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ORIGINAL ARTICLE

Suppressing tawny crazy ant (*Nylanderia fulva*) by RNAi technology

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Abstract The tawny crazy ant (*Nylanderia fulva*) is a new invasive pest in the United States. At present, its management mainly relies on the use of synthetic insecticides, which are generally ineffective at producing lasting control of the pest, necessitating alternative environmentally friendly measures. In this study, we evaluated the feasibility of gene silencing to control this ant species. Six housekeeping genes encoding actin (*NfActin*), coatomer subunit β (*NfCOP*β), arginine kinase (*NfArgK*), and V-type proton ATPase subunits A (*NfvATPaseA*), B (*NfvATPaseB*) and E (*NfvATPaseE*) were cloned. Phylogenetic analysis revealed high sequence similarity to homologs from other ant species, particularly the Florida carpenter ant (*Camponotus floridanus*). To silence these genes, vector L4440 was used to generate six specific RNAi constructs for bacterial expression. Heat-inactivated, dsRNA-expressing *Escherichia coli* were incorporated into artificial diet. Worker ants exhibited reduced endogenous gene expression after feeding on such diet for 9 d. However, only ingestion of dsRNAs of *NfCOP*β (a gene involved in protein trafficking) and *NfArgK* (a cellular energy reserve regulatory gene in invertebrates) caused modest but significantly higher ant mortality than the control. These results suggest that bacterially expressed dsRNA can be orally delivered to ant cells as a mean to target its vulnerabilities. Improved efficacy is necessary for the RNAi-based approach to be useful in tawny crazy ant management.

Key words artificial diet; gene silencing; housekeeping gene; *Nylanderia fulva*; RNAi; survival rate

Introduction

The tawny crazy ant, *Nylanderia fulva* (Hymenoptera: Formicidae), native to South America, was first reported in Texas in 2002 and has since invaded Alabama, Mississippi, Louisiana, Florida, and Georgia in the United States (Meyers, 2008; Gotzek *et al.*, 2012; Zhang *et al.*, 2015). In these states, *N. fulva* displaces the dominant red imported fire ant (*Solenopsis invicta*) and other regional ant and non-ant arthropod species, resulting in reduced local species diversity (LeBrun *et al.*, 2013). This competitive ant species can spread at the rate of 200 m per year (Meyers, 2008) due to its high fecundity and ability to form supercolonies (McDonald, 2012). Although in many cases, long-lasting effects are less likely because population explosions are often followed by rapid collapse (Wetterer *et al.*, 2014), new colony outbreaks in new regions are very destructive. For instance, short circuit of electronic appliances due to accumulation of *N. fulva* has caused great annoyance to residents and businesses, and even led to significant economic losses (Meyers, 2008). *N. fulva* is also a potential mechanical vector of animal

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and plant pathogens (McDonald, 2012), and tends seven families of honeydew-producing hemipterans on 10 plant species (Sharma *et al.*, 2013). The mutualism between *N. fulva* and hemipterans indicates that *N. fulva* can potentially become an agricultural pest.

Administration of chemical pesticides is the most effective method to control *N. fulva* (Meyers, 2008; McDonald, 2012; Calibeo *et al.*, 2017). Insecticides containing active ingredients such as deltamethrin, imidacloprid, β-cyfluthrin, and fipronil have resulted in high mortality in worker ants (Calibeo *et al.*, 2017). However, these broad-spectrum pesticides have shown high direct or indirect toxicity to fish and birds, threatening other nontarget organisms in the environment as well (Gibbons *et al.*, 2015). In addition, the high mortality of workers does not necessarily result in colony control as the high incidence of reproductive castes enables a rapid return of some pest populations. Despite recent progress in identification of a microsporidia parasite and isolation of an RNA virus from *N. fulva* (Plowes *et al.*, 2015; Valles *et al.*, 2016), more studies of their effects on this ant and assessment of environmental impacts need to be conducted before viruses become useful in biological control.

