Ant Trail Pheromone Biosynthesis Is Triggered by a Neuropeptide Hormone

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Abstract
Our understanding of insect chemical communication including pheromone identification, synthesis, and their role in behavior has advanced tremendously over the last half-century. However, endocrine regulation of pheromone biosynthesis has progressed slowly due to the complexity of direct and/or indirect hormonal activation of the biosynthetic cascades resulting in insect pheromones. Over 20 years ago, a neurohormone, pheromone biosynthesis activating neuropeptide (PBAN) was identified that stimulated sex pheromone biosynthesis in a lepidopteran moth. Since then, the physiological role, target site, and signal transduction of PBAN has become well understood for sex pheromone biosynthesis in moths. Despite that PBAN-like peptides (~200) have been identified from various insect Orders, their role in pheromone regulation had not expanded to the other insect groups except for Lepidoptera. Here, we report that trail pheromone biosynthesis in the Dufour’s gland (DG) of the fire ant, Solenopsis invicta, is regulated by PBAN. RNAi knock down of PBAN gene (in subesophageal ganglia) or PBAN receptor gene (in DG) expression inhibited trail pheromone biosynthesis. Reduced trail pheromone was documented analytically and through a behavioral bioassay. Extension of PBAN’s role in pheromone biosynthesis to a new target insect, mode of action, and behavioral function will renew research efforts on the involvement of PBAN in pheromone biosynthesis in Insecta.

Introduction
Pheromones are a subset of semiochemicals produced by organisms for communication within members of the same species. Since the first pheromone was identified over 50 years ago [1], pheromone research has been expanded tremendously in animal and plant taxa, especially insects [2,3]. While pheromone identification and their elicited behaviors are well known for a wide variety of insects, the regulation of pheromone biosynthesis is poorly understood and is limited to some lepidopteran moths [4–6]. For these moth species a neuropeptide hormone stimulates sex pheromone biosynthesis in female adults [7,8]. This neuropeptide was named pheromone biosynthesis activating neuropeptide (PBAN), it is synthesized in the subesophageal ganglion (SG) and released into the hemolymph to reach a target site, e.g., a pheromone gland [8–10]. PBAN/pyrokinin genes appear to be ubiquitous in insects and produce three or four peptides in addition to PBAN [11,12]. These peptides share a common functional epitope (FXPRL-NH2) or similar sequence at the C-termini [13,14], which characterizes the PBAN/pyrokinin family of peptides [8,15]. The PBAN/pyrokinin peptide family has been found in a variety of insect Orders, and to date over 200 PBAN/pyrokinin family peptides have been reported from over 40 species (from GenBank, unpublished). In addition to regulation of sex pheromone biosynthesis in female moths, several other physiological functions for this family of peptides have been demonstrated, for example: (a) induction of melanization in moth larvae [16,17]; (b) induction of diapause egg in moths [18,19]; (c) stimulation of visceral muscle contraction in cockroaches [20]; (d) acceleration of puparium formation in the flesh fly [21]; and (e) termination of development of pupal diapause in heliothine moths [22]. However, their involvement in the control of pheromone production has only been demonstrated for moth PBAN where it stimulates the biosynthesis of the sex pheromone [9,10].

Like other social insects, the fire ant, Solenopsis invicta, evolved complex pheromone communication systems for resource procurement, maintenance of social structure, and territoriality. Much is known about their behavior and chemistry, but regulation of pheromone production has not been elucidated. This study aims to utilize molecular techniques, e.g., inhibition of gene expression – RNAi, as well as chemical analysis and bioassays to elucidate the involvement of PBAN on biosynthesis regulation of the of fire ant trail pheromone – essential for resource retrieval and colony emigration [23].

