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Application of RNA Interference in Functional Genomics Studies of a Social Insect

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Summary

Social insects represent a group of organisms that have dual importance from perspectives relating to both basic and applied science. From a basic perspective, social insects serve as excellent model systems for studying social organization, behavioral ecology, neurobiology, and phenotypic plasticity. From applied perspectives, social insects play important roles in the pollination of agricultural crops, in the damage of human structures and commodities, and in cellulose processing in natural ecosystems. With the advent of insect sociogenomics research (and the ability to identify dozens or hundreds of relevant candidate genes from a single experiment) has come a great demand for functional genomics tools for application in gene characterization. To date, RNAi is one of the most powerful tools to have become available for such functional characterizations, and it has broad relevance across a range of insect sociobiology research topics.

Key Words: termite; hexamerin; honeybee; sociogenomics; RNAi.

1. Introduction

Insect sociality has evolved to varying degrees, ranging from what can be defined as primitive sociality to semi- and full-eusociality ***(1)***. All fully eusocial insects share several key characteristics. In particular, these characteristics include overlapping generations, a reproductive division of labor, and sterile or altruistic castes. Fully eusocial insects occur in the two insect orders Hymenoptera [bees/wasps/ants] and Isoptera [termites]. The Hymenoptera and Isoptera differ in a number of ways, most notably in metamorphosis, ploidy, social organization, and evolutionary history ***(1–3)***. Social insects provide excellent model systems for helping to better understand social organization

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in higher organisms, as well as for defining the molecular processes that underlie complex behaviors, behavioral ecology, and behavioral neurobiology ***(4)***. Because of the range of phenotypic caste differentiation capabilities exhibited by social insects, they also make excellent model systems for studying phenotypic plasticity and developmental polyphenism ***(5)***. Finally, because of the unique abilities of termites and their symbiotic gut fauna to digest cellulose, termites are potential sources of novel genes that can be applied in emerging bioenergy and biorefinery applications.

Several examples of the use of RNAi in social insects are currently available. In termites, RNAi has been used to characterize hexamerin proteins that serve a key proximate role in the status quo maintenance of altruistic worker castes ***(6,7)*** and to partially define the functions of an endogenous cellulase gene ***(6)*** and a behavior-related gene (M. A. S., unpublished). In the honeybee, which is a well-established behavioral model organism, RNAi has also been successfully applied. One example of the use of RNAi in honeybees is the dissection of vitellogenin-mediated behavioral polyethism; another is the roles of vitellogenin protein expression in a social ground plan ***(8–11)***. Other examples of the use of RNAi in honeybees include the characterization of genes linked to development ***(12)***, sex determination ***(13)***, and the neurological basis of behavior ***(14)***. These RNAi efforts have shed completely new light on some long-standing questions on insect sociality and are indicative of the great promise that RNAi holds for the future. In this chapter, we present protocols that have been developed in our recent work on RNAi in termites, which were inspired by Amdam et al. ***(8)*** and Beye et al. ***(13)***. Although we focus on termites, the methods and concepts we present should have broad application across all social insects.

2. Materials

2.1. Total RNA Isolation

Materials associated with the SV total RNA isolation kit (Promega, Madison, WI)

2.1.1. Materials Provided with the Kit

1. Spin column assemblies and elution tubes.
2. RNA lysis buffer.
3. RNA dilution buffer.
4. β-mercaptoethanol (48.7%).
5. DNase I (lyophilized).
6. 0.09 *M* $MnCl_2$.
7. Yellow core buffer.

8. DNase stop solution (concentrated).
9. RNA wash solution (concentrated).
10. Nuclease-free water.

2.1.2. Materials to Be Provided by the User

1. 2-mL Tenbroeck glass tissue homogenizer (Pyrex).
2. 75% and 95% ethanol, both RNase-free.
3. Benchtop minicentrifuge and high-speed microcentrifuge (e.g., Eppendorf 5415R).
4. Heating block.
5. Gel system for preparing and running agarose minigels.
6. Agarose (molecular biology grade).
7. Gel imaging system (e.g., Bio-Rad ChemiDoc™ XRS).
8. Spectrophotometer (e.g., Bio-Rad SmartSpec™ Plus).
9. RNA storage cocktail [1-μL glycogen solution (Ambion), 0.1 vol of 5 *M* ammonium acetate, and 2.5 vol of 100% ethanol (v = the volume of RNA solution to be stored)].

2.2. cDNA Synthesis and Target Gene Amplification

The following materials are used in association with the cMaster™ RT_{plus} PCR System and cMaster RT Kit (Eppendorf, Westbury, NY).

2.2.1. Materials Provided with the Kit

1. cMaster RT enzyme (15 U/μL).
2. cMaster PCR enzyme mix (5 U/μL).
3. RT_{plus} PCR buffer with 25 m*M* of $MgCl_2$ solution.
4. Prime RNase inhibitor solution.
5. 10X tuning buffer with 25 m*M* of $MgCl_2$ solution.
6. 10 m*M* of dNTP mix.
7. RNase-free water.

2.2.2. Materials to Be Provided by the User

1. Oligo$(dT)_{20}$ primer.
2. Gene-specific primers.
3. PCR thermal cycler, preferably one with gradient capabilities.
4. Benchtop minicentrifuge and high-speed microcentrifuge (e.g., Eppendorf 5415R).
5. Gel system for preparing and running agarose minigels.
6. Agarose (molecular biology grade).
7. 100-bp DNA ladder, sample loading dye, and 50X TAE (Bio-Rad, Hercules, CA).
8. Gel imaging system (e.g., Bio-Rad ChemiDoc™ XRS).
9. Spectrophotometer (e.g., Bio-Rad SmartSpec™ Plus).

2.3. Purification of Target Gene cDNA Fragment

The following are associated with the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA).

2.3.1. Materials Provided with the Kit

1. QIAquick spin columns.
2. Buffers PB, PE (concentrate), and EB.
3. Collection tubes.

2.3.2. Materials to Be Provided by the User

1. 100% ethanol and 100% isopropanol (both molecular biology grade).
2. Heating block.
3. Benchtop minicentrifuge and high-speed microcentrifuge (e.g., Eppendorf 5415R).
4. Gel system for preparing and running agarose minigels.
5. Agarose (molecular biology grade).
6. 100-bp DNA ladder, sample loading dye, and 50X TAE (Bio-Rad).
7. Gel imaging system (e.g., Bio-Rad ChemiDoc™ XRS).
8. UV trans-illuminator (preferably an inexpensive one for gel-chopping).
9. Spectrophotometer (e.g., Bio-Rad SmartSpec™ Plus).

2.4. Short Interfering RNA (siRNA) Synthesis and Purification

The following materials are used in association with the *Silencer*™ siRNA Cocktail Kit (RNase III) (Ambion, Austin, TX).

2.4.1. Materials Provided with the Kit

1. Nuclease-free water.
2. Transcription reaction filter cartridges.
3. siRNA purification units.
4. 2X wash solution.
5. T7 enzyme mix (aliquotted into single-use tubes) and 10X T7 reaction buffer.
6. Elution solution.
7. ATP, CTP, GTP, UTP solution (75 m*M* each).
8. 10X binding buffer.
9. RNase A.
10. RNase III (1 U/μL) and its 10X buffer.
11. DNase I (2 U/μL) and its 10X buffer.

