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# Knockdown of the Chromatin Remodeling Gene *Brahma* by RNA Interference Reduces Reproductive Fitness and Lifespan in Common Bed Bug (Hemiptera: Cimicidae)

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Research



# Knockdown of the Chromatin Remodeling Gene *Brahma* by RNA Interference Reduces Reproductive Fitness and Lifespan in Common Bed Bug (Hemiptera: Cimicidae)

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## Abstract

The common bed bug, *Cimex lectularius* L. (Hemiptera: Cimicidae) is a nuisance household pest causing significant medical and economic impacts. RNA interference (RNAi) of genes that are involved in vital physiological processes can serve as potential RNAi targets for insect control. *Brahma* is an ATPase subunit of a chromatin-remodeling complex involved in transcription of several genes for cellular processes, most importantly the homeotic genes. In this study, we used a microinjection technique to deliver double stranded RNA into female bed bugs. Delivery of 0.05 and 0.5 µg/insect of *brahma* dsRNA directly into hemocele resulted substantial reduction in oviposition. Eggs laid by bed bugs receiving both doses of *brahma* dsRNA exhibited significantly lower hatching percentage as compared to controls. In addition, *brahma* RNAi in female bed bugs caused significant mortality. Our results disclosed the potential of *brahma* RNAi to suppress bed bug population through injection of specific dsRNA, suggesting a critical function of this gene in bed bugs' reproduction and survival. Based on our data, *brahma* can be a promising RNAi target for suppression of bed bug population.

**Key words:** *Cimex lectularius*, RNAi, fecundity, egg viability, survival

The bed bug, *Cimex lectularius* L. (Hemiptera: Cimicidae) has re-emerged as a global pest that feeds primarily on human blood and impairs health. No specific data exist to attest bed bugs as a vector of human pathogens, but the existing ecological conditions, such cimids-bats interactions, repeated exposure of bed bugs to viruses and their gregarious mode of living can potentially increase their chances of transmitting vector-borne diseases (Adelman et al. 2013). The governing factors for bed bug infestation across the world are the evolution of insecticide resistance (Romero et al. 2007, Zhu et al. 2010, 2013; Tawatsin et al. 2011; Koganemaru et al. 2013; Dang et al. 2015), hitchhiking ability, high fecundity, and cryptic life cycle. Bed bugs might remain undetected until the population builds up to severe infestations (Miller 2010). The other control option such as heat treatment, ultracold treatment, and fumigation are economically prohibitive and may be less residual (Pereira et al. 2009). Therefore, it is prudent to search for innovative and environmental friendly bed bug management tactics.

RNA interference (RNAi) is a natural cellular process to defend from virus infections and regulate gene activity in most eukaryotic cells (Drinnenberg et al. 2011). The discovery of RNAi in the nematode, *Caenorhabditis elegans* Maupas (Fire et al. 1998), opened a new era for functional genomic studies in insects, plants and animals, and to use it as pest management tool. The dsRNA delivered into insects are transported into cell and processed by RNAi machinery. The endonuclease activity of Dicer cuts the dsRNA into 21–23 base pairs long small interfering RNAs (siRNAs). These siRNAs direct RNA-induced silencing complex (RISC) to complementary mRNA, and eventually facilitate the degradation process (Zamore et al. 2000). The degraded mRNA no longer synthesizes the protein and the gene activity is eventually silenced.

DNA encodes genetic information over enormously long linear DNA that densely folds into chromatic structure (Hargreaves and Crabtree 2011). Changes in the chromatin structure induced by chromatin remodeling protein enhance RNA polymerase access to the densely-packaged DNA for transcription (Armstrong et al. 2005). *Brahma* was first identified in *Drosophila* and is homologous to the yeast SWI2/SNF2 gene (Hargreaves and Crabtree 2011). SWI/SNF family protein has a catalytic subunit called ATP-dependent chromatin remodeling complexes that regulates transcriptional activation and repression of homeotic genes (Martens and Winston 2003, Tsukiyama et al. 1999). In *Drosophila*, along with homeotic gene transcription, *brahma* is essential for transcription of a majority of genes (Elfring et al. 1998, Holstege et al. 1998, Hargreaves and Crabtree 2011). An RNAi of chromatic remodeling genes in the western corn rootworm *Diabrotica virgifera virgifera LeConte* and the Neotropical brown stink bug *Euschistus heros* (Say) demonstrated

significantly reduced fecundity in both insects (Khajuria et al. 2015, Fishilevich et al. 2016). RNAi-based pest control approach has demonstrated high potency in several phytophagous insects both in laboratory and field settings (Baum et al. 2007, Mao et al. 2007, Zhang et al. 2015). In case of the hematophagous insects, the bottleneck to deliver the dsRNA in field conditions persists.