RNAi is a posttranscriptional gene silencing technology (Hannon, 2002). Exogenous dsRNA can be delivered into insects through feeding, soaking, and injection (Zhang *et al.*, 2013a). Once introduced into cells, the dsRNA is cleaved into 21–24 nt small-interfering RNAs (siRNAs) by dicer, an RNase III endoribonuclease. The antisense guide strand is incorporated into the RNA-induced silencing complex (RISC), which identifies and degrades the complementary mRNAs, preventing their translation (Zamore *et al.*, 2000; Elbashir *et al.*, 2001; Meister, 2013). As a useful tool in functional genomic studies, RNAi has been applied to insects in several orders, such as Coleoptera (Baum *et al.*, 2007), Lepidoptera (Turner *et al.*, 2006), Hymenoptera (Amdam *et al.*, 2003), Diptera (Dzitoyeva *et al.*, 2001), Hemiptera (Mutti *et al.*, 2006), Thysanoptera (Badillo-Vargas *et al.*, 2015), Orthoptera (Dong & Friedrich, 2005), and Blattodea (Cruz *et al.*, 2006).

Successful RNAi via injection of *in vitro*-synthesized dsRNA has been reported in some hymenopteran species, including European honey bee (*Apis mellifera*), turnip sawfly (*Athalia rosae*), red imported fire ant (*Solenopsis invicta*) and Florida carpenter ant (*Camponotus floridanus*) (Amdam *et al.*, 2003; Sumitani *et al.*, 2005; Yoshiyama *et al.*, 2013). Egg injection of dsRNA results in reduced *white* mRNA abundance and loss of embryonic eye pigmentation in turnip sawflies (Sumitani *et al.*, 2005). Silencing of the vitellogenin receptor gene in fire ant virgin queens causes failure of egg formation (Lu *et al.*, 2009). Increased mortality of larvae, pupae, and adults was also observed in this species when the expression of pheromone biosynthesis-activating neuropeptide gene was knocked down (Choi *et al.*, 2012). Injection of synthetic dsRNAs, although commonly used, is expensive and labor-intensive. Additionally, injection itself may cause stress to insects. For example, injection of sterile Ringer's solution induces immunity and stress-related genes in Florida carpenter ants (Ratzka *et al.*, 2011).

Oral delivery, on the other hand, can avoid mechanical damage to insects. The most recently developed, recombinant bacteria-based dsRNA expression system has substantially decreased the cost of dsRNA production (Kamath & Ahringer, 2003). Feeding insects with diet containing heat-inactivated bacteria that produce dsRNA have successfully silenced target genes in Colorado potato beetles (*Leptinotarsa decemlineata*) and cotton bollworm (*Helicoverpa armigera*) (Zhu *et al.*, 2011; Zhou *et al.*, 2013; Zhang *et al.*, 2013b; Zhao *et al.*, 2016). Here, we attempt to explore the feasibility of using this cost-effective dsRNA synthesis and delivery technique to control tawny crazy ants by silencing selected housekeeping genes.

Materials and methods

Insect collection and rearing

N. fulva colonies were collected from East Columbia, Texas. Queens, alates, brood, and workers as well as substrate surrounding the nests (leaves, soil, branches, and grass) were placed in 9 L buckets lined with talcum powder to prevent escape. The collected colonies were transferred to rectangular plastic containers (200 mm × 150 mm × 100 mm) using the drip method according to McDonald (2012). Vertical sides of the plastic containers were coated with fluon (BioQuip, Rancho Dominguez, CA, USA) to prevent ants from escape. Harborage inside the container was made with a disposable culture tube (13 mm × 100 mm) half-filled with water, which was plugged with a cotton ball and stoppered with a layer of Castone® plaster (Dentsply, York, PA, USA). Ants were also provisioned with water, 17.8% glucose, and dead crickets and kept in an environmental chamber (27 °C, 80% RH). Diet was replenished weekly.