2.4.2. Materials to Be Provided by the User

1. Target gene DNA fragment.
2. 100% ethanol (molecular biology grade).

3. PCR thermal cycler.
4. Benchtop minicentrifuge and high-speed microcentrifuge (e.g., Eppendorf 5415R).
5. Gel system for preparing and running agarose minigels.
6. Agarose (molecular biology grade).
7. Gel imaging system (e.g., Bio-Rad ChemiDoc™ XRS).
8. UV trans-illuminator (preferably an inexpensive one for gel-chopping).
9. Spectrophotometer (e.g., Bio-Rad SmartSpec™ Plus).

2.5. siRNA Injection

1. PUL-1™ micropipette puller (World Precision Instruments, Sarasota, FL) and borosilicate capillary tubes (World Precision Instruments Cat# 4879).
2. Silicone oil (Fluka Cat# 85411) and backfill syringes (Microfil™; World Precision Instruments Cat# MF34G-5).
3. For handling termites: soft larval forceps (Bio-Quip Cat# 4748; Rancho Dominguez, CA) and fine camel hairbrushes (available at any art supply store).
4. Nanoliter 2000™ injector (World Precision Instruments Cat# B203XYV).
5. Injection platform (custom manufactured by Optimec Inc., Gainesville, FL), micromanipulator (World Precision Instruments Cat# MMJR), tilting base (Cat# M3), and vacuum pump.
6. Binocular viewing scope and fiber-optic light source (ring lights are not recommended).

2.6. Quantitative Real-Time PCR

qRT-PCR is carried out using an iCycler iQ qRT-PCR detection system with iQ™ SYBR® Green Supermix (Bio-Rad). Total RNA isolation and cDNA synthesis can be obtained with the SV Total RNA Isolation System (Promega, Madison, WI) and iScript™cDNA Synthesis Kit (Bio-Rad), respectively.

1. qRT-PCR detection system (e.g., Bio-Rad iCycler-MyiQ).
2. iQ™ SYBR® Green Supermix (Bio-Rad).
3. 96-well thin-wall PCR plate (Bio-Rad).
4. 96-well PCR plate sealing mats (Bio-Rad).
5. Standard PCR thermal cycler.
6. Benchtop minicentrifuge and high-speed microcentrifuge with swing-bucker microplate rotor (e.g., Eppendorf 5415R centrifuge and A-4-81 rotor).
7. Gel system for preparing and running agarose minigels.
8. Agarose (molecular biology grade).
9. 100-bp DNA ladder, sample loading dye, and 50X TAE (Bio-Rad).
10. Gel imaging system (e.g., Bio-Rad ChemiDoc™ XRS).
11. UV trans-illuminator (preferably an inexpensive one for gel-chopping).
12. Gene-specific qRT-PCR PCR primers.

2.7. Protein Isolation and Quantification

1. Hemolymph isolation buffer: phosphate buffered saline (PBS) (10 m*M* monobasic sodium phosphate, 154 m*M* sodium chloride).
2. Whole-body isolation buffer: PBS containing 0.1% Triton X-100.
3. Protein assay reagents: Bradford kit (Bio-Rad); Lowry kit (Pierce, Rockford, IL).
4. Protein standards: bovine serum albumin (Sigma-Aldrich, Milwaukee, WI) diluted serially from 2.0 mg/mL to 0.0625 mg/mL, in either PBS or PBS-Triton isolation buffer.

2.8. SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Separating gel buffer (1.5 *M* of Tris, pH 8.8).
2. Stacking gel buffer (0.5 *M* of Tris, pH 6.8).
3. 30% Acrylamide/bis solution (Bio-Rad; premade).
4. Sodium dodecyl phosphate (SDS) (Bio-Rad; 10% in nanopure water).
5. Ammonium persulfate solution (Bio-Rad; 10% in nanopure water).
6. TEMED (Bio-Rad); use in hood.
7. SDS-PAGE running buffer: 0.025 *M* Tris, 0.192 *M* glycine, 0.1% SDS.
8. SDS-PAGE sample buffer (2X): 0.125 *M* Tris, 4% SDS, 20% v/v glycerol, 0.2 *M* DTT, 0.02% bromophenol blue.
9. Prestained molecular weight markers: Kaleidoscope™ broad-range prestained molecular weight markers (Bio-Rad).
10. Coomassie stain: 40% methanol, 5% acetic acid, 0.025% coomassie blue R250. Use in hood and dispose of properly.
11. Coomassie de-stain solution: 40% methanol, 5% acetic acid. Use in hood and dispose of properly.

2.9. Western Blotting

1. Nitrocellulose membranes that have worked well for us include Bio-Rad (Supported Nitrocellulose, Cat# 162-0116) and GE Healthcare (Hybond-ECL™).
2. Transfer buffer (0.25 *M* of Tris, 0.192 *M* of glycine, 20% methanol in nanopure water). Keep cold at –20 °C until use.
3. Ponceau stain: 0.1% Ponceau-S (Sigma P3504) in 5% acetic acid solution.
4. PBS: 0.1 *M* phosphate buffer, pH 7.4, 0.154 *M* NaCI).
5. PBST (PBS containing 0.1% Triton X-100).
6. Blocking solution: PBS containing 15 mg/mL of nonfat dry milk (Bio-Rad Cat# 170-6404).
7. Anti-JH primary antiserum was obtained from Dr. Walter Goodman, University of Wisconsin, Madison.
8. Anti-JH primary antibody solution: anti-JH antiserum diluted 1:1,000 in PBST.
9. Secondary antibody solution: goat-anti-rabbit alkaline-phosphatase conjugate (Bio-Rad Cat# 170-6518) diluted 1:1,000 in PBST.

10. BCIP/NBT development solution: two BCIP/NBT tablets (Sigma Cat# B5655) dissolved in 20 mL of nanopure water.

2.10. Developmental/Behavioral Assays

1. Assay dishes (Nunc tissue culture dishes; Fisher Cat# 1256593).
2. Juvenile hormone working dilution: Sigma Cat# J2000 diluted 0.50–0.67 µg/µL in analytical-grade acetone.
3. Paper towels (Georgia-Pacific; Singlefold™).

3. Methods

3.1. Identification of Knockout Target and Primer Design

Candidate genes to be targeted by RNAi can be identified in any number of ways; for example, by single gene approaches, subtractive library screens, array-based library screens, high-throughput EST sequencing, genome sequencing, etc. However, before moving into RNAi-based functional characterizations, several criteria for candidate genes should be met. These criteria include validating a link to a particular biological process as well as having determined developmental and tissue-based expression profiles. Identifying proteins encoded by target genes and being able to monitor these proteins are also highly advantageous. In particular, knowing when and where a gene is expressed is critical to being able to silence it and observe/quantify potential biological effects. Such biological effects include quantifiable changes during behavioral, developmental, or physiological processes.

