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# Phenotypic impacts of PBAN RNA interference in an ant, *Solenopsis invicta*, and a moth, *Helicoverpa zea*

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ABSTRACT

Insect neuropeptide hormones represent more than 90% of all insect hormones. The PBAN/pyrokinin family is a major group of insect neuropeptides, and they are expected to be found from all insect groups. These species-specific neuropeptides have been shown to have a variety of functions from embryo to adult. PBAN is well understood in moth species relative to sex pheromone biosynthesis, but other potential functions are yet to be determined. Recently, we focused on defining the PBAN gene and peptides in fire ants in preparation for an investigation of their function(s). RNA interference (RNAi) technology is a convenient tool to investigate unknown physiological functions in insects, and it is now an emerging method for development of novel biologically-based control agents as alternatives to insecticides. This could be a paradigm shift that will avoid many problems associated with conventional chemical insecticides. In this study, we selected the PBAN gene and its neuropeptide products as an RNAi target from two insect groups; a social insect, the fire ant (*Solenopsis invicta*) and a non-social insect, the corn earworm (*Helicoverpa zea*). Both insects are economically important pests. We report negative impacts after PBAN dsRNA treatment to suppress PBAN gene transcription during developmental and adult stages of both species, e.g. increased adult and larval mortality, delayed pupal development and decreased sex pheromone production in the moth. This is an important first step in determining the multiple functions of the PBAN gene in these two insects. This work illustrates the variety of phenotypic effects observed after RNAi silencing of the PBAN gene and suggests the possibility of novel biologically-based insect pest control methods.

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## 1. Introduction

Insect neuropeptides are the largest group of insect hormones. A variety of peptide families have been identified from insects (Gäde, 1997). One of these families is the Pheromone Biosynthesis Activating Neuropeptide (PBAN)/pyrokinin family defined by a conserved C-terminal pentapeptide (e.g: FXPRLamide) that is the active core fragment for peptide function (Kuniyoshi et al., 1992; Raina and Kempe, 1992). During the past two decades, PBAN has been one of the most intensively studied insect neuropeptide, because it regulates sex pheromone biosynthesis in many lepidopteran moths. PBAN was first identified from *Helicoverpa zea* adults (Raina et al., 1989), and now, the PBAN/pyrokinin peptide family has been identified from many insects and other arthropods. The studies have shown a broad range of physiological functions, for example: (1) stimulation of sex pheromone biosynthesis in female moths (Raina et al., 1989); (2) induction of melanization in moth larvae (Matsumoto et al., 1990; Raina et al., 2003); (3) induction of embryonic diapause in *Bombyx mori* (Suwan et al., 1994; Uehara et al., 2011); (4) stimulation of visceral muscle contraction in cockroaches (Predel and Nachman, 2001); (5) acceleration of puparium formation in the flesh fly (Zdarek et al., 1997); and (6) termination of development of pupal diapause in heliothine moths (Xu and Denlinger, 2003).

In moth species, PBAN is synthesized in the subesophageal ganglion (SG), released into the hemolymph, and acts on sex pheromone production in the pheromone gland. PBAN plus four or three additional peptides are encoded by the PBAN/pyrokinin gene. These additional peptides also share a common C-terminal FXPRLamide motif and have been named diapause hormone (DH), neuropeptide (NP)–α, –β, and –γ homologues (Choi et al., 2011). PBAN mRNA was expressed at similar levels in both female and male adult moths, although it is unclear if male moths produce pheromones or if these peptides elicit other functions (Bober and Rafaeli, 2010; Choi et al., 1998; Jing et al., 2007; Lee and Boo, 2005; Wei et al., 2008, 2004). The physiological mechanism of

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PBAN control over pheromone production is well understood for sex pheromone biosynthesis in a number of lepidopteran moths (Rafaeli, 2009; Rafaeli and Jurenka, 2003). However, thus far no other insect group has been shown to regulate pheromone biosynthesis using PBAN, although PBAN/pyrokinin peptides have been found in all insect groups thus far investigated. We recently identified and characterized the PBAN mRNA encoding four FXPRLamide peptides in the fire ant, *Solenopsis invicta* and sibling invasive ants (Choi et al., 2011, 2009; Choi and Vander Meer, 2009).

There are 17 land invertebrates listed among the world's 100 worst invasive alien species (Lowe et al., 2000). Ants represent 28% of these invertebrates, including fire ants. In the United States, imported fire ants infest more than 130 million hectares in 13 southern tier states and Puerto Rico and are continuing their spread (USDA-APHIS, 2009). The fire ant is estimated to be responsible for over $6 billion annually in damage repair, medical care, and control costs. The affected economic sectors are broad-ranging and include households, agriculture, and electric and communications (Lard et al., 2006). Current control methods rely on chemical insecticides in the form of toxic baits or drenches. There is a need for novel biologically-based control alternatives that are more species-specific and have less impact on the environment.