The phylogenetic relationship showed the *Rhodnius prolixus* Stål, a vector of the Chagas parasite, is the closest relative of bed bugs (Rosenfeld et al. 2016). The molecular approach of understanding behavior and biology of bed bugs has increased in recent years due to its increase in infestation and possibility of the transmission of disease pathogens. According to literature, most RNAi studies in bed bugs were focused on elucidating the mechanism for insecticide resistance (Zhu et al. 2012, 2013) or understanding gene functions (Gujar and Palli 2016a, 2016b; Tsujimoto et al. 2017). Moriyama et al. (2016) recently reported that the RNAi of *vitellogenin* gene reduced the reproduction capacity in female bed bugs. In this research, we used RNAi to determine the effect of *brahma* RNAi on reproductive fitness and lifespan in bed bugs.

## Materials and Methods

### Insects

A Harlan strain of bed bugs was reared on reconstituted human blood warmed to 35–36°C and maintained at 25 ± 1°C, relative humidity 55 ± 5%, and a photoperiod of 14:10 (L:D) h as per Montes et al. (2002). The bed bug colonies were maintained in glass jars containing Whatman filter paper (90 mm, Sigma-Aldrich, St. Louis, MO) and blood meal was provided weekly. These bed bugs were never exposed to any insecticide since collected in 1983 from a natural infestation site, Gainesville, FL.

### Sequence Analysis

The amino acid sequence of putative ATP dependent helicase *brahma* (NCBI GenBank accession no.: LOC106672117) was obtained and analyzed in Interpro (www.ebi.ac.uk/interpro). In addition, BLASTP was performed to search the protein database for *brahma* in other insects using bed bug *brahma* amino acid as the query protein.

### Gene Expression Across Different Life Stages

Total RNA was extracted from males, females, eggs and nymphs not exposed to any treatments to compare the baseline expression level of the *brahma* gene in the different life stages of bed bugs. Newly emerged males and females were selected for RNA extraction while nymphs were categorized into two groups, small nymphs (instars 1st–2nd) and large nymphs (instars 3rd–5th). Total RNA was extracted from bed bugs using an RNAeasy mini kit (Qiagen, Valencia, CA) and cDNA was synthesized from 0.5 µg of total RNA using a QuantiTect Reverse Transcription Kit (Qiagen).

### Synthesis of dsRNA

*Brahma* specific primers for dsRNA synthesis were derived from the putative ATP-dependent helicase *brahma* gene nucleotide sequence of the bed bug. A web-based software system, siDirect, was used to select genomic regions that yielded maximum siRNA (Naito et al. 2004). Sense and antisense primers were designed with Primer3plus software (Untergasser et al. 2012) and tailed with T7 polymerase promoter sequence (TAATACGACTCACTATAGGG) at the 5′ end to initiate in vitro transcription of dsRNA (Supplementary Table S1). The cDNA was used as template for the PCR. The PCR products were confirmed by sequencing and agarose gel electrophoresis. A non-target *Green Florescence Protein* (*GFP*) gene was used as negative control and was prepared from pIZT/V5-His vector (Invitrogen, Waltham, MA) using gene-specific primers. A 1.5 µg of PCR product was used as a template for dsRNA synthesis using a MegaScript High Yield Transcription Kit (Ambion, Life Technologies, Carlsbad, CA) in 20 µl reaction volume according to the manufacturer's instructions. The dsRNA was purified using an RNAeasy Mini Kit (Qiagen), eluted in ultra-pure water and quantified in a NanoDrop 8000 spectrophotometer (Thermo Scientific, Franklin, MA). The quality of dsRNA products was assessed by agarose gel electrophoresis.

### The dsRNA Injection and Phenotypic Inspection

Bed bugs used in this experiment were obtained from four different generations maintained in a growth chamber (Incubator I-35LL, Percival Scientific, Iowa). Newly emerged adult females and males from each colony were segregated and placed in glass jars for 2 d to allow mating. Males were removed and only females were selected for injection. Control groups included bed bugs injected with water, 0.5 µg of *GFP* dsRNA, and an additional control group of non-injected females. The treatment groups consisted of female bed bugs receiving 0.05 µg and 0.5 µg of *brahma* dsRNA. The injections were performed by piercing a fine capillary tube (44 × 1.2 mm) fitted with a nanoinjector (Nanoject II Auto-Nanoliter Injector, Drummond Scientific Company, Broomall, PA) on the ventral side behind the hind legs (Supplementary Fig. S2). After 12 h of recovery time in the growth chamber and subsequent feeding, the bed bugs were transferred to a small petridishes (50 mm × 11 mm) with a sterile pad for laying eggs (Advantec MFS, Inc., Dublin, CA). Only those bed bugs that fed on blood meal to repletion were transferred to the experimental unit. All treatments were replicated eight times, each consisting of four healthy bed bugs from four different colonies. Bed bugs were fed on a weekly basis and oviposition, hatching success and survival rate were recorded weekly for 4 wk. The bed bugs were dissected during first 3 wk to observe ovaries and visualized under Olympus SDF PlAPO 2X lens fitted to a stereomicroscope with Olympus S2 16 X lens (Olympus Imaging America Inc. Center Valley, PA). Any morphological changes in the bed bugs were assessed.