1. Once a candidate gene has been validated, we follow the protocol for PCR amplification of dsRNA templates as suggested by the Silencer™ kit (Ambion). For this protocol, a ~500-bp target gene region should be identified that has no sequence overlap with nontarget genes. In our efforts to silence hexamerin genes, we were able to target two highly conserved hexamerin genes with a single siRNA targeted to a homologous gene region, which had no sequence identity to other known termite genes or other nonhexamerin genes in public databases ***(6)***. Such dual silencing may not always be advantageous.
2. Using the Ambion protocol, primers are designed to a target gene region using any standard primer design software (e.g., "Primer3") ***(15)***. After designing gene-specific primers, the next step is to append T7 RNA polymerase recognition sites to the 5′ ends of the forward and reverse primers and then to verify that the primers have favorable thermodynamic characteristics. For the resulting ~42-mer primers, we validate them using the Web-based tool "NetPrimer" (Premier Biosoft International, Palo Alto, CA). Once primers are validated, they are ordered commercially.

3. At this stage, it is also necessary to develop RNAi-targeted fragments for control genes, in the same manner as that suggested above for target genes. Also included in the Ambion Silencer kit is a control template for the mammalian housekeeping gene *GAPDH*. The *GAPDH* template may also be used to synthesize control siRNAs to verify the specificity gene silencing; however, recent findings suggest that *GAPDH* siRNAs elicit off-target effects in termites (M. A. S., unpublished). As another control gene option, "scrambled" and miscellaneous control gene templates are also commercially available.

3.2. Total RNA Isolation

Isolation of total RNA is the first step in preparation of the dsRNA fragment. The following protocol is derived from SV Total RNA Isolation System:

1. Prepare solutions (add β-mercaptoethanol to RNA lysis buffer, add 95% ethanol to RNA Wash Solution and DNase Stop Solution), preheat a heating block to 70 °C, and aliquot DNase I enzyme (5 µL/tube, stored at –20 °C).
2. Remove termites from the colony and weigh them on a scale. Place approximately 30 mg of freshly collected termites into a sterile microcentrifuge tube (1.5 mL) and rinse with 75% ethanol before snap freezing (*see* **Note 1**).
3. Transfer 175 µL of RNA lysis buffer to a sterile glass tissue homogenizer (2 mL), and add 30 mg of frozen termites (–80 °C) into the homogenizer. Homogenize thoroughly on ice for about 30–60 sec before transferring lysed samples (lysate) to a 1.5-mL Eppendorf microcentrifuge tube.
4. Add 350 µL of RNA dilution buffer to the resulting lysate. Mix well by inverting three to four times. Place in the preheated heating block at 70 °C for 1–2 min.
5. Centrifuge for 10 min at 14,000 x g. Transfer the resulting supernatant to a fresh microcentrifuge tube by pipetting.
6. Add 200 µL of 95% ethanol to the supernatant, mix by pipetting, and then transfer this mixture to the spin column assembly.
7. Centrifuge at 14,000 x g for 1 min, and discard the pass-through in the collection tube. Add 600 µL of RNA wash solution to the spin column assembly. Centrifuge at 14,000 x g for 1 min.
8. Discard the pass-through, and pipette freshly prepared DNase incubation mixture (40 µL of Yellow Core Buffer, 5 µL of 0.09 *M* magnesium chloride, and 5 µL of DNase I enzyme per sample; mix by gentle pipetting) onto the membrane of the spin filter. Incubate for 15 min at room temperature.
9. After this incubation, add 200 µL of DNase stop solution to the spin filter, and centrifuge at 14,000 x g for 1 min. Next, add 600 µL of RNA wash solution and centrifuge at 14,000 x g for another 1 min.
10. Discard the pass-through, add 250 µL of RNA wash solution, and centrifuge at 16,000 x g for 2 min.
11. Discard the pass-through as well as the collection tube. Place the spin filter in a new elution tube. Add 100 µL of nuclease-free water onto the membrane of the

spin filter; allow to sit for 1 min, and then centrifuge at 16,000 x g for 1 min (*see* **Note 2**).

12. The quantity of the purified RNA can be determined by spectrophotometry (A_{260} method), and its quality determined by a combination of spectrophotometry (A_{260}/A_{280} ratio) and formaldehyde-agarose gel electrophoresis with ethidium bromide staining.
13. The eluted RNA is the template for subsequent cDNA synthesis, as described in the next section. Remaining unused RNA can be aliquoted in an RNA storage cocktail and stored at –80 °C.

3.3. Synthesis of the Target Gene cDNA Fragment

Total cDNA synthesis and PCR-amplification of the target gene fragment compose the second step in the preparation of the dsRNA fragment. The following protocols are derived from the cMaster™ RT_{plus} PCR System and cMaster RT Kit.

3.3.1. Total cDNA Synthesis from Total RNA

1. Remove all reagents from box except enzyme, and thaw on ice. Briefly mix and spin down all the reagents before use.
2. Set up *Mastermix I* [2 µL of RNase-free water, 2 µL of 10 m*M* dNTP mixture, 1 µL of oligo$(dT)_{20}$ primer, and up to 5 µL of total RNA] and *Mastermix II* (4.5 µL of RNase-free water, 4 µL of RT_{plus} PCR buffer with 25 m*M* of Mg^{2+}, 1 µL of cMaster RT Enzyme, and 0.5 µL of Prime RNase Inhibitor Solution). Mix well, snap centrifuge, and place the two mastermixes on ice.
3. Incubate *Mastermix I* in an iCycler at 65 °C for 5 min and then chill on ice. Next, transfer *Mastermix II* into the tube containing *Mastermix I* and mix thoroughly.
4. Place the mixed samples into a PCR thermal cycler set to 42 °C. Hold for 90 min, and then stop the reaction by heating to 85 °C for 5 min.
5. The quantity and quality of the total cDNA can be checked by spectrophotometry (A_{260} method) and agarose electrophoresis. On electrophoresis gels, the cDNA should appear as a smear from ~1.5 kb downward, with no small degradation products below 0.1 kb. The resulting cDNA can be stored in aliquots at –20 °C.

3.3.2. Amplification of Target Gene Fragment

1. Set up *Mastermix III* (3.5 µL of RNase-free water, 2.5 µL of 10 m*M* dNTP mixture, 1 µL of 10 µ*M* gene-specific forward primer, 1 µL of 10 µ*M* gene-specific reverse primer, and 2 µL of cDNA from the preceding procedure) and *Mastermix IV* (34.5 µL of RNase-free water, 5 µL of 10X tuning buffer with 25 m*M* of Mg^{2+}, and 0.5 µL of cMaster PCR Enzyme Mix). Mix well, snap centrifuge, and place the two master mixes on ice.
2. Combine *Mastermix III* with *IV*, spin briefly, and immediately place in a PCR thermal cycler (*see* **Note 3**). The temperature cycle includes an initial denaturing

step at 95 °C for 4 min, a five-cycle ramp (94 °C for 30 sec, 50 °C for 45 sec, and 68 °C for 1 min), 35 cycles of amplification (94 °C for 30 sec, 55 °C for 45 sec, and 68 °C for 2 min), and a final extension step at 68 °C for 7 min (*see* **Note 3**).

3. The quantity and quality of the gene-specific cDNA can be checked by spectrophotometry (A_{260} method) and agarose electrophoresis. On electrophoresis gels, the product should appear as a single, strong, sharp band. The resulting product can be stored at –20 °C before purification, as described in the following section.