*Helicoverpa* spp. are major insect pests of many economically important crops including corn, cotton, soybean, green and hot peppers, tomatoes, and potatoes throughout the world. The corn earworm, *H. zea*, is distributed in the New World, and is a well known moth species for the study of basic physiology and chemical ecology. Currently, control methods for *Helicoverpa* spp. rely on chemical insecticides and/or Bt transgenic plants, but control is increasingly more difficult due to insecticide resistance and non-target toxicity.

RNA interference (RNAi) technology is an emerging method in insect pest management because it has high potential to provide novel insect control methods for crop and other insect pests (Asokan, 2008; Huvenne and Smagghe, 2010). Although there are many technical and target gene selection challenges, RNAi for insect control represents a potentially new direction for insect pest management (Scharf et al., 2008).

The multifunctional roles already shown for PBAN/pyrokinin peptides translated by the PBAN mRNA provides an interesting RNAi target for both basic function studies and to assess potential for RNAi as a novel control method. We selected the PBAN gene for RNAi from two insect groups, the fire ant, *S. invicta* (social insect) and the corn earworm, *H. zea* (non-social insect), and investigated the physiological phenotypic impact(s) of RNAi silencing of PBAN gene transcription. Our findings demonstrate the effects of dsRNA-based interference of the PBAN gene on adults and developmental stages in *S. invicta*, and *H. zea*. This is the first step toward determining the potential of PBAN RNAi to control two diverse economically important insect pests.

## 2. Materials and methods

### 2.1. Fire ants

Field collected monogyne fire ant, *S. invicta*, colonies were collected around Gainesville, Florida, USA, and maintained in the laboratory using standard procedures (Banks et al., 1981).

### 2.2. Moths

Pupae of the corn earworm, *H. zea*, were purchased from Bio-Serv (Frenchtown, NJ, USA) and maintained at room temperature under a L/D regimen of 15:9 until they emerged as adults. Female pupae or virgin adults were used for dsRNA injection or sex pheromone analysis.

### 2.3. Cloning of Soi-PBAN gene and construction of dsRNA

Br-SGs were dissected from *S. invicta* female alates to isolate mRNA using the MicroFast mRNA purification kit (Invitrogen, Carlsbad, CA, USA), and used to synthesize cDNA with the GeneRacer cDNA synthesis kit (Invitrogen). The detailed cloning procedure was described previously (Choi and Vander Meer, 2009). The *S. invicta* PBAN (Soi-PBAN) dsRNA was synthesized from the full length Soi-PBAN cDNA (531-bp) using specific 5′-T7-appended PCR primers (5′-TAATACGACTCACTATAGGGACCGTCGACAACCGACTTAC-3′ and 5′-TAATACGACTCACTATAGGGGACTCTCAAGAGGTGGTGGC-3′) to amplify a 496-bp Soi-PBAN DNA fragment. This fragment served as the template for dsRNA synthesis using the MEGAscript RNA kit (Ambion, Austin, TX, USA). Green fluorescence protein (GFP) dsRNA served as the negative dsRNA control and was either purchased (Ambion) or was synthesized from a 546-bp GFP DNA template amplified by 5′-T7-appended primers, 5′-TAATACGACTCACTATAGGGACGTAAACGGCCACAAGTTC-3′ and 5′-TAATACGA CTCACTATAGGGTGCTCAGGTAGTGGTTGTCG-3′, using the same kit as above.

### 2.4. Cloning of the Hez-PBAN gene and construction of dsRNA

Br-SGs of 1–3- day old *H. zea* female moths were dissected to isolate mRNA for synthesis of cDNA using Invitrogen MicroFast mRNA purification kits and GeneRacer cDNA synthesis kits to clone the full length *H. zea* PBAN (Hez-PBAN) cDNA. The primer set: sense primer (5′-AAGATGTTCAATCAAACTCAGTTG-3′) and antisense primer (5′-AAATTATGTTGATCGCGTTTTGTTTGT-3′), was designed from the sequence deposited in GenBank (Accession number: U08109). PCR was performed with the following temperature program: 33 cycles at 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min. The PCR product was gel purified and cloned using TOPO TA cloning kit (Invitrogen) and sequenced. The obtained full-length sequence information was aligned and sequences compared with our partial sequence. To construct Hez-PBAN dsRNA, a PCR primer set was designed, 5′-T7-appended: 5′-TAATACGACTCACTATAGGGGTGTTTGCATTGTGTACCGC-3′, and 5′-TAATACGACTCACTATAGGGTATAGGAAGGGGTTGATGGC-3′, to amplify a 508-bp DNA fragment, which served as the template for dsRNA synthesis using the MEGAscript RNA kit (Ambion). The negative dsRNA control was dsGFP constructed by the same method described above for the fire ant. The length of Hez-PBAN dsRNA was synthesized from the full length of Hez-PBAN cDNA, 585-bp.