Cloning coding sequences (CDSs) of N. fulva housekeeping genes

Fifty worker ants were ground into powder in liquid nitrogen using a pestle installed onto a high-speed electronic drill (Bio Plas, San Rafael, CA, USA). Total RNA was

isolated using an RNeasy Mini Kit (QIAGEN, Hilden, Germany). A modification was made according to Valles *et al.* (2012) in which 200 μL of 0.5 mmol/L ethylenediaminetetraacetic acid (EDTA) (pH 8.5) was added into 600 μL extraction buffer RLT (QIAGEN). cDNA was synthesized from 2 μg of total RNA with random hexamer primers (Invitrogen, Carlsbad, CA, USA) and M-MuLV reverse transcriptase (NEB, Ipswich, MA, USA), and used as templates for cloning and gene expression analysis.

To clone CDSs of the six selected *N. fulva* genes, we took advantage of the fully sequenced Florida carpenter ant genome (Bonasio *et al.*, 2010) from the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/) and the partially assembled *N. fulva* genome (an ongoing project in coauthors Tarone and Vargo's labs). Primers used for CDS cloning (Table S1) were determined after BLAST search of carpenter ant CDSs against the *N. fulva* draft genome. If *N. fulva* gene sequences obtained (generally partial) contained either 5′ and/or 3′ ends of the target genes, primers were designed based on BLAST search results. For genes whose 5′ or 3′ regions were absent in the draft genome, degenerated primers were designed based on CDS of homologous genes from other ant species, including the Florida carpenter ant, red harvester ant (*Pogonomyrmex barbatus*) (Smith *et al.*, 2011), red imported fire ant (Wurm *et al.*, 2011), as well as two leaf-cutter ants (*Atta cephalotes* and *Acromyrmex echinatior*) (Nygaard *et al.*, 2011; Suen *et al.*, 2011). cDNAs prepared as described above were used as templates for PCR cloning. The resulting reverse transcription-PCR (RT-PCR) products were purified by QIAquick Gel Extraction Kit (QIAGEN) and subjected to DNA sequencing analysis.

Multiple sequence alignment and phylogenetic analysis

Nucleotide sequences of the *N. fulva* genes were translated by ExPASy Translate Tool (http://web.expasy.org/translate/). Putative amino acid sequences were used to search for homologous proteins from other insect and vertebrate species: the Florida carpenter ant, red imported fire ant, western honey bee (*Apis mellifera*), red flour beetle (*Tribolium castaneum*), Colorado potato beetle (*Leptinotarsa decemlineata*), common fruit fly (*Drosophila melanogaster*), zebrafish (*Danio rerio*), human (*Homo sapiens*) and house mouse (*Mus musculus*), by NCBI BLASTP (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins). Amino acid sequences were aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/), and the concatenated super alignment was then used to construct a phylogenetic tree (Gadagkar *et al.*, 2005). A phylogenetic tree was inferred by Neighbor-Joining with Jukes-Cantor pairwise estimated genetic distance as implemented in Geneious R11, and presented with bootstrap values based on 1000 replicates (Kearse *et al.*, 2012) (https://www.geneious.com/). NCBI accession numbers of all sequences used for phylogenetic analysis were shown in Table S2.

Expression of dsRNA in bacteria

T-tailed L4440 vector for dsRNA expression was constructed as described by Kamath and Ahringer (2003) and Zhu *et al.* (2011). cDNA fragments ranging from 115 to 380 bp for *N. fulva* genes were identified and PCR-amplified from their CDSs. Gene specificity was confirmed by BLAST analysis against the partial *N. fulva* genome sequences. PCR fragments of each selected gene were cloned into T-tailed L4440, sequenced, transformed into *E. coli* strain HT115 (DE3), and plated on LB-agar with 100 μg/mL ampicillin and 12.5 μg/mL tetracycline. Two inverted T7 promoters in L4440 transcribe the cloned gene fragment in opposing directions upon IPTG induction. DE3 is deficient in RNase III, allowing dsRNA accumulation of *N. fulva*-specific genes. Primers used in the cloning are shown in Table S1.

