A novel gene from the takeout family involved in termite trail-following behavior

Margaret A. Schwinghammer a,1, Xuguo Zhou b,2, Srinivas Kambhampati c, Gary W. Bennett a, Michael E. Scharf a,b,*

a Department of Entomology, Purdue University, West Lafayette, IN, USA
b Department of Entomology and Nematology, University of Florida, Gainesville, FL, USA
c Department of Entomology, Kansas State University, Manhattan, KS, USA

ARTICLE INFO

Article history:
Accepted 26 November 2010
Available online 4 December 2010
Received by I. King Jordan

Keywords:
Takeout
RNA interference
Behavioral gene
Trail pheromone
2-phenoxyethanol

1. Introduction

The takeout-homologous gene family is a well-studied family of insect genes that was initially characterized in Drosophila (Sarov-Blat et al., 2000; So et al., 2000). In Drosophila, takeout was found to have similarities to OBPs and hemolymph JHBP. Takeout and the related “moling” and “JP29” proteins found in Manduca sexta are all classified as hydrophobic ligand binding proteins (Touhara and Prestwich, 1992; Wojtasek and Prestwich, 1995; Shinoda et al., 1997; Saito et al., 2006; Robertson et al., 1999; Du et al., 2003; Hiruma and Riddiford, 2010). Expression of takeout mRNA in Drosophila is located in structures associated with feeding, specifically the head, cardia, crop, and antennae, and it has been linked to feeding behavior, food location, and longevity (Sarov-Blat et al., 2000; So et al., 2000; Wong et al., 2009; Gáliková and Flatt, 2010; Bauer et al., 2010; Benito et al., 2010). In Drosophila, starvation induces the expression of takeout mRNA; however, this process does not occur in mutants with disrupted circadian clocks, implying a link between takeout, circadian clocks, and the availability of nutrients (Sarov-Blat et al., 2000). Takeout mutants also display abnormal locomotory activities and die rapidly during periods of starvation, suggesting a connection between locomotory activity, survival, and the availability of food (Sarov-Blat et al., 2000). Takeout mRNA levels undergo a daily cycle, and takeout is down-regulated or undetectable in circadian mutant flies (So et al., 2000; Sarov-Blat et al., 2000). After 9–10 hours of starvation, takeout mRNA levels increase, while a 2-hour re-feeding period reverses these effects (Sarov-Blat et al., 2000). Based on the above findings, it has been suggested that takeout may contribute to an organism’s anticipation of food resource availability, or play a role in its response to conditions of changing food availability (Sarov-Blat et al., 2000; Wong et al., 2009). Additionally, wildtype flies exhibit daily

Abbreviations:
ANOVA, Analysis of variance; dRNA, double-stranded RNA; EST, expressed sequence tag; JH, juvenile hormone; JHB, juvenile hormone binding protein; PCR, polymerase chain reaction; K-W ANOVA, Kruskal–Wallis ANOVA; OBP, odorant binding protein; qRT-PCR, quantitative real-time PCR; NADH, reduced nicotinamide adenine dinucleotide; RNAi, RNA interference; siRNA, short-interfering RNA; TBE, tris-borate-EDTA.

* Corresponding author. O.W. Rollins/Orkin Chair, Entomology Department, 901 W. State St., Purdue University, West Lafayette, IN 47907-2089, USA. Tel.: +1 765 496 6710; fax: +1 765 494 0535.
E-mail address: mscharf@purdue.edu (M.E. Scharf).
1 Current address: CNA, 4825 Mark Center Dr, Alexandria, VA, USA.
2 Current address: Department of Entomology, University of Kentucky, Lexington, KY, USA.

0378-1119/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.gene.2010.11.012
fluctuations in takeout mRNA levels in cycling photoperiodic conditions as well as constant dark conditions, implying the daily cycling is a function of the insect’s internal clock rather than being externally light driven (So et al., 2000). Since the initial characterization of takeout in Drosophila, members of the takeout/ligand binding protein family have been identified in a number of insects, including termites (Ishida et al., 2002a; Hojo et al., 2005). A takeout-homologous gene with brain expression has also been linked to long-distance migration in the monarch butterfly, Danaus plexippus (Zhu et al., 2008).