### 2.5. Soi-PBAN dsRNA injection into fire ant adults and pupae

Soi-PBAN dsRNA was injected into the hemocoel of *S. invicta* adult workers and female alate pupae using a Nanoliter 2000TM System using pulled borosilicate needles (World Precision Instruments, Sarasota, FL, USA). Soi-PBAN dsRNA (20 ng/50 nL/ant), GFP dsRNA (20 ng/50 nL/ant), and water (50 nL/ant) were each injected into 10 young adult workers (brood tenders) and 25 female alate pupae (48–72 h after pupation). Workers were observed for mortality for 8 days post-injection. Injected pupae were observed for delay of cuticle melanization (cuticle tanning) and mortality until adult emergence, about 8 days.

### 2.6. Soi-PBAN dsRNA feeding to fire ant larvae

Soi-PBAN dsRNA (1 μg/μL) was dissolved in 10% sucrose solution (wt./vol.) and provided for a period of 12 days via a capillary tube to six nurse worker that were caring for ten 4th instar larvae.

Following the initial 12-day feeding period, the nurse workers were fed only 10% sucrose solution without dsRNA and observed for an additional 9 days. GFP dsRNA (1 μg/μL) and water in 10% sugar solution were used for negative controls. The fire ant larval mortality was recorded.

### 2.7. Hez-PBAN dsRNA injection into moth pupae and sex pheromone production in female adults

Hez-PBAN dsRNA (1 μg/3 μL/pupa) was injected into 4–5-day old female *H. zea* pupae and observed for 14 days during which adult emergence was expected. For controls, GFP dsRNA (1 μg/3 μl/pupa) was injected into 13 female pupae. Distilled water (3 μL/pupa) was also injected into 13 female pupae as an additional control. The pupae were then observed for mortality, development and adult emergence. Emerged female adults from the three groups were used to extract and quantify the amount of sex pheromone, *cis*-11-hexadecenal (Z11–16:Ald, major component) produced. The sex pheromone was extracted 2–4-h after lights-off in the 2nd–3rd scotophase after adult emergence. The *H. zea* pheromone glands were dissected and soaked in a hexane containing 100 ng *cis*-9-tetradecenal (Z9–14:Ald) as an internal standard. The gland extracts were analyzed by an Agilent 6890 N gas chromatography (GC) with a flame ionization detector and a DB-23 (J&W, Santa Clara, CA, USA) capillary column (30 m × 0.25 mm). The GC oven temperature was programmed at 80 °C for 1 min, then 10 °C/min to 230 °C and held for 8 min. The Z11–16:Ald was identified based on comparison of retention time with that of synthetic Z11–16:Ald (Bedoukian, Danbury, CT, USA).

### 2.8. Reverse transcriptase (RT)-PCR for Soi-PBAN and Hez-PBAN

After DNase treatment, total RNA was isolated using the PureLinkTM RNA Kit (Invitrogen) from the following fire ant tissues: adult worker tissues (head, Br-SG and abdomen), whole pupae (old pupae – completely melanized, <3-day old pupae – not melanized), whole larvae (2nd and 4th instar), and embryos (7-day old eggs). The 1st cDNA was synthesized from 5 μg total RNA from each of the above tissues using Soi-PBAN antisense primer (5′-CTAACTCGGATAGTTGCCTATAGTTTTCG-3′) and the fire ant 18S rRNA antisense primer (5′-ACGCTATTGGAGCTGGAATTACC-3′) by SuperScript RT® III (Invitrogen). One μL (1/20) was used for PCR amplification with Soi-PBAN primer set (5′-AGCGACGTG TCCGAG ATGATCGTCACCAG-3′ and 5′-CTAACTCGGATAGTTGCCTATAGTTT TCG-3′). PCR was performed as follows: 35 cycles at 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 1 min, then 72 °C for 5 min with *i*Taq polymerase (Bio-Rad, Hercules, CA, USA). A 100-bp fragment of the fire ant 18S rRNA gene was also amplified as a positive control using primers (5′-CCCGTAATCGGAATGAGTACACTTT-3′ and 5′-ACGCTATTGGAGCTGGAATTACC-3′). Then, part of each PCR product was checked for amplification using 1.5% agarose gel electrophoresis and visualized using GelRed™ (Biotium, Hayward, CA, USA) under a UV light. The expected PCR products were purified and cloned, then confirmed by DNA sequence (Interdisciplinary Center for Biotechnology Research, ICBR, University of Florida).