3.4. Purification of Target Gene cDNA Fragment

The following protocol is derived from the QIAquick Gel Extraction Kit™:

1. Prepare solution (add 100% ethanol to buffer PE), and preheat a heating block to 50 °C.
2. Run above DNA templates on a 1% low-melting-point agarose gel at 50 V, for 30–60 min depending on the size of the templates.
3. Excise the DNA fragment from the agarose gel on a UV trans-illuminator with a clean, sharp scalpel (*see* **Note 4**).
4. Weigh the gel slice in a clear 1.5-mL microcentrifuge tube, and then cut the gel slice into pieces using a sterilized toothpick. Add 3 vol of Buffer QG to 1 vol of gel (100 mg ~100 µL).
5. Incubate at 50 °C for 10 min, and vortex the tube every 2–3 min during incubation until the gel slice is completely dissolved.
6. Add 1 gel vol of isopropanol to the sample and mix by inverting.
7. To bind DNA, transfer sample to a QIAquick spin column and centrifuge at 16,000 x g for 1 min.
8. Discard the pass-through, add 0.5 mL of Buffer QG to a QIAquick spin column, and centrifuge at 16,000 x g for 1 min to remove the remaining agarose.
9. To wash, add 0.75 mL of Buffer PE to the spin column, let stand 5 min, and then centrifuge at 16,000 x g for 1 min. Discard the pass-through, and centrifuge for an additional 1 min.
10. Transfer the QIAquick spin column into a new 1.5-mL microcentrifuge tube.
11. To elute the purified DNA templates, add 30–50 µL of nuclease-free water onto the center of the QIAquick membrane, let stand for 1 min, and then centrifuge at 16,000 x g for 1 min.
12. The quantity and quality of purified DNA templates can be determined by spectrophotometry (A_{260} method) and agarose gel electrophoresis.
13. If necessary, the identity of these purified DNA templates can be verified by either direct sequencing or cloning.

3.5. Short Interfering RNA (siRNA) Synthesis and Purification

The siRNA preparation process involves *in vitro* transcription of the target gene fragment, cleaning and purification of dsRNA, RNase III digestion of dsRNA into siRNA fragments, and finally, siRNA purification.

3.5.1. In vitro *Transcription of Target Gene Fragment*

1. Prepare solution (add 100% ethanol into 2X wash solution), remove all reagents from box except enzymes, and thaw on ice. Vortex briefly and spin down all the reagents before use. Keep the 10X T7 reaction buffer at room temperature.
2. Set up transcription reaction at room temperature. The 20-μL reaction includes 2 μL of each dNTPs, 2 μL of T7 enzyme mix, 1 μg of target gene cDNA fragment, and nuclease-free water (*see* **Note 5**).
3. Mix gently by pipetting up and down. Spin down briefly in a minicentrifuge and then incubate at 37 °C for 2 h in a PCR thermal cycler.

3.5.2. *dsRNA Cleaning and Purification*

1. Set up dsRNA purification mixture. The 500-μL mixture includes 50 μL of dsRNA from step 5, 50 μL of 10X binding buffer, 150 μL of nuclease-free water, and 250 μL of 100% ethanol. Mix gently by pipetting, and briefly spin down before proceeding.
2. Set up dsRNA cleaning reaction on ice. The 50-μL reaction includes 20 μL of dsRNA from step 3 above, 21 μL of nuclease-free water, 5 μL of 10X digestion buffer, 2 μL of DNase I, and 2 μL of RNase A.
3. Transfer 500 μL of dsRNA purification mixture from step 6 onto the filter cartridge, and centrifuge at max speed in a microcentrifuge (16,100 x g) for 2 min.
4. Mix gently by pipetting up and down. Spin down briefly in a minicentrifuge, and then incubate at 37 °C for 1 h in a PCR thermal cycler.
5. Place the necessary number of transcription reaction filter cartridges in collection tubes.
6. Discard the pass-through, and wash the filter cartridge with 500 μL of wash solution by centrifuging at max speed in a microcentrifuge (16,100 x g) for 2 min. Perform this step twice.
7. Discard the pass-through, and centrifuge at max speed for an extra 30 sec to remove any traces of liquid.
8. Transfer transcription reaction filter cartridges to new collection tubes.
9. Preheat elution buffer in a PCR thermal cycler to 95 °C for 5 min. Transfer only the required volume of elution buffer to a microcentrifuge tube to avoid repeated reheating. Apply 50 μL of 95 °C elution buffer onto the filter cartridge, and centrifuge at max speed for 2 min. Repeat this step with another 50 μL of heated elution buffer, to yield approximately 100 μL of purified dsRNA.
10. The quantity and quality of the resulting purified dsRNAs can be determined by spectrophotometry (A_{260} method) and by agarose gel electrophoresis.

3.5.3. *RNase III Digestion and siRNA Purification*

1. Load the RNase III digestion reaction from step 15 onto the center of an siRNA purification unit and centrifuge at 14,000 x g for 8 min. Purified siRNA will pass

through the column into the new collection tube. The resulting purified siRNA should be quantified by spectrophotometry (A_{260} method) before injection into insects.
2. Mix gently by pipetting up and down. Spin down briefly in a minicentrifuge, and then incubate at 37 °C for 1 h on a PCR thermal cycler.
3. Pre-wet the siRNA purification unit by adding 50 µL of nuclease-free water to the top of the unit and centrifuging at 14,000 x g for 8 min.
4. Discard the collection tube from the bottom of the siRNA purification unit, and transfer the upper portion of the unit into a new collection tube.
5. Assemble the RNase III digestion reaction at room temperature. The 50-µL reaction includes 15 µL of RNase III, 5 µL of 10X RNase III buffer, 15 µg of purified dsRNA from step 12, and 15 µL of nuclease-free water.

3.6. Quantification, Dilution, and Storage of siRNA

After RNase III digestion, a pool of siRNA fragments ranging from 12 to 30 bp in length (majority = 12 to 15 bp) are produced from the target gene dsRNA. The actual amount of siRNA delivered to the target site is critical to the success of gene silencing experiments; thus, accurate quantification is important. Based on our experience with lower termites, there is an optimal concentration range to elicit gene silencing effects. Furthermore, the magnitude of biological impacts caused by siRNA injection in *Reticulitermes flavipes* follows an apparent dose-dependent pattern within the optimal siRNA concentration range (**Fig. 3a**).

1. Dilute the purified siRNA 1:25 into TE buffer (10 m*M* Tris-HCl, pH 8, 1 m*M* EDTA), and read the absorbance at 260 nm.
2. The following two equations give siRNA concentration in either µg/mL or µ*M*:

   a. siRNA concentration (µg/mL) = A_{260} × 25 (dilution factor) × 40 (µg/mL/absorbance unit).
   b. siRNA concentration (µ*M*) = siRNA concentration (µg/mL)/9 (conversion factor).

   [* The conversion factor is based on the average size of the siRNA population (we assume 13.5 bp). Further details are provided in the Silencer™ siRNA Cocktail Kit Protocol.]
3. Aliquot siRNA in working volumes (enough to accommodate one round/replication of injection), and only standardize siRNA concentrations among target genes immediately before injection (*see* **Note 6**).
4. siRNA can be stored at –20 °C for short-term storage or kept at –80 °C for long-term storage.

