the target tissue (Hagai et al., 2007). It has been suggested that JHBP acts as a JH reservoir in the hemolymph to keep the target cells saturated with JH despite oscillating titers (De Kort and Granger, 1996) and to scavenge excess JH. Maintenance of the JH balance throughout the reproductive cycle and removal of excess JH at the end of each cycle may be the reason queens had significantly higher transcript concentration of JHBP than virgins.

In addition, CYP4C9 was overexpressed in queens (see above). Similarly, a family 4 cytochrome P450 gene had high expression levels in female reproductives of *C. secundus* (Weil et al., 2007). Another gene from the same subfamily, CYP4C7, was found to metabolize JH and, thus, play a role in the reproductive cycle of a cockroach

(Sutherland et al., 1998; 2000). Expression of CYP4C7 in the corpora allata of *Diploptera punctata* is induced by an uncharacterized ovarian hormone that also coincidentally switches off JH synthesis to allow ovulation (Sutherland et al., 2000). Transcript levels of CYP4C7 fluctuate dramatically during the reproductive cycle of the female cockroach with low levels before to several days after mating, peak levels just before oviposition at day 7 and a gradual decrease until oviposition begins again. The capability of CYP4C7 to metabolize JH and its precursors (Sutherland et al., 1998) ensures clearance of JH at the end of each reproductive cycle (Sutherland et al., 2000).

Formosan subterranean termite queens have similar cycles of oviposition followed by several months of inactivity during which ovary size is reduced due to the absence of immature and mature oocytes (Raina et al., 2003). The queens in our study were collected toward the end of the first oviposition cycle (2 months after mating). At that time, expression of hexamerin and JHBP (both JH binding proteins) and CYP4C9 (putatively involved in catabolism of JH and its precursors) was high, which would result in reduced levels of JH and thus likely initiate the reproductive lag period. Further studies are warranted to elucidate the function of these genes in the reproductive cycle of termites, and the changes in transcript levels in relation to JH titers.

Surprisingly, the expression of vitellogenin, which is involved in ovary activation, egg production, and life history regulation (e.g., longevity, immunity) in honeybees (Koywiwattrakul et al., 2005; Koywiwattrakul and Sittipraneed, 2009) and the reproductive cycle in termites (Maekawa et al., 2010) was not significantly different between Formosan subterranean termite queens and virgins, although the gene (FK834516) was present in the EST library of the Formosan subterranean termite. Either expression changes were too subtle to detect, the reproductive cycle of the two-month old queens had entered a stage with decreasing egg production (Maekawa et al., 2010), or the particular genes for which probes were included on the microarray are not involved with ovary activation and the transition of virgin to functional queen in Formosan subterranean termites, but perhaps with non-reproductive caste differentiation (Scharf et al., 2005b; Tarver et al., 2010).

3.6. Overexpression in virgins

Whereas the majority (86%) of differentially expressed genes was upregulated in queens, only 13 (14%) showed higher expression in virgins (Table 5, Fig. 4) and only two of those had known homologues in Genbank. One matched to NADH:ubiquinone oxidoreductase iron-sulfur protein 7 (NADH-coenzyme Q reductase) described for the ant *Harpegnathos saltator* (Bonasio et al., 2010). This protein is a subunit of the mitochondrial membrane respiratory electron transport chain and thus responsible for providing energy via oxidative phosphorylation. It is possible that the upregulation of respiratory proteins in virgins reflects the aerobic demands on the alates during swarming, shedding of wings, and running in tandem with their mate. The second gene upregulated in virgins coded for a fibrillin-like protein with a von Willebrand factor type A domain which in general is known to mediate protein-protein interactions, including intramolecular adhesion, cell migration, signaling, transcription, and DNA repair. The significance of upregulation of this particular gene in virgins versus queens is unknown.

4. Conclusion

Transcriptome profiling at the whole-body level of Formosan subterranean termite virgins and egg-laying queens gave us first insight into genes that are upregulated during swarming and transformation into an egg-laying queen. Genes for which expression was higher in queens than in virgins included those with protein products related to energy metabolism (cellulases), protein storage, fat metabolism and juvenile hormone regulation (hexamerins, JHBP, CYP4C9), innate immunity (GNBP), and detoxification (GST-like protein, CYP4C9). These results likely reflect the metabolic requirements of egg production, and of survival during a time when resources of the incipient colony are mostly diverted toward reproduction. Most importantly, several gene products (hexamerin, JHBP, CYP4C9) appear to affect juvenile hormone titers via binding or catabolism and thus are directly linked to the down regulation of oviposition at the end of the reproductive cycle. We expect that further characterization of the genes in our library with no known function will yield additional insights into the reproductive biology of this insect. Future gene expression studies will compare expression between virgin kings and reproductively-active kings, and will also focus on individual tissues. Termite reproductive genomics is expected to shed light on important aspects of basic biology of an ecologically and economically important group of social insects outside of the Hymenoptera (bees, ants, and wasps) and uncover novel avenues for more effective and environmentally friendly pest management by using the discovery of vital genes and pathways to develop novel products and biotechnologies to solve pest problems.

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