RT-PCR from total RNA isolated from Br-SGs of workers (>20 ants/treatment) or whole pupae (>20 pupae/treatment) after they were injected with 1 μg PBAN dsRNA or water and incubated for 24 h, 48 h and 72 h to measure Soi-PBAN gene suppression by Soi-PBAN RNAi. Each total RNA (20 ng) was used to amplify a 501-bp DNA fragment of PBAN with the Soi-PBAN and 18S rRNA primer sets described above. The one-step RT-PCR was performed as follows: 1 cycle at 50 °C for 30 min, 40 cycles at 94 °C for 30 s, 50 °C for 30 s, and 68 °C for 1 min, then 68 °C for 5 min using RT/Taq mix polymerase (Invitrogen). Then, part of the PCR products were checked and confirmed by the same procedures described above.

RT-PCR for the Hez-PBAN gene by Hez-PBAN RNAi was performed with total RNA isolated from Br-SGs of 1–2-day old female adults after water, dsGFP RNA (dsGFP) or Hez-PBAN dsRNA (dsPBAN) was injected twice into 3–4-day and 7–8-day old pupae. Total RNA (20 ng) was used to amplify a 586-bp DNA fragment of PBAN with Hez-PBAN specific primer set (5′-GATGTTCAAT CAAACTCAGTTGT-3′ and 5′-TTATGTTGATCGCGTTTTGTTTG-3′). *H. zea* actin RNA (342-bp) was amplified with primer set (5′-GAT-CTGGCACCACACCTTCT-3′ 5′-TAACCCTCGTAGATGGGCAC-3′) for the positive control. The one-step RT-PCR was performed as follows: 1 cycle at 50 °C for 30 min, 35 cycles at 94 °C for 30 s, 52 °C for 30 s, and 68 °C for 1 min, then 68 °C for 5 min using RT/Taq mix polymerase (Invitrogen). Part of the PCR products were checked and confirmed by the same procedures described above.

### 2.9. Statistic analysis

All data obtained were analyzed with Kaplan–Meier Survival or *t*- tests from GraphPad Prism (ver. 4) programs.

## 3. Results and discussion

The objective of this study was to test the hypothesis that RNAi silencing of the PBAN gene will have a negative phenotypic impact on the hymenopteran and lepidopteran species investigated. The fire ant, *S. invicta*, and the corn earworm, *H. zea*, were selected as representatives of social and non-social insects and because they are economically important pests.

### 3.1. Phenotypic impacts on fire ant workers

Injection of workers with Soi-PBAN dsRNA resulted in significantly increased adult mortality when compared to controls (PBAN vs. GFP: Kaplan–Meier Survival, $\chi^2 = 4.038$, d$f = 1$, $P = 0.044$; PBAN vs. water: Kaplan–Meier Survival, $\chi^2 = 6.720$, d$f = 1$, $P = 0.010$) (Fig. 1). This was an important first step in documenting a phenotypic effect resulting from RNAi of the PBAN gene and potential for reducing the colony worker force. Fire ant workers comprise about 98% of all adult stages in fire ant colonies. They are sterile, but essential for tending brood and the queen, resource procurement, and colony defense. They communicate via complex pheromone

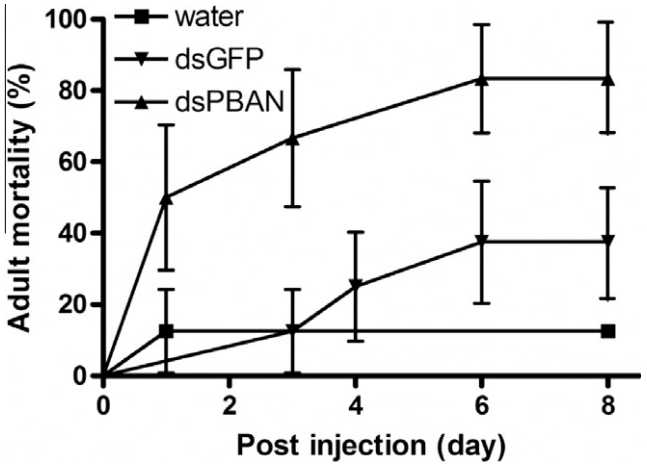


**Fig. 1.** Mortality of fire ant workers after Soi-PBAN dsRNA injection. Soi-PBAN dsRNA (20 ng), water (nuclease free, 50 nL/worker), or dsGFP (20 ng/worker) were injected into hemocoel of major workers. Data were analyzed by Kaplan–Meier survival curve comparison (PBAN vs. GFP, $P = 0.044$; PBAN vs. water, $p = 0.010$, respectively, $n \geqslant 10$). Soi-PBAN dsRNA treatment and water control were repeated three times (×3) and GFP was repeated one time (1×).

signals, e.g., recruitment and alarm pheromones. Reduction in worker numbers reduces the overall capabilities of the colony and makes them less competitive with neighboring nonspecific and heterospecific colonies.