3.7. siRNA Injection (Fig. 1)

1. Pull borosilicate capillary tubes into injection syringes using a micropipette puller. We have found it necessary to optimize the puller parameters “delay” and “heat”

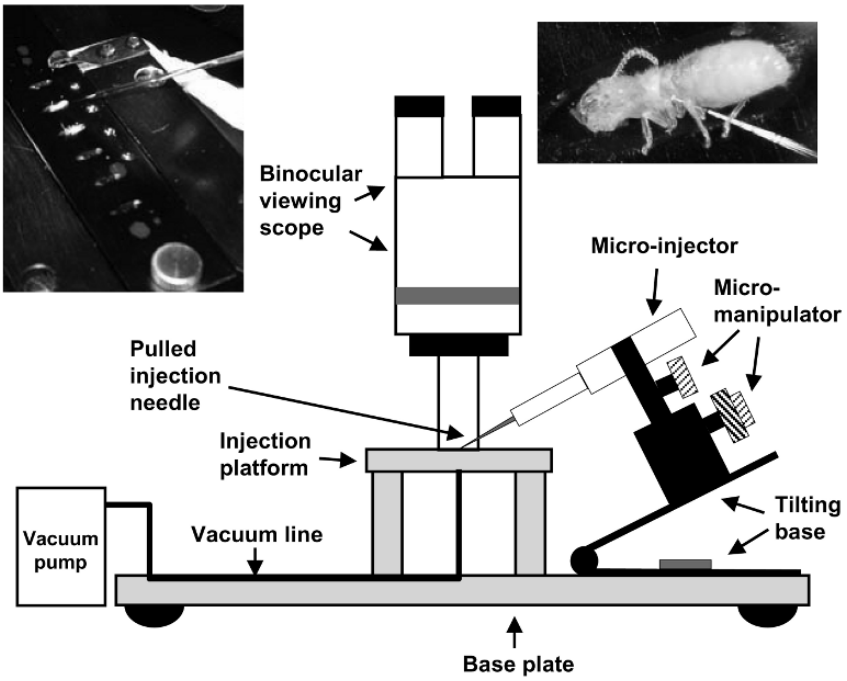

Fig. 1. Schematic drawing showing the components of the siRNA/dsRNA injection apparatus. *Inset at upper left*: termites being held by vacuum force atop the injection platform. The injection needle can be seen near the top right corner of the picture. *Inset at upper right*: magnified view of worker termite receiving an injection via the lateral thorax. Note how the termite is being held in place by vacuum force, which results in no long-term detrimental effects after injection.

for each type of microcapillary tube. For the #4879 tubes in our PUL-1™ puller, we use pulling parameters of delay = 4 and heat = 10 (*see* **Note 7**).

2. Just before use, completely back-fill an individual pulled capillary tube with silicone oil using a fine micropipette filling syringe. When back-filling, it is helpful to leave a small air bubble at the end of the injection tip.
3. Next, while holding the injector in one hand, depress the "empty" button on the microinjector control box with the other hand until the plunger protrudes about 0.3–0.5 cm. If the plunger is out too far, use the "fill" button to draw it back into the injector.
4. Load a back-filled capillary tube into the injector by pushing the unpulled end of the capillary tube into the injector until there is a slight "click." Tighten the threaded clamp until it is relatively tight, and then clamp the injector into the micromanipulator.

5. While viewing under the scope, use fine surgical scissors to cut ~0.25 cm from the tip of the syringe. Cut at an angle for a sharper tip. For insects with a hard cuticle, it may be necessary to cut more than 0.25 cm, so as to have a tip that is rigid enough to penetrate the cuticle without bending.
6. Use the "empty" button to expel all air and some oil from the syringe tip. Use a rolled-up lab tissue to sponge excess oil. If the oil flow becomes too strong, depress the "fill" button until the oil flow stops.
7. At this point, the syringe is ready to fill with siRNA solution. Place a sterile Petri plate lid or piece of Parafilm™ onto the injection platform. Pipette 1–2 μL of siRNA solution to the lid/parafilm. Lower the syringe into the droplet using the micromanipulator. Using the "fill" button, draw up the siRNA solution. Do not hold the button for more than 3 sec at a time; rather, hold it down 3 sec, and then wait 3 sec for the suction to catch up. A good way to monitor the filling process is by watching the meniscus line between the oil and aqueous solution as it is being drawn into the syringe.
8. When drawing up siRNA solution, it is also important to note the end of the syringe/capillary tube where it is clamped into the injector. If air bubbles are seen entering the syringe, the clamp must be tightened.
9. At this point, the system is ready for injection. Immediately prior to injection, termites can be placed on ice to slow their movement. Chilling in this manner allows for much easier handling. We have not found the chilling process to have any detrimental effect to the termites, nor does it alter gene expression ***(6)*** (X. Z., unpublished). Also, handling the insects as carefully as possible is critical, as bioassays after injection can last as long as 25 days (*see* notes). After chilling, soft larval forceps are used to transfer termites from the chilling dishes to the injector. Immediately after injections, a fine camel-hair paintbrush is used to transfer termites from the injector to assay dishes (*see* **Notes 8** and **9**).

3.8. Knockout Verification at the mRNA Level (Fig. 2)

3.8.1. qRT-PCR Primer Design and Selection

1. The *Primer3* Web tool ***(15)*** is used to design gene-specific qRT-PCR primers. Some parameters are revised from the Primer3 default settings to accommodate requirements for qRT-PCR. These parameters include, but are not limited to, product size range (150–250 bp), primer size (18–23 bp), primer Tm (60 ± 1 °C), primer GC% (40–60%), max self-complementarity (set to 5), max 3′ self-complementarity (set to 1–2), and max poly X (set to 3).
2. We test primer thermodynamic characteristics with the Web-based tool "NetPrimer" (Premier Biosoft International). Once primers are validated, a local biotechnology company is used for primer synthesis (Genomechanix, Gainesville, FL).
3. Before conducting any qRT-PCR analyses, we first experimentally validate qRT-PCR primer pairs. Here, we look at final PCR product profiles with both melting curves, and on agarose electrophoresis gels (*see* **Note 10**). Melting-curve analysis measures melting temperatures (Tm) of double-stranded DNA molecules. The

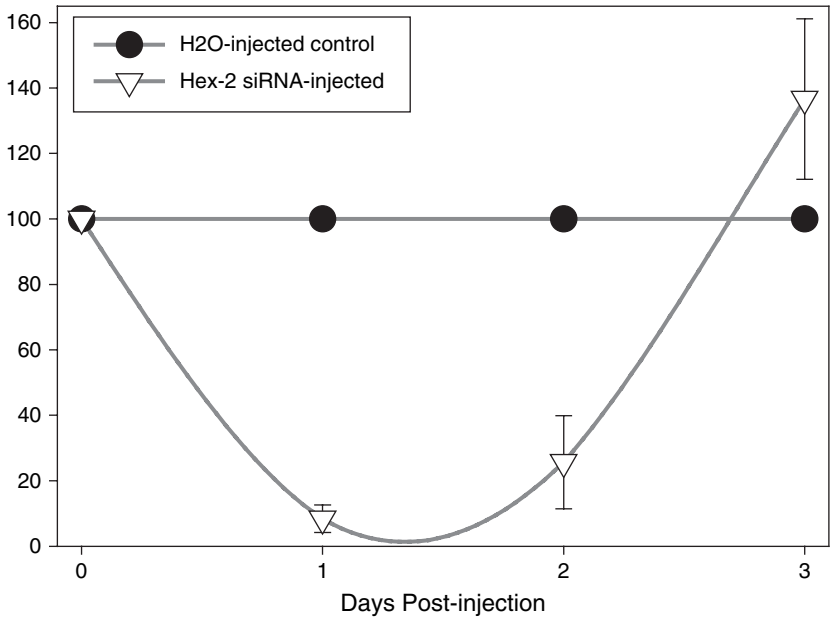

Fig. 2. The time course of gene silencing after RNAi, as determined by quantitative qRT-PCR. Shown are relative hexamerin expression levels at days 1–3 following siRNA injection, relative to day 0. Closed circles represent gene expression for water-injected controls, while open triangles represent the percentage of hexamerin expression relative to water-injected controls on each respective day. Results represent the avg. ± std. error for five individuals, each determined in triplicate. For the data set shown, injection of 500 picograms of hexamerin-2 siRNA results in a high degree of Hexamerin-2 gene silencing for greater than 48 h.