Materials and Methods
Fire ant colonies
All S. invicta samples were from monogyne (single functional queen) colonies collected in the Gainesville area (FL, USA) by nest excavation or by rearing colonies from newly mated queens. All colonies were maintained as described previously [24]. No specific permits were required for the described field collections and the collections did not affect endangered or protected species. The newly mated queens were collected in an area not protected in any way.
SolinPBAN and the fire ant trail pheromone

To test if Solenopsis invicta PBAN (SolinPBAN) stimulates trail pheromone biosynthesis, a saline control or SolinPBAN dissolved in saline was injected (10 pmol/50 nL/ant) into worker ants of approximately the same size (by inspection) and age (collected from the foraging area of rearing tray - age is related to task) using a Nanoliter 2000TM injector (World Precision Instruments) fitted with custom-pulled borosilicate needles. After injection, the fire ant workers were kept in a small plastic container with food and water until dissection and extraction of the trail pheromone for quantitation of the recruitment pheromone or for bioassay. A preliminary experiment indicated that the amount of trail pheromone 0, 1, 2, 4, 6 and 12 hours post PBAN injection was greatest after 6 hours (See Fig. S1B). This “incubation” time was chosen for the highly replicated (N=35) saline vs. PBAN injection comparison (Fig. 1C).

Gland dissection

All dissections were performed using a Zeiss Stemi SV 6 stereo microscope at 25× magnification. A drop of water was placed on the surface of a homemade silicon filled Petri dish (85 mm dia.). A decapitated ant was held at the anterior end of the gaster just below the petiole in a drop of water with ≥5 Dumont jewelers’ forceps. With a second pair of forceps the last segment of cuticle on the dorsal side of the gaster was gently opened and removed, exposing the sting. The sting was grasped and gently pulled away, yielding the sting (S), the poison gland/sac (PG), and the Dufour’s gland (DG). Forceps were cleaned in 10% sodium hypochlorite solution (bleach) between each dissection to avoid microbial and cross contamination. The DG is a small gland usually covered in fat bodies that is located at the base of the sting. The gland was pinched off at its base with the forceps, and checked to confirm its presence under microscope, then placed in an RNase free micro-centrifuge tube on Dry-Ice and kept at −80°C until RNA extraction. The Petri dish was cleaned with 10% bleach solution (bleach) between dissections. For GC analysis, a DG was placed in a 100 µL vial insert containing 50 µL hexane and 50 ng C16 as internal standard (IS). The insert was placed in a 2 ml vial containing a small amount of hexane, which acted to slow solvent evaporation. Samples were held at −20°C until analysis.

Trail pheromone analysis

A GC (Agilent 6890N) equipped with a 30 m × 0.25 mm i.d. DB-1 column, a flame ionization detector (FID) (Agilent), and autosampler (Agilent N10149) was temperature programmed 40°C to 285°C at 10°C/min with 5 min hold. Z,E-α-farnesene was identified based on prior GC-mass spectral and retention time comparison (Fig. 1C). The fire ant PBAN receptor (Invitrogen). 3′-RACE was performed using primers 5′-TGTGATGCTCTTACGTGACGGTGTCA-3′ (from nucleotide 1106) and 5′-TAGTAAATTGGTGCGGTGTTGCGGACCTT-3′ (from nucleotide 588) by the 5′-RACE kit (Invitrogen), 3′-RACE was performed using primers 5′-TTYTATYTGGGCGNCC NTYCGA-3′ (from nucleotide 1199), 5′-CACCACCGTGAATCCGACTTCTACAATAA-3′ (from nucleotide 1336) and 5′-TCAAGTCAATTTGGTGCGGACCTT-3′ (from nucleotide 1392). Then PCR products were inserted into a subcloning vector (TOPO-TA, Invitrogen) and confirmed the nucleotides by DNA sequence. Sequences of the receptor DNA and corresponding amino acids were analyzed by Genetyx software ver. 10 (Genetyx Corporation) for 30 s, 50°C for 30 s, and 72°C for 1 min. The PCR product was directly sequenced by Interdisciplinary Center for Biotechnology Research (ICBR, University of Florida), and then the sequence result was used to design further gene-specific primers to find 5′- and 3′- ends of the fire ant PBAN-R cDNA. 5′-RACE (GeneRacer kit, Invitrogen) was performed using primers 5′-ATGCAYCANCNCANAAYTAY-9TAYYTNTT-3′ (MHTATNYYLF) and 5′-GCRTGRR-9AANGGNCCCRGCADATRAARAA-3′ (FFICWAPFHA) mostly conserved in TM2 and TM7 domains from insect PBAN-Rs [25–27]. PCR was performed for 35 cycles at 95°C