### 3.2. Phenotypic impacts on fire ant pupae

Female alate pupae were injected with Soi-PBAN dsRNA to maximize the probability of observing a phenotypic effect. Pupal cuticle melanization (tanning) (Fig. 2A), and mortality (unsuccessful eclosion to adults) (Fig. 2B) were monitored. Pupal cuticle melanization was significantly reduced (unpaired *t*-test, two-tailed, $P = 0.013$) in treated young pupae (14.7%) as compared to water controls (44.2%) for the first 8 days (Fig. 2A). Female alate pupae mortality (54.5%) was significantly greater for the PBAN dsRNA treatment compared to mortality for the water control (28.9%) (Kaplan–Meier Survival, $\chi^2 = 3.984$, $df = 1$, $P = 0.045$). Injection can damage young pupae and be a source of mortality (Fig. 2B). These data expand the phenotypic effects of RNAi of the PBAN gene to reduced pupal melanization and abnormal eclosion to adults (mortality). A major characteristic of fire ant colonies is their high reproductive rate. Each single queen (monogyne) colony can produce up to 5000 winged (alate) reproductive males and/or females for up to 5 years (Morrill, 1974). Since there can be 100–150 colonies per hectare (Tschinkel, 2006) and mating flights are coordinated in time based on environmental conditions, the number of newly mated queens invading unoccupied territory is huge. PBAN family peptides have been implicated in pupation and melanization processes in moths (Matsumoto et al., 1990; Raina et al., 2003), thus it is not unexpected that RNAi of the PBAN gene in fire ant female sexual pupae led to aberrant development to adults.

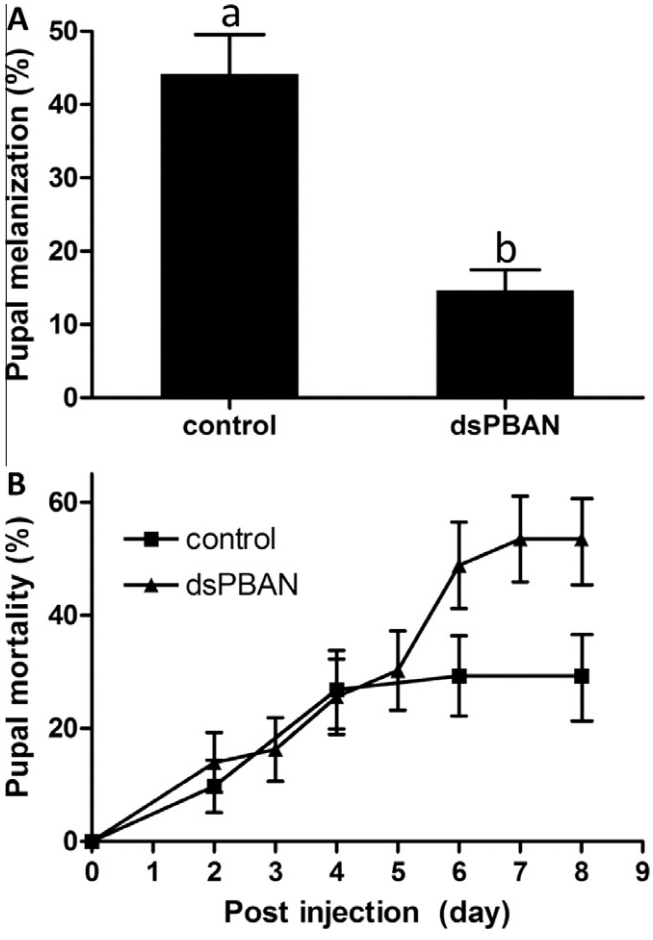


**Fig. 2.** Pupa melanization (tanning process) effect **(A)** and pupal mortality **(B)** of the fire ant after Soi-PBAN dsRNA injection. Young female pupae, 24–48 h-old, were injected with Soi-PBAN dsRNA (20 ng/50 nL/pupa) or a water control (50 nL/pupa) and observed for 8 days. Pupa melanization was significantly reduced compared to the negative water control. The mean + s.e.m. ($n \geqslant 25$) are shown. Bars on different letters are significantly different (unpaired *t*-test, two-tailed, $P = 0.013$, $n \geqslant 25$). Cumulative pupal mortality was recorded for 8 days until adult emergence. Soi-PBAN dsRNA treatment and water control were repeated three times (×3). Data were analyzed by Kaplan–Meier survival curve comparison ($P = 0.045$, $n \geqslant 25$).