number of peaks identified in the melting curve corresponds to the number of amplified PCR products, which reflects the specificity of a particular primer set. If there are undesired product bands visible on gels (e.g., primer-dimer or unspecific bands), or if there are multiple peaks in a melting curve, this will lead us to exclude that particular primer set from use in qRT-PCR.

3.8.2. Reference Gene Selection

1. Improper reference gene selection can dramatically impact the outcome of qRT-PCR experiments. Based on our experiences and those of other researchers ***(7,16–19)***, there is no universally applicable reference gene for use in all qRT-PCR experiments. In the case of *R. flavipes*, we have identified three broadly useful (to date) reference genes from an EST database. These validated termite reference genes include β-*actin* (accession no. DQ206832), *HSP-70* (accession no. CK906362), and *NADH-dh* (accession no. BQ788168). Each of these reference

genes has been determined to have stable expression in whole-body homogenates under different treatments; however, only *NADH-dh* is a suitable reference in tissue-specific treatments ***(6,7,16)***.

2. For insects other than *R. flavipes*, several candidate housekeeping genes should be selected from the target organism. These can be easily obtained from an EST database, or by PCR with primers targeted to conserved gene regions. The importance of reference gene selection cannot be overemphasized.
3. From the gene sequence, design and select appropriate qRT-PCR primers for these candidate reference genes as described in steps 1 and 2 of the preceding section.
4. Under experimental conditions, examine candidate reference gene expressions alongside a target gene that serves as the positive control. Each target and reference gene reaction should be replicated three times on a plate. A reference gene is considered suitable if its expression does not change significantly under different experimental treatments, in particular, during knockout of a target gene by RNAi. In qRT-PCR experiments, the stability and suitability of candidate reference genes are evaluated by the changes of CT (threshold cycle) value before and after treatments (*see* below).
5. Several spreadsheet-based analysis tools have recently been developed to aid in reference gene selection and to ensure accurate normalization of qRT-PCR data. Examples of these analysis tools include *BestKeeper* ***(18)***, *geNorm* ***(20)***, and *NormFinder* ***(17)***.

3.8.3. Quantitative Real-Time PCR (qRT-PCR)

1. Turn on the real-time PCR system at least 20 min before starting a PCR run. Remove reagents from a –20 °C freezer and keep on ice. Briefly mix and spin down all the components before use.
2. For our purposes, we have found that real-time PCR reaction volumes of 20 µL provide the same reproducibility as 50-µL reactions, at a fraction of the cost. However, we do not recommend reaction volumes lower than 15 µL (X. G. Z., unpublished). A standard 20-µL reaction includes 10 µL of iQ™ SYBR® Green Supermix, 7 µL of nuclease-free water, 1 µL of 10 µ*M* forward and reverse primers, respectively, and 1 µL of DNA template.
3. Prepare a master mix that contains iQ™ SYBR® Green Supermix, primers, and nuclease-free water (*see* **Note 11**). Next, load 1 µL of DNA template into the respective wells of a 96-well thin-wall PCR plate, followed by adding 19 µL of the master mix. Keep the PCR plate on ice during the loading process, and seal the plate with a transparent 96-well PCR plate sealing mat immediately after loading is finished.
4. Transfer the PCR plate to a benchtop high-speed centrifuge with microplate swing bucket, and spin briefly to ensure that all PCR reagents are collected at the bottom of plate. This procedure enhances the reproducibility of replicate samples by reducing the frequency of erratic readings.

5. Place the PCR plate into the block of the real-time PCR system and immediately start the PCR program (*see* **Note 12**). The real-time thermal cycle program includes the initial denaturing step at 95 °C for 10 min, 45 cycles of 94 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 30 sec, an extension step at 72 °C for 10 min, and the final melting-curve step (90 cycles of temperature reduction from 90 °C to 50 °C at a rate of 0.5 °C/10 sec).
6. The threshold cycle (CT) is the cycle that individual PCR amplification crosses the threshold value and begins logarithmic amplification. CT values will appear alongside PCR amplification curves when the real-time run ends. At this point, CT values can be transferred to a spreadsheet to accommodate further analyses with spreadsheet-based analysis tools.
7. At the end of the run, assay plates can be stored at –20 °C for future analysis of PCR products by agarose gel electrophoresis. This is particularly useful when testing a new primer set.

3.8.4. qRT-PCR Data Analysis

1. Currently, there are several methods of calculating relative expression levels (REL) from real-time data. Pfaffl ***(21)*** proposed the following equation, where E represents individual PCR amplification efficiency:

$$\mathrm{REL} = (E_{\mathrm{target}})^{\Delta \mathrm{CTtarget(control-treatment)}} / (E_{\mathrm{reference}})^{\Delta \mathrm{CTreference(control-treatment)}}.$$

2. However, if target and reference genes have similar efficiencies, i.e., $E_{\mathrm{target}} = E_{\mathrm{reference}}$, then the above equation can be simplified as

$$\mathrm{REL} = \Delta CT_{\mathrm{target(control-treatment)}} / \Delta CT_{\mathrm{reference(control-treatment)}}.$$

3. The latter equation was developed by Livak and Schmittgen ***(22)*** and is referred to as the $2-^{\Delta\Delta \mathrm{CT}}$ or ΔΔCT method. We have adopted this method for analysis of our own real-time PCR data. Based on our experiences with iCycler-MyiQ real-time PCR detection system and iQTM SYBR® Green Supermix, PCR amplification efficiencies for both target genes and reference genes consistently stayed at similar values around 2 (calculated by REST 2005 version 1.9.9 software, ©2005 Corbett Research Pty Ltd. and Michael W. Pfaffl; X. G. Z., unpublished). Therefore, it is acceptable to use this simplified $2^{-\Delta\Delta \mathrm{CT}}$ method, at least with a highly stable and reproducible real-time detection system (*see* **Note 13**).
4. Relative expression levels for specific target genes are normalized to the appropriate reference gene(s) and calculated using the $2-^{\Delta\Delta \mathrm{CT}}$ method ***(22)*** with three independent replicates.
5. As described later (**Section 3.11**), nonparametric statistics are used to compare expression levels among controls and treatments.