![Figure 1. PBAN stimulates trail pheromone biosynthesis in the fire ant.](image-url)
and PredictProtein software [28]. The ORF of PBAN-R cDNA was amplified using the sense primer of 5'-GTCGCGCGCGCTAAAGCAGAATTTTCGAGTAA-TACG-3' (from nucleotide 255 including the NotI site) and 5'-AGGCTCTAGATTGTCTTAATTCGAGGC-GACGTTCT-3' (from nucleotide 2122 including the XbaI site). The PCR product was ligated into a pIBV5His vector (Invitrogen) for expression in Sf9 cells as described previously [25].

SolinPBAN-R/peptide binding assays
All peptides used in this study were synthesized or purchased from Sigma Genosys or Peninsula Laboratories. The preparation of cells, peptides, and Fluo-4AM with hymenopteran saline [29] followed a previously described method [25]. Cell fluorescence intensity in a 96-well cell-culture was measured using a plate reader (BMG's NovoStar) equipped with filters (excitation: 485 nm and emission: 520 nm), and one pipetter and two injectors. Fluorescence measurements from each well were taken every 10s. After 30s PBAN or FXPRL peptide (10 μM) was added by pipetter and fluorescent changes were measured for up to 3 min. Then, 1 μM ionomycin (5 μL) was added into the cells to obtain a maximum fluorescence reading. The effect of ligand-exposure was expressed relative to the maximum value obtained with ionomycin. Data were analyzed using Microsoft EXCEL as described previously [25] and measured EC50 values of ligands were determined using GraphPad Prism 4.03 software.

RNAi suppression of SolinPBAN and SolinPBAN-R gene expression
Fire ant PBAN (=SolinPBAN) dsRNA was constructed with 5’-T7-appended PCR primers (5’-TAATACGACCTAGATACGTGAGCGTACGACGAAACCCGACTTAC-3’ and 5’-TAATACGACTAGATGGGACTCTCAAGAGGTGGTGC-3’) to amplify a 506-bp PBAN DNA fragment, which serves as the template for dsRNA synthesis using the MEGAscript RNA kit (Ambion). Fire ant PBAN-R (SolinPBAN-R) dsRNA was constructed by specific primers (5’-TAATACGACCTAGATACGTGAGCGTACGACGAAACCCGACTTAC-3’ and 5’-TAATACGACTAGATGGGACTCTCAAGAGGTGGTGC-3’) to amplify a 510-bp DNA template for PBAN-R dsRNA synthesis. GFP dsRNA was constructed by specific primers (5’-TAATACGACCTAGATACGTGAGCGTACGACGAAACCCGACTTAC-3’ and 5’-TAATACGACTAGATGGGACTCTCAAGAGGTGGTGC-3’) to amplify a 546-bp DNA template for

Figure 2. Phylogenetic tree of receptors for PBAN, PK2 and neuromedin U (NmU). The tree was made by Genetyx-Tree software (ver. 10) with the neighbor-joining distance phylogram method using bootstrap 1,000 replicates. Accession number of species: Acromyrmex echinatior (EGI70561), Acyrthosiphon pisum (XP_001950091), Aedes aegypti (XP_001657210), Anopheles gambiae (AAAX84798), Apis mellifera (NP_001091688), Bombus terrestris (XP_003395149), Bombyx mori (NP_001036977), Camponotus floridanus (EFN62034), Culex quinquefasciatus (XP_001861460), Drosophila melanogaster (CG8795), Helicoverpa armigera (AAW47417), Helicoverpa zea (AAP93921), Heliolthis virescens (ABU93812), Manduca sexta (ACQ90219), Mus musculus (NP_034471), Nasonia vitripennis (XP_000600587), Pediculus humanus (XP_002424393), Plutella xylostella (AAY34744), Solenopsis invicta (JX657040), Spodoptera exigua (ABY62317), Spodoptera littoralis (ABD52277), Tribolium castaneum (EZ97726).
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GFP dsRNA using the same kit as above. The purified dsRNAs were dissolved in nuclease-free water and injected into workers using the same injector as above. Adult workers were injected with 1 μg (50 nL) of SolinPBAN or SolinPBAN-R dsRNA and incubated for 24, 48, and 72 h. After injection, fire ants were kept as described above until Br-SG or DG dissection. Br-SGs were dissected to isolate total RNA for SolinPBAN transcription by RT-PCR. Dissected DGs were used for trail pheromone analysis and for isolation of total RNA and determination of SolinPBAN-R transcription levels by RT-PCR as described below.