To express dsRNA, a single bacterial colony was inoculated into 1 mL of 2× YT with 100 μg/mL ampicillin and 12.5 μg/mL tetracycline followed by an overnight incubation at 37 °C with shaking (200 r/min). One microliter of the culture was transferred into 5 mL 2× YT with 100 μg/mL ampicillin, which was continuously incubated as above. From this overnight culture, 1 mL was transferred into 200 mL 2× YT with 100 μg/mL ampicillin. When the OD_{600} reached 0.4, isopropyl β-D-thiogalactopyranoside (IPTG) was added into the culture to a final concentration of 1 mmol/L. The culture was further incubated with shaking for another 5 h at 37 °C. Bacterial cells were harvested by centrifugation at 8000 r/min for 5 min and then heat-inactivated by incubation at 80 °C for 20 min.

To visualize dsRNAs in bacterial cells, 2 mL of bacterial cells before and after IPTG induction were harvested for individual constructs. Total RNA of the recombinant bacteria was extracted by RiboZol^{TM} (AMRESCO, Solon, OH, USA), and 4 μg of which was separated on a 1.5% agarose gel. Bacteria harboring the vector without an insert served as control.

Artificial diet preparation and bioassays

To incorporate the heat-inactivated bacterial cells into the artificial diet, the above cell pellet (~0.5 mL) from

200 mL culture (~2×10^{10} cells) was resuspended thoroughly with 1.3 mL of 27.5% glucose solution, minimal but sufficient to ensure maximal amount of bacteria and even distribution of dsRNA. The cells/glucose mixture was transferred to a culture tube (10 mm × 13 mm) containing 0.2 mL of warm, unsolidified 10% agar, bringing the total volume to 2 mL. Well-mixed suspension (by pipetting up and down at least 10 times) was distributed in 150 μL aliquots in 200 μL tubes prior to solidification. This artificial diet, made of 25% bacterial cells (v/v), 17.8% glucose, and 1% agar, was stored at 4 °C for bioassays. Control diet was prepared the same way but mixed with bacteria transformed with the empty vector.

N. fulva workers used in the bioassays were deprived of diet for 6 h before experiments began. Worker ants (30 per treatment) were placed in a plastic container (200 mm × 150 mm × 100 mm) with harborage, water, and artificial diet prepared as described above. Diet was replenished every 2 d. Living ants were counted at day 9. After counting, ants were collected and immediately frozen in liquid nitrogen. Samples were kept in –80 °C for gene expression analysis. Each construct was tested 12 times (4 biological replications × 3 technical replicates).

Quantitative RT-PCR

Effects of gene silencing were assessed by quantitative RT-PCR (RT-qPCR). Isolation of total RNA and cDNA synthesis was performed as mentioned above. SYBR Green Mastermix (BioRad, Hercules, CA, USA) was used in qPCR reactions, which were run on a CFX384 Real-Time System (BioRad). Primer sequences are provided in Table S1. The gene encoding for 60S ribosomal protein L4 (*NfRPL4*) was used as an internal control. Amplification specificity of each primer set was confirmed by examining the dissociation curve. The relative mRNA expression was calculated as described previously (Zhu-Salzman *et al.*, 2003).

Statistical analysis

SPSS software (v.20.0; SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Survival rates (means ± SE) were analyzed by one-way analysis of variance (ANOVA) and separated by Duncan's Multiple Range Test. Percentage data were transformed to arcsine-square-root to normalize the distribution. Relative gene expression data were analyzed by the independent samples' Student's *t* test.

Results

Phylogenetic relationship based on selected housekeeping genes

To determine whether feeding tawny crazy ants diet containing heat-inactivated, dsRNA-expressing bacteria could be a viable RNAi method, we selected six housekeeping genes, *NfActin*, *coatomer subunit β* (*NfCOPβ*), *arginine kinase* (*NfArgK*), *V-type proton ATPase subunits A* (*NfvATPaseA*), *B* (*NfvATPaseB*), and *E* (*NfvATPaseE*), as silencing targets based on their biological functions and on RNAi efficiency previously reported in different insect species (Baum *et al.*, 2007; Zhu *et al.*, 2011). Actin forms microfilaments, the major component of the cytoskeleton, involving not only cell shape and motility, but also cell division and signaling (Pollard & Cooper, 2009; Dominguez & Holmes, 2011). COPβ is a component of non-clathrin-coated vesicles, functioning in intra Golgi transport (Peter *et al.*, 1993), especially protein trafficking in the cell (Price & Gatehouse, 2008). ArgK is a phosphotransferase that catalyzes the reversible phosphorylation of L-arginine by ATP, crucial for the energy metabolism and buffering in insects and other invertebrates (Newsholme *et al.*, 1978; Chamberlin, 1997). vATPase is an ATP-driven proton pump with multiple subunits in plasma membrane to establish a pH gradient (Jefferies *et al.*, 2008).