Trail following behavior in termites is an experimentally tractable and important social behavior for which no genetic basis has yet been identified. Subterranean termites live in eusocial colonies, spending most of their lives underground and in complete darkness (Wilson, 1971). The vast majority of individuals in subterranean termite colonies are eyeless and therefore must use chemical trails to navigate through the colony’s network of underground tunnels, and to communicate and convey messages for exploration, defense, and food harvesting among nest mates (Stuart, 1967; Moore, 1966; Matsumura et al., 1968; Wilson, 1971). The functions of termite pheromones secreted from sternal and labial glands have been investigated and are well understood. Sternal gland secretions containing ZZE-dodecatrienol function as trail pheromone, which is used for orientation and signaling for communal exploitation of food resources (Matsumura et al., 1968). During the exploitation of a food source, secretions from the labial gland containing the active compound hydroquinone are released onto food, stimulating feeding by conspecific nestmates (Reinhard and Kaib, 1995). The transfer of food and its transportation back to the nest occur primarily on trails where a high concentration of sternal gland secretions have been deposited; whereas, gnawing behavior is concentrated at sites where labial gland secretions are present (Reinhard and Kaib, 1995). Interestingly, some lower termite species of the genera Reticulitermes and Coptotermes will readily follow trails of fresh ball-point pen ink containing the compound 2-phenoxethanol (Chen et al., 1998).

RNA-interference (RNAi) is an important functional genomics tool for the study of gene function. RNAi, also called post-transcriptional gene silencing or transgene silencing (Hannon, 2002), is a process that uses dsRNA which shares a homologous sequence with a target gene to interfere with the gene’s expression (Fire et al., 1998). The biological origin of the RNAi pathway is thought to be associated with the immune response, protecting organisms from exogenous genetic material (Gura, 2000). Several recent studies have demonstrated that RNAi is achievable in termites and that it can reveal important insights into termite biochemistry, physiology, sociality, and behavior (Zhou et al., 2006a, 2006b, 2007, 2008; Scharf et al., 2008; Korb et al., 2009). As behavior is the outward manifestation of gene expression, this study used RNAi to investigate the influence of a termite takeout homolog hypothesized to be involved in trail following behavior. Additionally, this study examined how the RNA-interference pathway can be enlisted to examine the roles of specific genes in the modulation of downstream behaviors and caste differentiation processes (Robinson et al., 2005; Smith et al., 2008).

This paper reports sequence, expression, and functional data for the deviate gene of the termite Reticulitermes flavipes, which has the highest homology to members of the takeout family of ligand binding proteins. Given the various behavioral and physiological roles for takeout family members in Drosophila and other insects, this research was conducted as a candidate gene study into the potential roles of R. flavipes deviate. Information presented here includes tissue and caste distribution of the deviate transcript; deviate transcript expression changes in response to JH, temperature, feeding and light-dark conditions; and deviate silencing by RNAi. To test the hypotheses that the deviate gene is linked to trail-following, foraging, and/or feeding behavior, bioassays were designed and conducted to investigate trail following behavior in association with RNAi-attenuated deviate gene expression. Additionally, to test the hypotheses that deviate might play roles in regulating or mediating temperature-dependent caste differentiation (Scharf et al., 2007), studies were conducted to investigate the impacts of temperature and JH on expression, and by using RNAi to silence deviate expression in combination with JH-dependent caste differentiation bioassays. A recent publication by Korb et al. (2009) provided the first evidence of a termite behavioral gene by using RNAi to silence a gene encoding a putative odorant-releasing enzyme linked to suppression of aggressive behaviors in Cryptotermes termites. Here, we provide a second and very different example of a termite behavioral gene: one with ubiquitous expression that mediates trail following accuracy, and that is responsive to JH, temperature, feeding, and light-dark conditions.

2. Materials and methods

2.1. Termites

Termites were collected from logs at a site on the Purdue University campus (West Lafayette, Indiana, USA). Termites were identified as R. flavipes based on soldier morphology (Nutting, 1990). Workers, soldiers, and nymphs were isolated from logs and housed in plastic containers with moistened wood and paper towels at ca. 22 °C and 97% relative humidity, in full darkness. These laboratory colonies became reproductively competent within a few weeks and were fully self-sufficient thereafter.

2.2. Deviate gene sequencing and sequence analyses

The full-length cdNA sequence for the deviate gene (Genbank accession no HQ003932) was assembled from multiple overlapping clones from several different cdNA libraries (Wu-Scharf et al., 2003; Tartar et al., 2009; Steller et al., 2010) (accession nos. BQ788174, G0902234, FLG39426, G0900869, G0901038, G0901465, FLG37875, GO899218, FLG39883, FLG38424, FLG37493, and FLG36085). The majority of the ORF sequence was verified by PCR amplification of an 864 bp fragment of the cdNA spanning cdNA nucleotides 27–824 (Fig. 1), using the forward and reverse primers 5′-CCCCCTCGATTGTTTATGG-3′ and 5′-GAAGGGCAACTGAGAACA-3′. Database searches were made using NCBI (http://www.ncbi.nlm.nih.gov/) using blastn and blastx under default settings. Peptide translations were made using SDSC Biology Workbench (http://workbench.sdsc.edu/). Signal peptide and N-glycosylation analyses were performed using the SignalP and NetNGlyc 1.0 servers available at http://www.cbs.dtu.dk/services/ and http://www.cbs.dtu.dk/services/NetNGlyc/.. Protein alignments and cladograms were generated using ClustalV in the Lasergene software package (DNAsStar; Madison, WI). Cladogram analyses were performed with 100 bootstrap replicates of parsimony analysis using PAUP* 4.0b10.