**RT-PCR for SolinPBAN and SolinPBAN-R**

Total RNA was isolated after DNase treatment from the following fire ant tissues: head, Br-SG and abdomen, the PDS (PG, DG, and sting together), PG, DG, sting, and ventral nerve cord (VNC) using the PureLinkTM RNA Kit (Invitrogen) (Fig. 2C). Generally, each total RNA was used to synthesize cDNA using 3'-RACE kit or an antisense primer (5'-TGTAAACGCGC CGAATCCGATCCC GTGAAT-3') by SuperScript RT® III (Invitrogen). The 1st cDNA was synthesized from 5 μg total RNA from the head, Br-SG, and adult body minus head, 1 μL/(1/20) was used for PCR amplification. The 1st cDNA was synthesized from total RNA (~1200 ng) from PG, DG, sting, and VNC, and 1 μL/(1/20) was used for PCR amplification. Each cDNA was used to amplify a 313-bp DNA fragment of SolinPBAN-R with a specific primer set (5'-CACCCGGT-GAATCCGCAATTCCGAATATCGAAT-3') and 5'-TGTAAACGCGCGAATCCGATCCC GTGAAT-3'). A 100-bp fragment of the fire ant 18S RNA was also amplified for a positive control as described previously [30]. PCR was performed as follows: 35 cycles at 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min.

**RT-PCR for SolinPBAN expression** was performed with total RNA isolated from Br-SGs of workers (>15 ants/treatment) after they were injected (1 μg/50 nL/ant) with PBAN dsRNA, nuclease-free water, or in a separate experiment dsGFP and incubated for 24 h, 48 h and 72 h. The total RNA (20 ng) was used to amplify a 501-bp DNA fragment of PBAN with a PBAN specific primer set (5'-AGGAAATTCGTGGAAATCGTGC-3' and 5'-GTTTGCGTAAATCGTGCA-3'). A 100-bp fragment of the fire ant 18S RNA gene was also amplified as a positive control using primers (5'-CCCCTATCTGGAATGATGATACACAGT-3' and 5'-AGGCTATTGGAGCGTCCAATT-3'). The one-step RT-PCR was conducted with 1 μL of the above PCR products and the same primers using the following cycles: 1 cycle at 50°C for 30 min, 40 cycles at 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min, then 72°C for 5 min using RT/Taq mix polymerase (Invitrogen). Then the second PCR was conducted with 1 μL of the above PCR products and the same primers using the following cycles: 1 cycle at 95°C for 3 min, 33 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min, then 72°C for 5 min using iTaq DNA polymerase (Bio-Rad). Then, part of the PCR products were checked for the amplification using 1.5% agarose gel electrophoresis and visualized using ethidium bromide under a UV light. The expected PCR products were purified and cloned, then confirmed by PCR following the manufacturer’s procedure. Then, PCR amplification was performed with 2 μL of the 1st strand cDNA above as a template to amplify a 313-bp DNA fragment of PBAN-R with the specific primer set (5'-CACCCGGTGAATTCGTGGAAATCGTAAATAA-3' and 5'-TGTAACGCGCCGTAATCCGATCCC GTGAAT-3'). A 100-bp fragment of the fire ant 18S rRNA was also amplified as a positive control as previously described. The PCR condition was 95°C for 3 min, 33 cycles at 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min, then 72°C for 5 min using iTaqDNA polymerase (Bio-Rad). The expected PCR products were purified and cloned, then confirmed by their DNA sequence at ICBR.
Reduced trail pheromone production and the orientation bioassay