### 3.3. Phenotypic impacts on fire ant larvae

Delivery of the dsRNA by injection is impractical for pest control applications, thus, other dsRNA delivery methods into the target ants are necessary. Unlike non-social insects, fire ant larvae cannot feed themselves. Nurse workers feed larvae by regurgitating liquid food (trophallaxis) obtained from foraging and/or reserve workers. We directly fed a fire ant PBAN dsRNA sucrose solution to nurse workers that were tending worker brood. The results showed that 80.0% of the treatment larvae died before pupation compared to ~30.0% mortality in the control groups after 21 days (PBAN vs. GFP: Kaplan–Meier Survival, $\chi^2 = 5.836$, $df = 1$, $P = 0.0157$; PBAN vs. water: Kaplan–Meier Survival, $\chi^2 = 4.175$, $df = 1$, $P = 0.0410$; GFP vs. water: Kaplan–Meier Survival, $\chi^2 = 0.2202$, $df = 1$, $P = 0.6389$) (Fig. 3). During the first 12-days of the experiment, mortality was similar between the groups.

These results demonstrate that PBAN dsRNA can be ingested by fire ant workers, transferred to larvae, and result in a phenotypic effect (larval mortality). The greatest mortality was observed after RNAi feeding was stopped, suggesting that treated larvae were unable to recover (data not shown). Food-based baits are well developed for pest social insect control and could be a way to practically use Soi-PBAN dsRNA in controlling the fire ant. This is an important first step toward development of a fire ant bait formulation that can be further evaluated under continually more application-oriented experimental conditions. Some nurse workers died during the course of the experiment, but the numbers were not significantly different amongst groups (data not shown).

The PBAN gene products have been shown to have multiple functions in a variety of insects; however, the physiological mechanism of these peptides is unknown in the fire ant. In fact, PBAN/

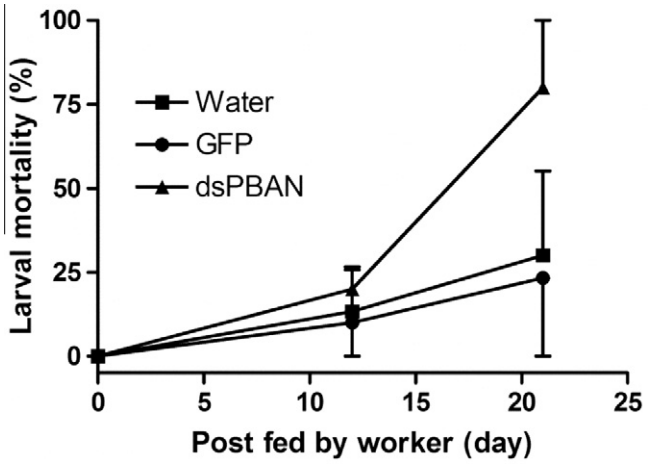


**Fig. 3.** Mortality of fire ant worker larvae. Nurse workers were fed Soi-PBAN dsRNA (1 μg/μL), GFP dsRNA (1 μg/μL), or water with 10% sucrose solution. Accumulated mortality was recorded 12- and 21-days post treatment. The experiment was repeated three times. Data were analyzed by Kaplan–Meier survival curve comparison (PBAN vs. GFP, $P = 0.0157$; PBAN & water, $p = 0.0410$; GFP vs. water, $P = 0.6389$, $n \geqslant 10$). Means values ± s.e.m. are shown.

pyrokinin peptides are produced in all stages including larval, pupal and adult stages, as well as the different sexual forms (Choi et al., 2011). This suggests that the peptides produced from PBAN/pyrokinin genes may have stage specific functions; however, direct evidence is needed. While we succeeded in observing phenotypic effects of PBAN RNAi in the fire ant, the precise cause of the lethal effects in adult workers, pupae and larvae is unknown. To understand these phenotype changes, we needed to investigate the stage specific PBAN gene expression after dsRNA treatment. For example, we found that PBAN dsRNA impacts fire ant larval development; therefore, we expect PBAN gene expression in the larval stage. The GFP dsRNA treatment was no different than the water control (unpublished data), thus we have confidence that the fire ant PBAN dsRNA has a specific rather than a random RNA interference effect.