3.9. Knockout Verification at the Protein Level (Fig. 3)

3.9.1. Protein Isolation

1. Hemolymph *(23)*: Place termites on a piece of two-sided tape under a viewing scope. Clip either a leg or the abdominal cuticle with fine dissecting scissors. Collect the hemolymph with a 10-µL capillary tube, and then dispense into ice-cold 1X PBS. We use a ratio of 1 µL of PBS per individual termite; for example, if bleeding 15 termites, use 15 µL of PBS. Take an aliquot for the proteins assay and freeze the remaining portion at –20 °C.
2. Whole body *(7)*: Homogenize termites (10 termites per 2.0 mL) in 1X PBS containing 0.1% Triton X-100 using a Potter–Elvehejem Teflon-glass motorized homogenizer. Centrifuge for 15 min at ~13,000 x g and at 4 °C to pellet cellular debris. Filter the supernatant through glass wool, take an aliquot for the protein assay, and freeze the remainder at –20 °C until use.
3. Determine the protein content of the samples using a standard protein assay (e.g., Bradford, Lowry, BCA), relative to bovine serum albumin standards prepared in the protein isolation buffer. Follow the manufacturer's instructions. Also, *see* **Note 14** for details on assay method differences.

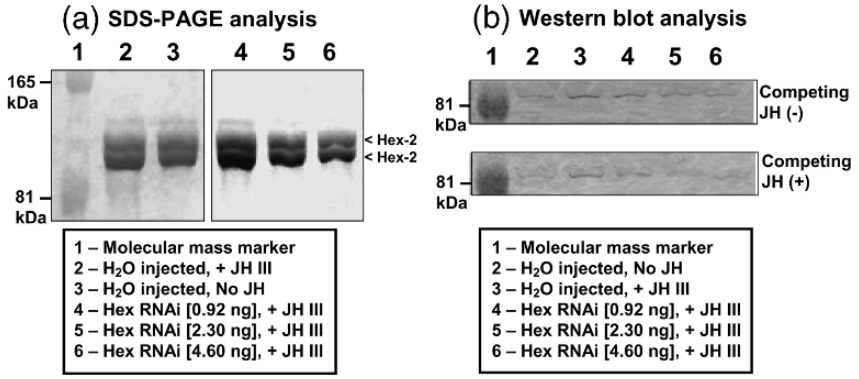

Fig. 3. (a) SDS-PAGE and (b) Western blotting analysis of hexamerin proteins under various experimental treatments. Lane designations are provided in each figure. (a) A dose-response experiment testing a range of hexamerin siRNA quantities (0.92, 2.30, and 4.60 ng) on Hex-1 and Hex-2 protein expression (top and bottom bands, respectively) at 9 days after siRNA injection. Controls shown at the left are water-injected controls, with and without juvenile hormone III (JH III) treatment. All siRNA treatments were held with ectopic JH III. (b) Western blotting results for the same protein preparations as shown in (a). Western blotting used anti-JH antiserum to identify JH III bound to the Hex-1 protein. The upper and lower blots represent primary antibody incubations that were performed in the presence (top) and absence (bottom) of competing JH III. Note reduced intensity in the lower blot, indicating that immunoreactivity is reduced in the presence of competing JH.

3.9.2. SDS-PAGE

1. Prepare 7–8% SDS-PAGE minigels following the manufacturer's instructions. Typically, for examining hexamerin proteins (~80–85 kDa) in the hemolymph, we use gels containing 8% acrylamide (*see* **Note 15**). Because of the strong hexamerin abundance, we have found 7% acrylamide gels to provide better resolution when examining hexamerins from whole-body or tissue homogenates. For proteins in other size ranges, the percentage acrylamide should be adjusted accordingly.
2. Prepare to load gels with 10 μg of protein per lane, by mixing the appropriate volume of protein at a 1:1 ratio with 2X SDS-PAGE sample buffer. Heat-treat proteins in sample buffer at 95 °C for 5 min, and then snap-cool on ice before loading onto gels. A standard PCR-thermal cycler works well for this denaturing step.
3. Molecular weight markers are loaded at 3–5 μL per lane and do not need to be heat-treated before loading.
4. Run gels at ~150 V for ~1 h, or until the dye front reaches the bottom of the gel.
5. Stain gel overnight in Coomassie stain (*see* **Note 15**). De-stain with Coomassie de-stain solution until bands become visible at a desirable level.

3.9.3. Western Blotting

1. Prepare and run an SDS PAGE minigel (7–8%) as above, but stop at step 4, i.e., do not stain. Load 5 μg of protein per lane.
2. A wet transfer cell is recommended for transferring gels to nitrocellulose membranes. We use a Bio-Rad mini Protean™ transfer cell, but any make or model of wet transfer cell should work.
3. While the gel is running, prepare 1 L of transfer buffer. Keep cold at –20 °C until use.
4. After the gel is finished running, immediately place it in ~20 mL of transfer buffer for about 5 min. While the gel is soaking, place the nitrocellulose membrane in transfer buffer, also for 5 min. Next, assemble the transfer apparatus according to the manufacturer's protocol. Avoid air bubbles, as they will inevitably end up blocking the transfer of target immunoreactive bands.
5. Transfer at either 100 V for 1 h or at a constant 30 mA for 12–16 h. We perform transfers in a 4 °C cold room.
6. When removing the membrane after transfer, use scissors to cut away one corner of the membrane so you always know which side is up. At this point, the molecular weight markers transferred to the membrane should be readily visible. Also, reversible Ponceau staining can help verify the transfer ***(24)***.
7. Rinse the membrane briefly in PBS, and then place in a blocking solution. The membrane can be left on a rocking platform for 1 h or placed at 4 °C overnight, whichever is more convenient.
8. Ideally, optimal antibody dilutions should be determined in advance using "curtain gels" and a strip blotting procedure that compares ratios of protein

loading to primary and secondary antibody dilutions ***(25)***. For the particular antibody that we use, optimal conditions involve 5 µg of protein per lane with 1:1,000 dilutions of both primary and secondary antibody.

9. To assess RNAi effects, we have used a primary antibody that detects juvenile hormone (JH) that is bound to hexamerin proteins ***(7,26)***; however, these procedures should apply to any antibody that could be used to assess RNAi effects at the protein level.
10. Transfer the membrane directly from the blocking solution to the primary antibody solution. Use a dish that provides for complete membrane coverage by solution. Rock gently for 1 h at room temperature.
11. Rinse membrane three times in ~50 mL of PBST, with gentle rocking. Change to a clean container after each rinse. The rinse steps do not need to last longer than 2 min.
12. Transfer membrane to 50 mL of secondary antibody solution. Rock gently at room temperature for 1 h.
13. Rinse membrane five times in ~50 mL of PBST for no longer than 2 min (as with step 11). Change to a clean container after each rinse.
14. While the membrane is on its first rinse of five (in step 13), prepare the BCIP/NBT development solution. Stir with a magnetic stirring bar until the BCIP/NBT tablets are completely dissolved.
15. Place the membrane in a small, clean plastic dish. Add the BCIP/NBT solution, gently rock, and observe closely for the bands to become visible. Be very careful to prevent the membrane background from becoming too dark.
16. Once the bands are clearly visible, transfer the membrane immediately to ~100 mL of fresh nanopure water. After 10 min, dry the membrane on clean filter paper and photograph as soon as possible. Store the developed membrane in darkness in a sealed container.