A queen right colony collected and maintained in the laboratory as described above for approximately 4 weeks was selected for the bioassay. The trail orientation bioassay was modified from a previously described assay [31,32]. A colony cell (14 cm Petri dish with a central hole in the lid and a layer of moistened Castone® in the bottom) containing the queen, brood and workers was transferred to a clean rearing tray (8 cm tall; Fig. S4), then across another tongue depressor (7 cm) to a second platform (same size as the first) holding food (a dead cricket and 10% sugar water in a cotton ball). A piece of paper (size of platform) was placed on the second platform and the ants then allowed to form a natural trail from their colony cell to the food platform. Bioassays were conducted by replacing the original paper with a test sheet (size of platform). Test sheets had two curved pencil lines (ca. 10 cm) drawn such that they met at the mid-point of the short sides of the paper. The test papers were prepared for a bioassay by streaking one pencil arc with 10 μL hexane (10 μL Hamilton® syringe) and the other arc streaked with a DG extract (concentration expressed as DG equivalents). A bioassay was scored positive if at least one foraging ant followed the treatment arc to the food source within 1 min. The test paper was then removed and the original paper was returned to the platform allowing the ants to return to their natural trail between bioassays.

Results and Discussion

The DG is the source of the fire ant trail pheromone [33], which is released through the sting (Fig. 1A). The most abundant volatile trail pheromone component is Z,E-α-farnesene (Fig. S1A) [34]. This component is responsible for the orientation of the ants along a pheromone trail. Our earlier studies on the trail pheromone [34] coupled with our recent characterization of the PBAN/pyrokinin family of peptides from fire ants [11,29,30,35], provided the foundation for the discovery that Solenopsis invicta PBAN (SolinPBAN) regulates trail pheromone biosynthesis in the DG (Fig. 1B).

SolinPBAN and the fire ant trail pheromone

Many moth sex pheromones are biosynthesized in epithelial glands located in the last abdominal segments, usually with no lumen [5,36]. This type of gland/pheromone system facilitated bioassays needed to determine the effect of PBAN by measurement of pheromone present or absence in decapitated or neck-ligated female moths [7,8]. However, exocrine glands with a lumen, e.g. DG [37], maintain pheromone levels within a range through biosynthetic activation and deactivation. This variability presents challenges to measure differences from experimental hormone stimulation. To determine if SolinPBAN stimulates trail pheromone production in the DG we measured Z,E-α-farnesene levels in adult worker DGs after SolinPBAN or saline (control) injections. To maximize potential treatment and control differences we first determined that 6 h was an optimal post injection incubation period (Fig. S1B). A large-scale experiment using this incubation time then showed that SolinPBAN injected worker ants produced significantly greater amounts of trail pheromone than ants injected with saline (Fig. 1C). About 85% of SolinPBAN treatments had trail pheromone levels above the normal range, as defined by the mean saline control, and presumably closer to the DG capacity limit (Fig. 1C). This result strongly supports SolinPBAN stimulation of trail pheromone biosynthesis in the DG, and suggests that a receptor for SolinPBAN could be located in the DG as a potential SolinPBAN target site.

The fire ant PBAN receptor

Insect PBAN receptors are classified in the G-protein-coupled receptor (GPCR) superfamily and many have been identified by sequence homology [12,26,38,39] and/or by functional expression in insects [25,27,40–47]. To investigate the potential SolinPBAN target site(s) in fire ants and neuropeptide binding preferences, we identified the SolinPBAN receptor (SolinPBAN-R) using degenerate primers (deposited the nucleotide and amino acid sequences in GenBank with accession number, JX657040). As expected SolinPBAN-R is closest to other ant PBAN receptors, followed by bees, and then moths (Fig. 2), but SolinPBAN-R transmembrane (TM) domains are highly conserved with those from different insect Orders (Fig. S2).