We performed phylogenetic analyses using translated amino acid sequences of the above genes from tawny crazy ants and its homologues in three other hymenopterans (including two ant species), two coleopterans, one dipteran, as well as three vertebrates (Table S2). As expected, ant species were clustered together and they were phylogenetically closer to insects than vertebrates. In addition, ants share higher sequence similarity with honeybee (Hymenoptera) than insects from other orders (Fig. 1). Within Formicidae, higher similarity was shared between *N. fulva* and the carpenter ant (Fig. 1) with 89%–93% identity at DNA and 91%–99% at protein levels. Of all the target genes, actins showed highest amino acid sequence similarities and thus most conserved among different species (96%–99% to *N. fulva*), whereas ArgK sequences displayed the greatest divergence (43%–96% to *N. fulva*) (Table S2). Conserved sequences are indicative of the conserved roles of these selected genes. The phylogenetic relationship generated in our study is highly consistent with the classical taxonomic classification (Wiegmann *et al.*, 2009; Trautwein *et al.*, 2012), suggesting that analysis based on these selected housekeeping genes can provide a reliable estimation of *N. fulva*'s phylogenetic position.

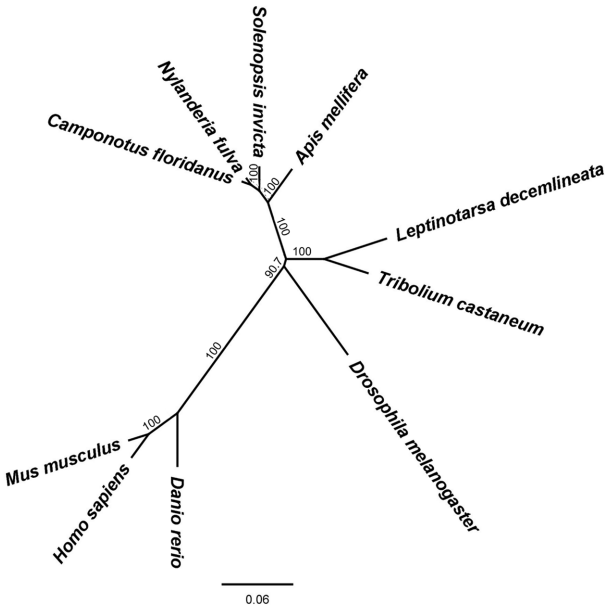

Fig. 1 A phylogenetic tree based on homology of selected genes. Concatenated alignment of amino acid sequences of actin, COPβ, ArgK, vATPaseA, vATPaseB, and vATPaseE homologs from *N. fulva*, Florida carpenter ant (*Camponotus floridanus*), red imported fire ant (*Solenopsis invicta*), western honey bee (*Apis mellifera*), red flour beetle (*Tribolium castaneum*), Colorado potato beetle (*Leptinotarsa decemlineata*), common fruit fly (*Drosophila melanogaster*), zebrafish (*Danio rerio*), human (*Homo sapiens*), and house mouse (*Mus musculus*). Ten animal species were used to construct phylogenetic trees with the neighbor-joining method (Geneious), which was based on genetic distances. The confidence values (%) of each clade on the phylogenetic trees, which represent the support for each clade, were based on 1000 bootstrap replicates. The scale bar indicates the levels of genetic changes (the number of amino acid substitutions/the length of the sequence).