2.3. Tissue and caste expression profiles

Visualization of conventional PCR products on agarose gels was used to examine deviate expression across five body regions (whole body, antennae, head, thorax, and abdomen), and three caste phenotypes (workers, soldiers, and nymphs). Total RNA was isolated from homogenized termite samples using the RNaseasy Mini Total RNA Kit (Qagen; Valencia, CA). Three RNA isolations were made per tissue and caste phenotype from two replicated colonies. Per replicate, fifteen whole termites or body regions (head, thorax, or abdomen), or 40–50 individual antennae were used. RNA quality and quantity were determined by gel electrophoresis and spectrophotometry. cdNA was synthesized from total RNA using the Invitrogen SuperScript First-Strand Synthesis System (Invitrogen; Carlsbad, CA). PCR was performed using 1 μl of cdNA, iQ SYBR Green Supermix (Bio-Rad: Hercules, CA) and deviate gene specific primers. The deviate
forward and reverse primer sequences, respectively, were 5’-AGGCCAGTTGTGTCACAT-3’ and 5’-GTGAAGGCGGCCGCGATCG-3’; NADH-dh (BQ788168; Wu-Scharf et al., 2003) was used as a reference gene. The NADH-dh forward and reverse primer sequences, respectively, were 5’-GCTGGGGGTGTTATT CATTCCTA-3’ and 5’-CAATAGTGATGACCTGGCCGT-3’. PCR products were visualized on 1.75% agarose-TBE gels containing 0.25% Synergel (Diversified Biotech; Boston, MA).

2.4. Light–dark assays and quantitative real-time PCR methods

Bioassays were conducted in 35 mm diam. Petri dishes using 15 termites per dish from three replicate colonies as described previously (Scharf et al., 2008). Three treatments were tested, all at 27 °C: continuous darkness, continuous light, or 12:12 hr light:dark. For the continuous darkness treatment, all manipulations were performed in a dark room under red light. After 15 days, termites were frozen at −80 °C. Total RNA was isolated from frozen whole termites using the SV Total RNA Isolation System, which included on-column DNAse treatment (Promega; Madison, WI). cDNA was synthesized from total RNA using the iScript cDNA synthesis kit (Bio-Rad). qRT-PCR was performed using iQ SYBR Green Supermix (Bio-Rad; Hercules, CA), according to each manufacturer’s protocol. cDNA and translated amino acid sequence for the NADH-dh gene (Genbank accession No. HQ003932). Nucleotides are shown in lower case letters and amino acids in capital letters above the reference gene. The expression of two reference genes (β-actin (DQ206832; Zhou et al., 2006b) and NADH-dh (BQ788168; Wu-Scharf et al., 2003) was used as a reference gene. To test the effect of starvation and feeding on deviate gene expression, groups of termites were held without a food source for 0, 5, 10, or 15 days and compared to termites held under similar conditions but with food. In the “fed” treatment, 15 termites from three replicate colonies were placed in 35 mm diam Petri dishes with one disk of double-ply paper towel as described previously (Scharf et al., 2008). In the starvation treatments, termites were placed in Petri dishes as above, but with one double-ply disk of moistened paper towel attached to the inside-top cover of the dish (inaccessible to termites). Assays were replicated across three colonies per treatment and ran for 15 days at 27 °C. Every 5 days, individual Petri dishes were opened and 100 μl of distilled water added to the paper towel disks either lining the bottom of the dish (control treatment) or the top of the lid (starvation treatment). At 5, 10, and 15 days, termites were removed from assay dishes and frozen at −80 °C. Termite mortality never exceeded 1 individual per replicate. Total RNA was isolated, cDNA synthesized, and qRT-PCR performed and analyzed as described above (Section 2.4).

2.5. Starvation assays

To test the effect of starvation and feeding on deviate gene expression, groups of termites were held without a food source for 0, 5, 10, or 15 days and compared to termites held under similar conditions but with food. In the “fed” treatment, 15 termites from three replicate colonies were placed in 35 mm diam Petri dishes with one disk of double-ply paper towel as described previously (Scharf et al., 2008). In the starvation treatments, termites were placed in Petri dishes as above, but with one double-ply disk of moistened paper towel attached to the inside-top cover of the dish (inaccessible to termites). Assays were replicated across three colonies per treatment and ran for 15 days at 27 °C. Every 5 days, individual Petri dishes were opened and 100 μl of distilled water added to the paper towel disks either lining the bottom of the dish (control treatment) or the top of the lid (starvation treatment). At 5, 10, and 15 days, termites were removed from assay dishes and frozen at −80 °C. Termite mortality never exceeded 1 individual per replicate. Total RNA was isolated, cDNA synthesized, and qRT-PCR performed and analyzed as described above (Section 2.4).