### Table 1. Binding activity of PBAN/pyrokinin and related peptides to SolinPBAN-R.

| Species       | Peptide* | Amino acid sequence** | ECso***  |
|---------------|----------|-----------------------|----------|
| S. invicta    | SolinPBAN| GSGEDLSYGDAVYEVDHHPLFVPRL | 18 nM    |
|               | SolinDH  | TSQDIAAGMWFGRPL       | 125 nM   |
| H. zea        | HelzePBAN| LSDDMPATPADQEMYRQIDPSRKYFSPL | 56 nM    |
|               | HelzePG24(DH) | NDVXDDAASAGAHS/GL/FGPRPL | >1 μM    |
| D. melanogaster| DromePK2   | SPVFPRPL              | 163 nM   |
|               | DromePK1  | TGPSSASGLFGPRPL       | >1.0 μM  |
| L. maderae    | LPK       | PETSFPRPL             | 36 nM    |

*Peptides: PBAN (pheromone biosynthesis activating neuropeptide), DH (diapause hormone), PGN (PBAN-encoding gene neuropeptides), PK (pyrokinin), LPK (leucokopyrin).

**All peptides are amidated in the C-termini.

***EC50: half-maximal effective concentration.

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cells [25], which enabled binding specificity studies by measurement of calcium-based fluorescence intensity (Fig. 3A and Table 1). The two types of peptides can be distinguished by their different binding affinities (half-maximal effective concentration, EC\textsubscript{50} to SolinPBAN-R. SolinDH was 7 times less active than SolinPBAN in binding with SolinPBAN-R (Fig. 3A) and the EC\textsubscript{50} values of other PK1 peptides from *Drosophila* and moth were >50 time less active than SolinPBAN (Table 1), supporting preferred SolinDH binding to a PK1 receptor [12,48,49]. On the other hand, the low active than SolinDH (Table 1), supporting preferred SolinDH binding to a PK1 receptor. The EC\textsubscript{50} value of SolinPBAN was 7 times less than HelzPBAN (18 nM vs. 56 nM, respectively), indicative of at least family ligand/receptor fidelity. The strong binding affinity of SolinPBAN confirmed SolinPBAN-R as the receptor for SolinPBAN in the fire ant. The SolinPBAN/pyrokinin gene is expressed in all life stages in fire ant, from egg to adult [50]; however, the disposition of the four SolinPBAN/pyrokinin peptides in each life stage, as well as the possibility of additional receptors is unknown and currently under investigation.

We localized SolinPBAN-R in the ant by measuring receptor gene transcription levels in various tissues (Fig. 3B). Strong SolinPBAN-R expression was detected in the Br-SG and body (Fig. 3B). Most interesting was the result obtained from the body, because the DG resides in the abdominal part of the body of the fire ant (Fig. 1A). Further investigation of the abdomen showed SolinPBAN-R expression predominantly in the DG and moderately in the poison gland/sac (PG) (Fig. 3C). Strong PBAN-R expression in the DG supports it as a target site for SolinPBAN in the fire ant. Expression of SolinPBAN-R in the Br-SG (Fig. 3B) and moderate expression in the PG (Fig. 3C) suggests other physiological roles for the SolinPBAN family of peptides. In an earlier study the expression of SolinPBAN mRNA was detected in all developmental stages, from embryo to adults [50], indicating probable stage-specific functions in the fire ant.

RNAi suppression of SolinPBAN and SolinPBAN-R gene expression

To further test the hypothesis that SolinPBAN is involved in fire ant trail pheromone biosynthesis, we used RNA interference (RNAi) to knock down SolinPBAN and SolinPBAN-R in adult workers (Fig. 4). Worker ants injected with SolinPBAN dsRNA were incubated for 24, 48, and 72 h prior to DG dissection for trail pheromone analysis. The SolinPBAN RNAi significantly inhibited trail pheromone production at 48 h, but results at 24 and 72 h post-injection were not significantly different from controls (Fig. 4A). SolinPBAN gene transcription levels were determined from dissected Br-SGs from worker ants at 24, 48, and 72 h post-injection of SolinPBAN dsRNA. SolinPBAN expression was inhibited at all three time periods (Fig. 4B), with maximum effect at 48 h, which corresponded to the time of greatest pheromone reduction (Fig. 3A). SolinPBAN transcription was clearly inhibited after 24 h post-injection; however it is possible that residual SolinPBAN and/or SolinNPs synthesized in the SG prior to RNAi injection stimulated some pheromone production in the DG during the first 24 h. Previous research using moth PBAN RNAi gave a similar result for reduction of sex pheromone production in *H. zea* [50]. Injection of the non-specific dsRNA control, GFP dsRNA, did not reduce transcription of SolinPBAN (Fig. S3).