### 3.4. Soi-PBAN gene expression in fire ants

We investigated Soi-PBAN transcription levels from eggs to adult workers using RT-PCR (Fig. 4A). The PBAN gene is clearly expressed in all developmental stages, with similar expression levels observed from larvae to adults. Interestingly, Soi-PBAN mRNA was detected in 7-day old eggs, indicating possible PBAN gene involvement in the embryo development in fire ants. Fire ant PBAN mRNA translates four neuropeptides including Soi-PBAN and Soi-DH (diapause hormone) (Choi and Vander Meer, 2009), but physiological roles for these peptides are unknown. In moths that exhibit embryonic diapause, e.g., the silkworm, PBAN-DH mRNA was expressed in mature eggs to induce diapause development (Uehara et al., 2011; Xu et al., 1995). However, fire ants are not known to undergo diapause.

To investigate RNAi knock down of Soi-PBAN, fire ant workers were injected with Soi-PBAN dsRNA (1 μg/100 nL/ant) or water (100 nL/ant) and incubated for 24 h, 48 h, and 72 h prior to Br-SG dissection from adult workers. Soi-PBAN expression was inhibited at all three time periods; however, the maximum effect was at 48 h (Fig. 4B). To investigate RNAi knock down of Soi-PBAN in the pupal stage, young pupae (<3-day old) were injected with Soi-PBAN dsRNA (1 μg/100 nL/pupa) and incubated for 24 h, 48 h, and 72 h prior to isolation of total RNA from whole pupae. Interference of Soi-PBAN gene transcription was observed from three time periods 24 h, 48 h, 72 h post-injection; however, 72 h had maximum gene suppression (Fig. 4C). Therefore, our RNAi results indicate Soi-PBAN dsRNA directly interfered with PBAN gene expression, which led to increase mortality of adults and pupae, or delay of the pupal melanization. Investigation of Soi-PBAN gene expression of additional developmental stages, including larvae and eggs remain for future studies.

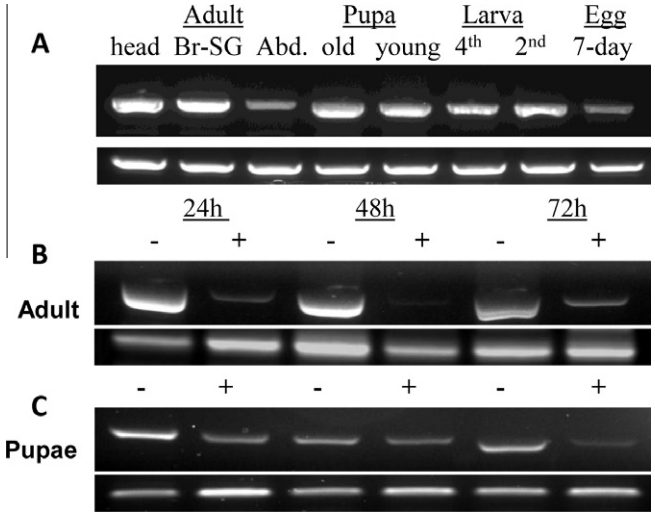


**Fig. 4.** Soi-PBAN gene expression in fire ants. **(A)** Soi-PBAN mRNA is expressed in adult worker – head, Br-SG and abdominal body (Abd.); pupal stages - old (completely melanized) and young (not melanized, <3-day old) pupae; larval stages – 2nd and 4th instars; embryo stage – 7-day old egg. **(B)** Suppression of Soi-PBAN gene expression from Br-SG of workers at 24 h, 48 h and 72 h post-injection of water (−) or 1 μg Soi-PBAN dsRNA (+). **(C)** Suppression of Soi-PBAN gene expression from whole body of worker pupae at 24 h, 48 h and 72 h post-injection of water (−) or Soi-PBAN dsRNA (+). Fire ant 18S rRNA was used for the positive control.

### 3.5. Phenotypic impacts on moth pupal mortality

The corn earworm, *H. zea*, provided us with an economically-important non-social insect system. Hez-PBAN dsRNA was injected into 3–4-day old female pupae, which were then monitored for phenotypic impacts. Hez-PBAN dsRNA injected pupae had significantly higher mortality, 53.8%, compared to the two control groups, dsRNA GFP (7.7%) (PBAN vs. GFP: $\chi^2$-test, $P = 0.0001$) and water (0.0%). This mortality mainly resulted from a failure of adults to emerge, rather than from impacts on pupal development. This means the pupae developed adult tissue, but failed to enclose from the pupal cuticle, or if they emerged, their wings and appendages did not expand. The similar phenotype also has been observed from another heliothine moth after dsRNA treatment (Choi personal observation). This phenotypic impact, pupal mortality, was observed from the PBAN RNAi, but not from the PBAN receptor RNAi (Choi personal observation), indicating that PBAN/PBAN receptor signal is not involved in the observed pupal mortality. Therefore the phenotypic result must be due to one or more of the remaining PBAN gene neuropeptide products, DH, (NP) –α, –β, or –γ during pupal development. The DH peptide has been demonstrated to be important for pupal diapause termination and normal adult development in heliothine moths (Sun et al., 2003; Xu and Denlinger, 2003); therefore, interference of pupa to adult development by PBAN RNAi is likely the result of none to low production of DH peptide, although this needs to be demonstrated. A similar phenotypic change has been observed from fire ant pupae (see Fig. 2). Experiments remain to be carried out on the quantification of peptide levels, with and without PBAN RNAi treatments.