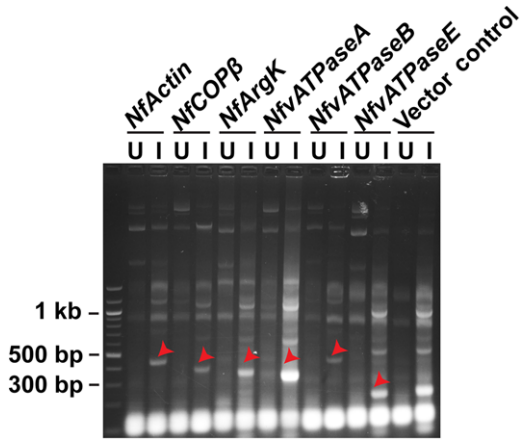

Fig. 2 Illustration of dsRNA production of target genes in bacteria. Total RNA of the bacteria was isolated using Ribozol^{TM}. Four micrograms of RNA before (U) or after (I) addition of IPTG was separated on a 1.5% agarose/TAE gel and visualized by ethidium bromide under ultraviolet light. Red arrows mark the positions of dsRNAs.

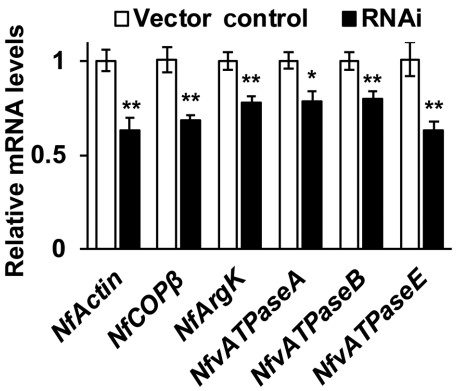

Fig. 3 Downregulation of target genes by RNAi in *N. fulva* workers on day 9 of bioassay. Data (mean ± SE, $n = 4$) were analyzed by the independent samples' Student's *t* test. Asterisks represent significant differences between vector controls and RNAi (*$P < 0.05$ or **$P < 0.01$).

Ingestion of bacterially expressed dsRNAs reduced target gene expression and impacted ant survival rate

Conserved housekeeping functions make these genes good targets for gene silencing. dsRNAs specific for all selected genes were successfully produced in bacterial cells (Fig. 2). RNAi molecular effects were determined by RT-qPCR after worker ants had fed on dsRNA-containing diets for 9 d. At this time point, no difference in survival rate was detectable whether or not the artificial diet fed to ants had bacteria transformed with empty L4440 vector, excluding mortality caused solely by bacterial incorporation (data not shown). Significantly reduced expression was shown in all target genes tested, ranging from 20% reduction in *NfvATPaseB* to 37% in *NfvATPaseE* (Fig. 3). Despite downregulation of all six genes, only those ants that fed on dsRNAs targeting *NfCOPβ* or *NfArgK* showed reduced survival rate (~15% reduction) compared to those feeding on control diet with bacteria transformed with empty vector (Fig. 4). Taken together, bacterially expressed dsRNA, when incorporated into artificial diet with its heat-inactivated bacterial host, without further purification, is sufficient to trigger the gene silencing mechanism and impact survival in *N. fulva*, but the potency was modest.

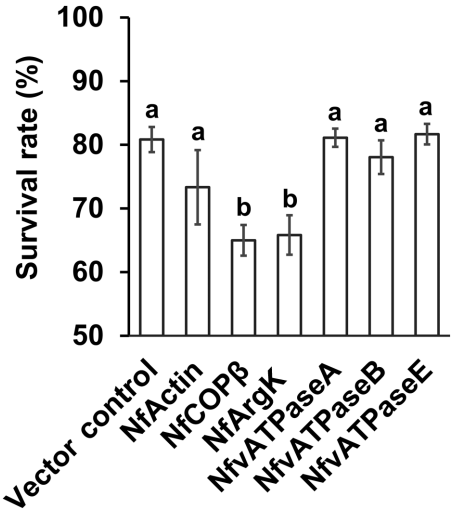

Fig. 4 RNAi-mediated down-regulation of *COPβ* and *ArgK* significantly reduced worker survival. The average survival rates of *N. fulva* workers were calculated based on data recorded on day 9 of the bioassay. Each treatment had three biological replicates, and each experiment was repeated four times. Data (mean ± SE, $n = 12$) were analyzed by one-way ANOVA ($F_{6, 77} = 5.94$, $P < 0.0001$). Means with the different letters were statistically significant (Duncan's Multiple Range Test, $P < 0.05$).