2.6. Temperature and JH assays

To test the effect of temperature and JH on deviate gene expression, groups of termites were held at 17, 22 and 27 °C for 15 days in the presence and absence of 150 μg ectopic JH III. This method is well-established for its ability to induce worker-to-presoldier morphogenesis (Zhou et al., 2006a; Scharf et al., 2007; Scharf et al., 2008). Assays were set up and run as described in preceding sections (2.4, 2.5) with 3 colony replicates per treatment. Environmental chambers were used to attain the three temperatures. Each assay paper received 150 μg JH III (Sigma-Aldrich; St. Louis, MO) and was left to dry for 30 min before placing in assay dishes. For control treatments, paper towel disks were normalized to those of two reference genes (β-actin and NADH-dh) using the BestKeeper method (Pfaffl et al., 2004). Relative mRNA expression. CT values for the deviate PCR reactions were normalized to those of two reference genes (β-actin and NADH-dh) using the BestKeeper method (Pfaffl et al., 2004). Relative expression levels of the deviate gene were then calculated using the 2−ΔΔCT method (Livak and Schmittgen, 2001).
received acetone alone. RNA was isolated, cDNA synthesized, and qRT-PCR performed and analyzed as described above (Section 2.4). All reported data were normalized to 27 °C data.

2.7. siRNA production

Total RNA was isolated and cDNA synthesized as described above (Section 2.4). A ~500 bp deviate gene cDNA fragment was PCR-amplified using the gene specific primers “deviate RNAi forward” (5′-AGCAGAGGCTCAATACAAG-3′) and “deviate RNAi reverse” (5′-CTGCCAAAGGTAACATCT-3′). siRNA was synthesized following the Silencer siRNA Cocktail Kit™ (Ambion; Austin, TX) protocol and Scharf et al. (2008), as described in detail elsewhere (Scharf et al., 2008). The concentration of the siRNA sample was measured by spectrophotometry and diluted to a final concentration of 0.5 ng per 360 nl.

2.8. Microinjection of siRNA and RNAi knockdown quantification

Microinjections were performed using a custom-designed platform and vacuum manifold, a Nanoliter 2000™ injector (WPI Inc.; Sarasota, FL), and custom-pulled glass capillary needles back-filled with silicone oil (Zhou et al., 2006a; Scharf et al., 2008). Worker termites were isolated from lab colonies and placed in dishes on ice shortly before injection, which slowed movements and made termites easier to handle during the injection process. Termites quickly became active again after the chilling and injection procedure and non-injected termites were also chilled for the same amount of time.

siRNA solution (36 nl containing 0.5 ng siRNA; Zhou et al., 2006a) was injected into the lateral thorax, above the coxae of the legs (Zhou et al., 2006a; Scharf et al., 2008). Controls were injected with nuclease-free nanopure water alone. After injection, groups of 15 termites were placed in 35 × 10 mm dishes with double-ply disks of moistened paper towel lining the bottoms of the dishes. Five of these dishes were then placed inside a covered plastic box, lined with moistened paper towels. Non-injected termites, termites injected with deviate siRNA were removed from holding dishes 0, 1, 2, and 3 days after injection. RNA was isolated, cDNA synthesized, and qRT-PCR performed and analyzed using two reference genes as described above (Section 2.4).

2.9. RNAi bioassays

2.9.1. JH bioassays

In the first set of RNAi bioassays, siRNA-injected and control termites from three replicate colonies were subjected to JH bioassays and observed for presoldier differentiation as described previously (Section 2.6; Zhou et al., 2006a).

2.9.2. Trail following bioassays

In the second set of RNAi bioassays, termites from three replicated colonies were tested for their ability to follow simulated trails prepared with black pen ink containing the artificial trail pheromone 2-phenoxyethanol (Chen et al., 1998). To create a template for the trail, a dark 30 cm line was drawn in pencil on a 21.5 × 35.5 cm (11 × 14”) sheet of paper. Additionally, two dark outside “deviation” lines were drawn 0.5 cm on either side of this line. A fresh sheet of paper was placed on top of the template sheet and a ruler used to draw a straight 30 cm line with a black ink BiC™ Round Stick (medium point) (Clearwater, FL) (Suppl. Fig. 1). A 3.7 cm base diameter plastic cup with a small exit hole on the side facing the line was placed over the termite (for containment) until the trail was located. To test the ability of termites to cross a break in the simulated trail, a 2 cm gap was created at the end of the trail. Ink trails were less than 30 s old at the time termites were introduced. Fresh sheets of paper were used for each trail, and no trail was used more than once. Seventy-five termites per replicate each received deviate siRNA injections, injections of nuclease-free water, or were chilled but remained un-injected. Termites from each treatment were divided into five small Petri dishes, for a total of 15 termites per dish. Disks of double-ply paper towel were placed at the bottom of each dish and moistened with 100 μl of distilled water. The 5 dishes from each treatment were placed inside separate plastic boxes lined with moistened papers towels and the boxes were stored in a growth chamber at 27 °C for 15 days.