3.10. Developmental and Behavioral Assays

Once specific knockout of the target gene/protein can be verified as described in the preceding section, RNAi can be used to investigate the biological roles played by a particular target gene. Below, we provide bioassay protocols for investigating developmental and behavioral effects of target gene silencing. However, virtually any bioassay that could be used in investigating any aspect of sociobiology could be applied in dissecting gene function. In these studies, target silencing results are compared statistically against results for water- and control gene-injected controls.

1. Developmental assays: We have developed and validated presoldier induction assays for use in investigating a key developmental transition in termites: the worker-to-presoldier transition ***(6,23,27)***. Under natural conditions, this transition is induced by elevated JH titers in worker termites. This process is readily duplicated under laboratory conditions by ectopic JH exposure ***(27)***. After RNA injections, worker termites are placed on paper towel disks that have been treated with

150 μg of JH and 50 μL of water (as a moisture source) and are then monitored for 25 days. Typically, in this assay configuration, presoldier differentiation begins on day 14 and proceeds through days 23–24. Thus, assays typically are not run longer than 25 days. It should also be noted that external JH application is used to force the majority of individuals to make the developmental presoldier transition. We have also observed that hexamerin-RNAi-induced differentiation is also possible in the absence of ectopic JH, but at lower (constitutive) levels ***(6)***. Hexamerin genes and protein expression are JH-inducible, which led us to deduce that ectopic JH application to see maximal developmental impacts is necessary. However, for genes that are JH-suppressible, this suggests that a bioassay is necessary that involves holding injected individuals without ectopic JH.

2. Behavioral assays: As noted above, virtually any well-developed assay for investigating insect social behavior can be applied in investigating the function of candidate behavioral genes in any social insect. We are currently using three configurations of assays to investigate feeding behavior, trail following, and soil tunneling. After siRNA injection, these assays involve (respectively) monitoring (1) the amount of cellulose-based substrates that are consumed by termites, (2) the ability of termites to follow trails that consist of extracts containing their own trail pheromone, and (3) the ability of termites to build soil tunnels.

3.11. Experimental Design Considerations and Statistical Analysis of Data

1. Our experimental designs for injection-based RNAi studies include three replicates of 15 termites, injected on three consecutive days. In our studies, conducting three replicates has provided sufficient statistical power; however, if feasible, five replicates are recommended.
2. Effects typically should be verified across at least three colonies from widely spaced geographic locations. Termite (and other social insect) colonies are notoriously variable in their physiology, behavior, and ecology. Therefore, consistent results across multiple colonies are required to document the importance of a given candidate gene with respect to a particular developmental transition or behavior.
3. For quantification of knockout effects at the protein level, we have found it very useful to perform at least three independent replicate gels or blots. From each gel or blot, densitometry analyses can be performed to determine the percent of relative intensity of target bands across all treatments, on each gel or blot. For densitometry analysis, the public domain software tool "ImageJ" is available through the NIH at http://www.rsb.info.nih.gov/ij/.
4. For statistical analysis of phenotypic differentiation, SDS-PAGE, Western blotting, or RNA expression, we have used the nonparametric Kruskal–Wallis test in SAS (SAS Software, Cary, NC). With this test, an experiment-wise mean separation procedure can first be conducted to examine for significant variation at a specified p-value ($p < 0.01$, 0.05, or 0.1). If significant experiment-wise variation is observed, more detailed pairwise comparisons are performed.

4. Notes

1. For long-term storage, whole termite samples can be held at –80 °C for several months before RNA isolation.
2. In order to maximize total RNA yields, perform a second elution from spin columns with 100 µL of nuclease-free water. This option, however, should be considered against potential disadvantages created by overdilution of the eluted RNA.
3. When PCR-amplifying dsRNA templates with gene-specific primers that contain appended T7 RNA polymerase recognition sites, the PCR may require excessive optimization. For this purpose, we recommend the use of a PCR thermal cycler with gradient capabilities to test a number of Tm profiles at once. Additionally, Tm will likely vary among different primer sets.
4. To reduce the risk of damaging the DNA during the gel-purification process, limit the exposure time to UV light and use a less intense UV setting while cutting the DNA bands from the gel.
5. Our target gene fragments (Hex-2, Cell-1, and *takeout*) are ~500 bp in length; therefore, we generally use 1.0 µg of cDNA template in the *in vitro* transcription reactions (although reactions using 0.2 µg have provided identical data). If target gene fragments are smaller than 500 bp, template quantities in the 0.2–0.5-µg range are recommended.
6. If using nuclease-free water to standardize siRNA concentrations, do not reuse the remaining thawed siRNA for subsequent rounds of injection. Repeated freeze-and-thaw cycles are likely to reduce the integrity of siRNA samples.
7. After pulling injection needles, the tips will be melted closed and will need to be cut before use.
8. Becoming proficient at injection takes a great deal of practice. We find that working very deliberately and taking frequent breaks helps the process go smoothly.
9. When performing injections, injection tips may become plugged with either the siRNA or termite hemolymph (for example, if you work too slowly and the water in the RNA solution at the injection tip evaporates). If the tip plugs, carefully cut a minute portion from the tip with surgical scissors. Also, to guard against losing precious siRNA, it is good practice never to load the siRNA all at once.
10. Agarose gel electrophoresis or melting-curve analysis used alone may not be sufficient to fully validate a qRT-PCR primer set. For example, larger amplicons (>250 bp) sometimes can cause double peaks in the melting curve even when nonspecific bands are absent on agarose gels. Also, electrophoresis artifacts such as a blur migrating ahead of a gene-specific band can be visible on electrophoresis gels in association with shorter amplicons (<150 bp). For these reasons, we set the optimal product size (amplicon) at 150–250 bp when designing primers. Furthermore, although agarose gel electrophoresis and melting-curve analysis individually have their limits, the combination of two

methods provides the best chance of identifying an optimal qRT-PCR primer set that will provide accurate results.

11. When out of the freezer, SYBR Green reagents need to be covered with aluminum foil at all times due to light-induced degradation of SYBR Green. Also, aliquoting of PCR reagents eliminates repetitive freeze-and-thaw cycles that could lead to reduced performance.
12. Monitor initial qRT-PCR runs closely, particularly with new primers sets and/or genes. This will allow for better customization of real-time PCR protocols. If a low-copy number gene is encountered, additional cycles can be added. Alternatively, if a high-copy number gene is encountered, the number of RT-PCR cycles can be reduced.
13. PCR amplification efficiency is closely related to the concentration of the starting DNA template. If the DNA template is too dilute, PCR amplification efficiency will likely be unacceptable (i.e., it will deviate from 2). In this case, Pfaffl's first calculation method ***(21)*** should be used to take into account the different PCR amplification efficiencies among target and reference genes. To avoid this problem, we conduct preliminary experiments to determine optimal cDNA concentrations prior to conducting actual real-time experiments.
14. When determining the protein content for SDS-PAGE, we have found that the Lowry and BCA methods underestimate protein content in termite preparations by ~50% relative to the Bradford method. Thus, while we have found 10 μg per lane sufficient to achieve the desired band intensity with the Bradford method, we load 20 μg per lane to achieve similar results with the Lowry and BCA methods.
15. The acrylamide and methanol that are used as part of SDS-PAGE procedures are neurotoxins and should be handled with appropriate care. Methanol/acetic acid staining solutions should be used in a fume hood at all times. All used gels and waste staining/de-staining materials should be disposed of in accordance with institutional guidelines.

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