The effect of SolinPBAN-R RNAi on trail pheromone production was measured 24, 48, and 72 h after workers were injected with SolinPBAN-R dsRNA or a nuclease-free water control. Pheromone production was significantly reduced at 24 and 48 h sampling times (Fig. 4C). At 72 h post injection, pheromone production was decreased but not significantly. To determine if SolinPBAN-R RNAi was affecting SolinPBAN-R transcription in DGs, we dissected DGs from worker ants injected with SolinPBAN-R dsRNA after 24, 48, and 72 h post-injection and observed SolinPBAN-R expression levels (Fig. 4D). SolinPBAN-R expression in DGs was reduced for all time periods, especially evident at 24 h and 48 h post-injection (Fig. 4D), corresponding to when the greatest pheromone reduction...
injected into the Br-SG and the Br-SG of control workers were injected with saline. (Fig. S3). The RNAi treatment resulted in significant reduction in pheromone concentration for treatments versus saline controls at six incubation times. Means (± s.e.m., $P=0.0158$, $n=10$) are shown. A six h incubation period after SolinPBAN injection showed a significant increase in ZEF.

**Figure S2 Alignment of PBAN and NmU receptors.** Ant (S. invicta), bee (A. mellifera, NP_001091688), fly (D. melanogaster, CG8795), mosquito (A. gambiae, AAX84798), moth (H. zea, AAP93921), beetle (T. castaneum, EEZ97728), louse (P. humanus, XP_002424393), aphid (A. Psam, XP_001950091) and mouse (M. musculus, NP_034471). The alignment was made by Genetyx-Tree software (ver. 10). The transmembrane domains for the S. invicta PBAN-R are indicated by double dashes above the aligned sequences. Dashed lines indicate spaces needed to optimize alignment.

**Figure S3 GFP RNAi effects on SolinPBAN and SolinPBAN-R gene expression in workers.** Gene transcription levels of SolinPBAN expression in the Br-SG (upper) and SolinPBAN-R expression in DGs (middle) of workers at 24, 48 and 72 h post-injection of GFP dsRNA. Expression of the fire ant 18S rRNA, the positive control, is shown in the bottom row (see Materials and Methods for details).

**Figure S4 Photo of orientation bioassay.** Ants are forced to develop a pheromone trail up the ramp and across the platform to the food reward. Bioassays are performed by placing the paper on the platform with another paper streaked with control and treatment trails, then observing the behavior of the previously trailing ants (see Materials and Methods for details).

**Movie S1 Positive (≠ control) trailing bioassay.** A hexane extract of Dufour’s glands (DG) from saline injected workers was applied to the trailing bioassay curve marked ‘T’ (10 μL of a 0.001 DG equivalent extract). Positive trailing behavior is indicated here by the ants readily following the experimental Control trail.

**Movie S2 RNAi treatment (≠ SolinPBAN-R (SolinPR) dsRNA) trailing bioassay.** A hexane extract of Dufour’s glands (DG) from dsPBAN-R injected workers was applied to the trailing bioassay curve marked ‘T’ (10 μL of a 0.001 DG equivalent extract). Negative trailing behavior is indicated here by the ants not detecting the experimental Treatment trail.

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**Author Contributions**

Conceived and designed the experiments: MC RVM. Performed the experiments: MC RVM. Analyzed the data: MC RVM. Contributed reagents/materials/analysis tools: MC RVM. Wrote the paper: MC RVM.

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