Four parameters were measured each time a termite was tested: the time (seconds) for the termite to run the length of the 30 cm trail, the ability (yes or no) of termites to cross a gap in the trail, the time (seconds) for termites to cross the gap, and the number of times termites wandered 0.5 cm off the center ink trail and touched either of the outside deviation lines (Suppl. Fig. 1). The latter deviation data were converted to relative proportions for reporting (i.e., proportion of deviations). Data collection began after a termite was placed on the sheet of paper and moved out from the area under the cup onto the ink trail. No termite was tested more than once. Eighty termites were randomly sampled per replicate, and three replicates were tested for each of the three treatments (deviate injected, water injected, and non-injected). Behavioral bioassays were conducted 1, 2, 3, and 4 days after RNAi injections.

2.9.3. Distance feeding bioassay

In the distance feeding bioassay (Suppl. Fig. 2), 150 termites from a single colony each received deviate siRNA injections, injections of autoclaved water, or were chilled but not injected. Termites from each treatment were divided into five 100 × 20 cm Petri dishes (for a total of 30 termites per treatment group). This dish contained no food source but had a moistened paper towel disk, which termites can derive nutrition from, attached to the lid (inaccessible to termites). This dish was connected to a second dish by 1 m of clear 3.2 mm Tygon™ tubing. Disks of double-ply paper towel were placed in the bottoms of the second dishes (accessible to termites) and moistened with 100 μl of distilled water. Dishes from each treatment were randomly placed inside separate plastic boxes lined with moistened papers towels, which were housed in an environmental chamber at 27 °C for 15 days. Paper in the second dish was replaced on days 5, 10, and 15, and pre- and post-weights recorded.

Termites were restricted to the first Petri dish for the first 48 hours after injection to ensure that they did not begin foraging before the RNAi effect reached maximal levels (see Section 3.5). This was accomplished by clamping the tubing just outside of the first dish. After 48 hours clamps were removed and termites allowed to move freely between both dishes.

2.10. Statistical analyses

Statistical analyses were conducted using JMP 6.0 (SAS Institute; Cary, NC, USA). Analysis consisted of non-parametric K-W ANOVA tests (Zhou et al., 2006a), replicated as described in each methods and results section. All bioassay treatments were independently replicated on three colonies, except in the distance-foraging assay, in which only a single colony was used.

3. Results

3.1. cDNA sequences and amino acid translations

The full-length deviate cDNA and translated amino acid sequences are shown in Fig. 1. The cDNA sequence contains 43 nucleotides of 5′-untranslated region (UTR) ahead of the ATG start codon, an 851 bp open reading frame (ORF), and 148 nucleotides of 3′ UTR between the “tag” termination codon and the poly-A tail. The 3′ UTR contains an apparent polyadenylation signal “AATAAA” and a putative terminal
poly-A tail. The translated deviate protein sequence contains 283 amino acids and a 16 amino acid signal peptide MLQFVLAALLATLA, which is suggestive that the mature protein is soluble and secreted. Two cysteine residues putatively involved in creating a disulfide bridge involved in ligand binding are present near the N-terminus. Four predicted N-glycosylation sites are present in the mature protein at N52, N82, N137 and N179.

A ClustalV multiple alignment of insect takeout and JHBP amino acid sequences is shown in Fig. 2. The alignment shows the closest database matches obtained from blastx searches of the Genbank nr database using the deviate cDNA sequence as a query. Sequences included in the alignment consist of R. flavipes deviate and 13 other species from Isoptera, Diptera, and Lepidoptera. The alignment shows only low degrees of amino acid identity between deviate and the other 12 sequences; i.e., the highest identities are only 9.1–14.1%. Only the two cysteine residues involved in creating a ligand binding pocket are conserved across all 13 family members. A cladogram (Fig. 3) generated from the Fig. 2 alignment (parsimony analysis, 293 total characters, 6 characters constant, 67 characters parsimony uninformative, 220 parsimony informative characters) shows deviate as a distinct outgroup that clusters nearest to JHBPs of M. sexta and B. mori. These results indicate that R. flavipes deviate is a highly novel member of the takeout/JHBP protein family.

3.2. Constitutive expression among tissues and caste phenotypes

Constitutive expression studies were conducted using conventional PCR and agarose electrophoresis (Fig. 4). In general, ubiquitous deviate mRNA expression was identified among all tissues and body regions (including antennae) of workers, soldiers, and nymphs. Only worker gels are shown for brevity.

3.3. Deviate expression responses to light–dark conditions and feeding

In light–dark experiments, qRT-PCR data from continuous light and dark treatments were normalized to 12:12 hr light:dark conditions.
expression data (Fig. 5A). A ~2.5-fold increase (K-W ANOVA; \( p = 0.0794 \)) in deviate mRNA expression occurred in continuous light treatments relative to continuous darkness. Deviate expression was also not different between continuous light and 12:12 hr light: dark conditions (K-W ANOVA; \( p > 0.1 \)).