### 3.6. RNAi impacts on sex pheromone production and PBAN gene expression in the female moth

Although some adults successfully emerged after pupal injection of Hez-PBAN dsRNA, those female adults produced significantly lower amounts (23%) of the major sex pheromone component (Z11–16:Ald) when compared to adults that emerged from the GFP dsRNA or water controls (PBAN vs. GFP, $P = 0.019$; PBAN vs. water, unpaired *t*-test, two-tailed, $P = 0.031$) (Fig. 5A). This is the first direct evidence that suppression of PBAN gene transcription results in sex pheromone reduction in a moth species. To investigate RNAi knock down of Hez-PBAN gene expression, the Hez-PBAN transcription level was measured by RT-PCR using female adults that had emerged from pupae injected with Hez-PBAN dsRNA. Hez-PBAN gene expression was clearly inhibited from dsRNA treatment compared to two control groups (water and GFP) (Fig. 5B). Decreased pheromone production has also been demonstrated in moths using the PBAN receptor-targeted RNAi (Bober and Rafaeli, 2010; Lee et al., 2011; Ohnishi et al., 2006). The timing of RNAi application is critical to effectively attenuate target gene expression and to observe and measure specific phenotypic and/or behavioral changes.

Insect neuropeptide hormones are involved in many important functions such as regulation of fat body homeostasis, feeding, digestion, excretion, circulation, reproduction, metamorphosis,

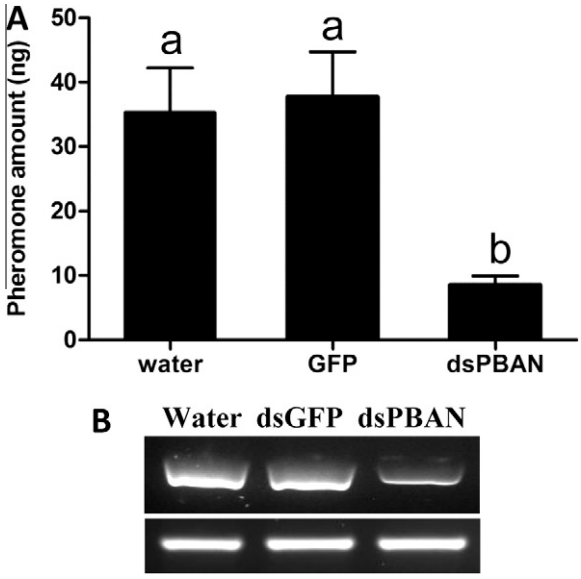


**Fig. 5.** Effect of PBAN RNAi on sex pheromone production and Hez-PBAN gene expression in *H. zea* female adults. (A) Hez-PBAN dsRNA (1 μg/3 μL), water (3 μL) or GFP (1 μg/3 μL) was injected into 4–5-day old pupae. Sex pheromone was extracted 2–4 h after light-off from 2–3-day old female adults. Bars with different letters are significantly different (unpaired *t*-test, two-tailed, PBAN vs. GFP, $P = 0.019$; PBAN vs. water, $P = 0.031$, $n \geqslant 5$). (B) Suppression of Hez-PBAN gene expression from the Br-SG of 1–2-day old female adults after water, dsGFP RNA (dsGFP) or Hez-PBAN dsRNA (dsPBAN) was injected into the pupal stage. *H. zea* actin RNA was used for the positive control as described detail in Methods.

and behavior (Clark et al., 2004; Gäde and Goldsworthy, 2003). Specific peptides were first determined based on their regulation of single physiological functions; however, these peptides now appear to be involved in more than one function, and more than one peptide can regulate a single physiological process. Our results give a glimpse of the multiple functions of the PBAN family of neuropeptides and the potential of RNAi in pest insect control. Although dsRNA insecticides are at an early stage of development, they may provide solutions to many of the problems associated with conventional pesticides. RNAi technology applied to insect neuropeptide hormones is a promising strategy for the development of alternative insect pest control tools, as well as finding unknown physiological function(s) for newly-discovered insect genes.

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