Discussion

Bacterially expressed dsRNA has demonstrated great potential in control of some pests such as the Colorado potato beetle, in which 60%–90% knockdown of gene expression has been achieved (Baum *et al.*, 2007; Zhu *et al.*, 2011; Bolognesi *et al.*, 2012). Tawny crazy ants that were subjected to the same RNAi procedure only showed less than 40% reduction (Fig. 3). Although it is difficult to assess the amount of dsRNA each ant ingested, the dsRNA concentration in ant diet is much higher than the beetle diet. This estimation was based on (1) the assumption that homologous genes and the same expression system was used, and therefore the comparable levels of dsRNA expression in equal volume of bacterial culture and (2) the consideration of diet preparation procedures for the two insects. Incomplete and/or transient gene knockdown and resulted limited impact on insect performance no doubt undermines its promise for crazy ant management.

Different insects or the same insect species at different stages have demonstrated variable sensitivity to feeding RNAi (Huvenne & Smagghe, 2010; Baum & Roberts, 2014). For instance, coleopterans such as western corn rootworm *Diabrotica virgifera virgifera* and Colorado potato beetle *Leptinotarsa decemlineata* are very susceptible to ingested dsRNA, whereas higher dsRNA concentration and lower RNAi efficiency have been observed in lepidopterans and hemipterans (Baum & Roberts, 2014; Christiaens *et al.*, 2014; Singh *et al.*, 2017). When ingested, dsRNA stability during the passage of the insect alimentary canal could contribute to the success of RNAi (Baum & Roberts, 2014; Christiaens *et al.*, 2014; Singh *et al.*, 2017). Degradation of dsRNA molecules by extracellular nonspecific nucleases in the insect saliva or gut lumen have been demonstrated to degrade dsRNA ingested by the silkworm *Bombyx mori* and locust *Schistocerca gregaria*, possibly resulting in ineffectiveness of dsRNA delivery (Arimatsu *et al.*, 2007; Luo *et al.*, 2013; Wynant *et al.*, 2014). Such nonspecific nucleases homologous sequences have been identified in five insect orders (Singh *et al.*, 2017). We also located a putative DNA fragment (data not shown), suggesting that tawny crazy ants could also possess this function. Measurements of pH values and cation composition of different compartments in the digestive canal, particularly the crop and midgut may help understand the marginal RNAi efficacy in tawny crazy ants, as shown in other insect species (Forconi & Herschlag, 2009; Valles *et al.*, 2012; Baum & Roberts, 2014; Mamta & Rajam, 2017). Poor incorporation of dsRNA into insect cells and/or low activity of RNAi machinery could also reduce the efficacy of RNAi (Christiaens *et al.*, 2014; Yoon *et al.*, 2016; Singh *et al.*, 2017; Yoon *et al.*, 2017). In response to ingested dsRNA molecules, expression patterns of key genes in dsRNA uptake machinery such as *Che*, *AP-50* and *Sid-1*, as well as RNAi core genes such as those encoding for Dicers and Argonautes will likely give some clues about the barriers of ant RNAi. Moreover, knowledge on tissue-specific expression of the RNAi target genes in midgut, hemolymph, and head may reveal how RNAi spreads in tawny crazy ants.

Gene silencing efficacy determines implementation of RNAi in pest control. For the RNAi technology to be widely used in insect control practice, improvement has to be made to facilitate orally ingested dsRNA uptake by gut cells from the lumen and systemic movement to other tissues beyond digestive system. Besides factors like concentration, length and sequence of dsRNAs, recent explorations of various delivery agents and methods and stacking RNAi (Whyard *et al.*, 2009; Yu *et al.*, 2015; Whitten *et al.*, 2016) represent potential effective tools to increase RNAi success in many recalcitrant insect species.

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Disclosure

The authors declare that they have no conflict of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Annotation of target genes and primers used for CDS cloning, dsRNA synthesis, and RT-qPCR.

Table S2. Genbank accession numbers of all the sequences used in bioinformatic analysis.