For feeding/starvation experiments conducted in complete darkness, qRT-PCR data from days 5, 10, and 15 were normalized to day 0 (Fig. 5B). Significant differences in deviate mRNA expression were found between day 0 and day 5 fed (K-W ANOVA; \( p = 0.0116 \)), and day 5 fed and starved (K-W ANOVA; \( p = 0.0006 \)). At day 10, both fed and starved treatments continued to show significant differences from day 0 (K-W ANOVA; \( p = 0.0044, p = 0.0035 \), respectively), but were not different from each other. At day 15, starved and fed treatments had identical expression, and starved treatments were different from day 0 (K-W ANOVA; \( p = 0.0059 \)), but fed treatments were not.

3.4. Temperature and JH impacts on deviate expression

For these experiments, qRT-PCR data from 17 °C and 22 °C treatments were normalized to 27 °C treatment expression data (Fig. 6). Only marginal differences (K-W ANOVA; \( p < 0.1 \)) were found between the two temperatures under baseline conditions, but the temperature effect was significant in JH treatments (K-W ANOVA; \( p < 0.02 \)). Likewise, there were no differences in deviate mRNA expression at 17 °C (K-W ANOVA; \( p > 0.1 \)), but at 22 °C deviate expression decreased to ~0.14× in the presence of ectopic JH (K-W ANOVA; \( p = 0.02 \)). In agreement with previous findings for other JH-responsive genes and proteins (Scharf et al., 2007) these results indicate that temperature and JH interact significantly to impact deviate expression.

3.5. Methods development and attenuation of deviate mRNA levels after deviate siRNA injection

Before proceeding with behavioral bioassays, it was first necessary to optimize RNAi conditions. The optimal concentration of deviate siRNA injected into the termites was identified to ensure that gene silencing was effective, quantifiable, and produced no unintended or detrimental side-effects. For this purpose termites were injected with either deviate siRNA (36 nl containing 0.5 ng siRNA) or water, or left non-injected. Consistent with earlier work (Zhou et al., 2006a), no significant mortality was observed in association with the injection process.

After initial test injections of siRNA in workers, qRT-PCR results showed an expected reduction in deviate gene expression when compared to pre-injection expression levels and water-injected controls (Fig. 7A). Expression levels were normalized to two control genes (\( \beta\)-actin, NADH-dh), which were unaffected by deviate silencing. One day after RNAi injections, deviate mRNA expression dropped to 53.6% of pre-injection expression levels (K-W ANOVA; \( p < 0.05 \)); however, at 2 and 3 days after siRNA injections, deviate expression returned to levels identical to water-injected controls, which did not show significant changes in deviate or control gene expression over the 3-day test period.

3.6. Impacts of deviate RNAi on JH-dependent worker-to-soldier caste differentiation

Worker termites were subjected to a model bioassay in which ectopic exposure to JH was used to force caste differentiation in deviate siRNA injected and control individuals. JH III induced presoldier development in all treatments (deviate siRNA injected, water injected, and non-injected), as expected, starting at assay
day 10. Presoldier differentiation steadily increased over the course of the assay (Suppl. Fig. 3); however, no significant differences were observed among the three treatments. Also, as expected, no presoldier development occurred in non-injected control treatments in the absence of ectopic JH. These results do not suggest roles for deviate in either mediation or attenuation of JH-dependent caste differentiation.

3.7. Impacts of deviate RNAi on trail following and foraging

Next, trail following bioassays were conducted in siRNA injected treatments and controls, using worker termites and 2-phenoxethanol as an artificial trail pheromone. Termites completed trails in ~15–25 s, and there were no differences among RNAi treatments and controls in the time it took to complete trails (Suppl. Fig. 4). However, significant differences between deviate siRNA and control treatments did occur in the number of deviations termites made from the center trail line (Fig. 7B). Specifically, 1 day after injections, deviate siRNA injected termites made >4-fold more deviations than water (K-W ANOVA; $p = 0.0051$) and non-injected (K-W ANOVA; $p = 0.0116$) controls. On day 2, deviate siRNA injected termites once again made significantly more deviations than both water (K-W ANOVA; $p = 0.0209$) and non-injected (K-W ANOVA; $p = 0.0093$) controls. There were no significant differences between controls and treatments on day 3 and beyond.

No significant differences were observed for other trail-following parameters between termites injected with deviate siRNA and controls for the ability to cross a 2 cm gap in the trail (Suppl. Fig. 5) and the time to cross a 2 cm gap in the trail (Suppl. Fig. 6). There was also no correlation between the number of deviations a termite made from the center trail line and the time it took termites to run the length of the trail. In distance feeding bioassays (Suppl. Fig. 7), there were no significant differences among treatments within any of the days, and no differences between days 1 and 5, and days 10 and 15. In distance feeding assays, deviate siRNA-injected treatments exhibited identical feeding levels to controls (K-W ANOVA; $p > 0.05$), indicating that deviate gene silencing did not affect general vigor in terms of long-term mobility and foraging.

4. Discussion

4.1. Overview

Findings of this study indicate that the R. flavipes deviate gene and translated amino acid sequences are highly unique relative to all other known takeout family members. The takeout gene family is large and diverse, with members that participate in odorant and JH binding, as well as circadian output, circadian signaling, and feeding behavior.
Thus, at the onset of this study we set out to investigate each of these aspects. We found that *R. flavipes* deviate is responsive to light–dark conditions, feeding, temperature and JH, and plays a significant role in trail following accuracy, but not JH-dependent caste differentiation. In the sections that follow, each of these topics is discussed relative to termite biology and what has been previously reported for other insect groups.

4.2. The deviate gene and protein of *R. flavipes*

In database searches, the deviate gene and its predicted translation product were most similar to takeout-homologous genes, OBPs, JHBPs from multiple insect species. BLAST searches using the translated deviate amino acid sequence identified proteins of known or investigated function including takeout and takeout-like genes in species including *Aedes aegypti* (Bohbot and Vogt, 2005), *Phormia regina* (Fujikawa et al., 2006), *Apis mellifera* (Hagai et al., 2007), and *Drosophila melanogaster* (So et al., 2000; Sarov-Blat et al, 2000) (Fig. 2). Other deviate-homologous genes with characterized functions included a solider-specific protein from *Naustitermes takasagoensis* (Hojo et al., 2005), JHBP in *M. sexta* (Du et al., 2003), a circadian-clock controlled gene precursor in *Drosophila yakuba* (Thackery and Kyriacou, 1990), and several antennal OBPs occurring across multiple orders of insects, including termites (Ishida et al., 2002a). *R. flavipes* deviate shares little homology with other known termite takeout family members as reported previously (Ishida et al., 2002a; Hojo et al., 2005). The fact that this is the first deviate homolog identified from a hemimetabolous insect may also explain its uniqueness.

The takeout gene and related OB and JHBPs are typically 230–250 amino acids long and contain two cysteine residues located near the N-terminal end of the peptide sequence (Saito et al., 2006; Robertson et al., 1999; Wojtaszek and Prestwich, 1995). These cysteines form a disulfide bridge that creates secondary structure involved in ligand binding (Wojtaszek and Prestwich, 1995). Another feature common to many insect OBPs is the presence of six cysteine residues located throughout the protein (Ishida et al., 2002b; Robertson et al., 1999; Bohbot and Vogt, 2005). In addition to cysteine residues, two conserved ligand binding fragments were identified in *M. sexta* JHBP (Touhara and Prestwich, 1992; Saito et al., 2006). The first binding motif is located closer to the N-terminus of the peptide sequence and the second includes the C-terminus. Some takeout and OBPs also contain signal peptide sequences (Saito et al., 2006; Sarov-Blat et al., 2000; Hojo et al., 2005).

*R. flavipes* deviate is shorter in length than most takeout family members, possesses a secretory signal peptide, and possesses only a single cysteine residue beyond its two highly conserved N-terminal cysteine residues. Interestingly, in addition to having expected antennal expression, deviate mRNA expression was also observed throughout the bodies of workers, soldiers and nymphs. Deviate ESTs were sequenced from *R. flavipes* alate (Steller et al., 2010) and gut cDNA libraries (Tartar et al., 2009; Tarver et al., 2010) further supporting that it has roles in multiple castes and tissues. These findings suggest that the *R. flavipes* deviate gene and protein are highly unique relative to all other known insect takeout homologues, and perhaps perform unique social functions in termites.

4.3. Links to circadian physiology, feeding, temperature, and JH

4.3.1. Circadian physiology

In light–dark studies, marginally significant differences in deviate expression were found between light and dark treatments (Fig. 5A). The *Drosophila* takeout gene has been described as a circadian clock-regulated output gene (McDonald and Rosbash, 2001; Benito et al., 2010). Specifically, takeout gene expression fluctuates with daily circadian rhythm patterns and is undetectable in circadian mutants, which suggests a role in circadian-associated functions (Sarov-Blat et al., 2000). In addition, recent findings suggest that takeout also plays a role in lifespan regulation (Gáliková and Flatt, 2010; Bauer et al., 2010). Consistent with takeout links to circadian biology, the current study suggests that, although subterranean termite workers are eyeless, they have the ability to sense changes in photoperiod and alter deviate gene expression accordingly. The alteration of deviate gene expression under different light–dark regimes suggests that worker termites possess photosensory capabilities, and that deviate may play a role in photoperiod-regulated behavior and/or physiology.

4.3.2. Feeding

In feeding studies, although deviate expression changed significantly through time in both treatments and controls, the most substantial differences in deviate expression occurred in day 5 fed vs. starved treatments (fed = significantly higher expression; Fig. 5B). In *Drosophila*, alternatively, takeout expression increased during periods of starvation (Sarov-Blat et al., 2000). While these results indicate changes in gene expression over time when worker termites are faced with starvation conditions, they may also be associated with removal from colony conditions. In this respect, it is noteworthy that there may be a “colony-release”-associated reduction in deviate expression resulting from our experimental design, which over-shadows changes in gene expression due to starvation (Zhou et al., 2006a, 2007). It was also previously suggested that *Drosophila* takeout plays a role in foraging behavior by directing flies to make a choice between reproduction and foraging (Sarov-Blat et al., 2000). This is seemingly contrasted in worker termites, which have no reproductive capabilities. Thus, deviate and takeout apparently play very different roles in directing feeding behavior of termites and *Drosophila*, respectively.

4.3.3. JH–temperature interactions

JH plays complex caste-regulatory roles in termites (Zhou et al., 2006a, 2007; Scharf et al., 2007; Tarver et al., 2010). Because the deviate gene shows some structural similarities to JH binding proteins, we hypothesized that, similar to hexamerin proteins (Zhou et al., 2007; Scharf et al., 2007), deviate may participate in caste regulation via modulation or mediation of JH signaling. In studies involving ectopic exposure to JH concentrations capable of inducing caste differentiation at different temperatures, both factors were found to interact significantly to influence deviate mRNA expression (Fig. 6). In a companion study, significant differential expression of deviate mRNA (called ‘TO-F’) was noted among workers exposed to JH, live soldier, and soldier head extract treatments (Tarver et al., 2010). These findings suggested that deviate expression levels are influenced by endogenous JH and colony primer pheromone titers, which in turn are influenced by a number of extrinsic factors (Zhou et al., 2007; Scharf et al., 2007; Tarver et al., 2010). However, in deviate RNAi studies conducted here (Suppl. Fig. 1), no significant impacts on JH-dependent presoldier differentiation were observable, suggesting that deviate does not directly interact with JH to influence caste differentiation.

4.4. Evidence linking the deviate gene to trail-following behavior

Trail following bioassays were used to investigate changes in termite behavior following deviate silencing using 2-phenoxyethanol as an artificial trail pheromone (Chen et al., 1998). In addition to trail following bioassays, long-term distance feeding bioassays were also used to assess whether deviate silencing impacted general vigor and foraging in the presence of natural trail pheromone (ZZE-dodeca-trienol; Matsumura et al., 1968). Because odorant and ligand binding proteins are associated with the reception and transport of chemical signals within the insect body, and insects with mutations in these proteins have been shown to exhibit aberrations in movement
(Sarov-Blat et al., 2000; Meunier et al., 2007), we examined whether similar responses occur in *deviate* silenced termites. Our results indicate that trail following accuracy is associated with *deviate* expression, but not any other parameters investigated (e.g., speed, gap crossing). These findings provide evidence that *deviate* may encode an OBP involved in the perception of trail pheromone. However, the uniqueness of the translated *deviate* protein sequence and its expression outside the antennae suggests it may have been co-opted to perform functions related to the subterranean eusocial lifestyle of *R. flavigas*.

In the distance bioassay, *deviate* silenced termites did not display impairment in their ability to move toward or feed upon a food source. However, this observation must be considered in the context of the experimental design, which did not test the ability of termites to make behavioral foraging choices, i.e., termites were not subjected to conditions which required them to make foraging or navigational choices. Nonetheless, because high levels of survival occurred and equal food consumption was observed between treatments and controls, the distance bioassay provides good evidence that termite vigil is not impacted by *deviate* silencing.

### 4.5. Summary and conclusions

This research tested four main hypotheses related to the involvement of the *R. flavigas deviate* gene in trail following, foraging, food consumption, and caste differentiation. With the exception of the trail following hypothesis, all other hypotheses were rejected. Worker termites injected with *deviate* siRNA did not exhibit differences from controls in total food consumption or in the timing of food consumption. In distance-foraging experiments termites from all treatments consumed similar amounts of paper (cellulose). Finally, although we observed *deviate* expression to be responsive to JH, no impacts on JH-dependent caste differentiation were observable after deviate RNAi. Thus, evidence obtained in this study is most consistent with the hypothesis that the *deviate* gene encodes a ligand-binding protein involved in odorant perception and trail following accuracy.

In conclusion, functional aspects of the *R. flavigas deviate* gene were investigated here and RNAi techniques were used to obtain findings that link *deviate* and termite behavior. We have concluded that *deviate* encodes a ligand binding protein that plays a role in the perception of termite trail pheromone, or potentially, in the following accuracy.


Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jgene.2010.11.012.

### References