### Gene Knockdown Study

Total RNA was extracted from female bed bugs 2 and 4 d after dsRNA injection and mRNA levels were quantified. In addition, RNA was also extracted from eggs deposited by the females in the treatment and control groups (15 eggs/sample) to assess *brahma* expression in eggs.

### Real-time Quantitative PCR

The quantification of transcript levels of *brahma* was done by Real-time Quantitative PCR (RT-qPCR) using SYBR green in a 7500 Fast System real-time PCR detection system (Applied Biosystems, Grand Island, NY). The descriptions of primers used for RT-qPCR are specified in Supplementary Table S1. Total RNA extraction and cDNA preparation was carried out as mentioned earlier. The primers were designed outside the dsRNA sequence of target gene and efficiencies were evaluated using serial fivefold dilutions. The cycling parameters were 40 cycles each consisting of 95°C for 5 s, and 58°C for 30 s, as described in manufacturer's protocol. Melt curve analysis ruled out primer dime formation and nonspecific product formation. In addition, the amplified products were run in 1.7% agarose gel to visualize product, and a single band of expected size confirmed

the amplification of target. Relative quantification of transcripts was calculated using a comparative 2– ΔΔCT method (Livak and Schmittgen 2001). The mRNA levels were normalized using the internal control *ribosomal protein L8* (*rpL8*) (Zhu et al. 2010).

### Statistics

The data on oviposition and hatching success were analyzed separately as repeated measures analyses of variance (ANOVA). Repeated measures ANOVA provides treatment and time effects, and interaction between them. Data on gene expression and mortality were analyzed as one-way (ANOVA) ($P < 0.05$) and means were compared using Tukey Kramer's adjustment. All analyses were done with PROC GLIMMIX in SAS 9.4 (2013) (SAS Institute, Cary, NC).

## Results

### Bioinformatics

The bed bug putative ATP-dependent helicase *brahma* gene consists of the two transcript variants X1 and X2. The amplified fragment of gene for *brahma* dsRNA synthesis covers both the transcript variants. BLASTP analysis of *brahma* amino acid sequence revealed *brahma* is highly conserved across insects (Supplementary Table S3). The protein sequence analysis of the *brahma* gene in InterPro revealed that the *brahma* protein hits the SMARCA2, a subunit of SWI/SNF complex (Supplementary Fig. S4).

### *Brahma* Gene Expression Pattern Across Different Life Stages

The baseline expression of bed bug *brahma* was highest in females followed by eggs, small nymph (instars 1st–2nd), males and large (instars 3rd–5th). Data analysis showed significantly higher transcript level of *brahma* in females as compared to all other life stages, but no significant differences were observed across other life stages ($F = 16.52$; df = 10, 4; $P < 0.001$; Fig. 1).

### Effect of *Brahma* RNAi on Fecundity and Survival

*Brahma* RNAi caused distinct reduction in oviposition in the initial 2 wk and completely shut down the reproduction after that time. ANOVA showed a significant effect of *brahma* RNAi ($F = 199.98$; df = 33.7, 4; $P < 0.0001$), and time ($F = 15.08$; df = 109, 3; $P < 0.0001$) on oviposition. In the first and fourth week, the number of eggs laid by uninjected bed bugs was significantly higher relative to other control groups (water and *GFP*), suggesting a significant effect of the injection on oviposition (Fig. 2A). The eggs laid were further investigated for hatching success. ANOVA of data on egg hatch revealed significant effect of *brahma* RNAi ($F = 733.40$; df = 35, 4; $P < 0.0001$) and time ($F = 77.21$; df = 105, 3; $P < 0.0001$). Simple effect analysis was conducted to compare treatment means in each week (Fig. 2B). In the first 2 wk, significantly fewer eggs were hatched in the treatment groups when compared to the control groups, but no significant differences were observed among control groups or between two treatment groups. In the third and fourth weeks, no eggs were laid by treatment groups to determine hatching percentage. The dissection of bed bugs showed normal development of eggs within the ovaries in *GFP* treated bed bugs. Similarly, *brahma* dsRNA treated bed bugs also had ovaries with normal egg development in the first week, but no eggs were observed in the second and third week (Fig. 3A). The *brahma* RNAi also caused a remarkable abdominal inflation, and this morphological change was evident in the third week (Fig. 3B). Survival of female bed bugs from control groups and treatment groups were also monitored for 4 wk. The *brahma* RNAi caused significant mortality in bed bugs as compared to the control groups ($F = 130.97$; df = 35, 4; $P < 0.0001$; Fig. 4).

### *Brahma* Gene Knockdown

Quantification of the *brahma* transcript level by RT-qPCR and analysis showed significant knockdown of *brahma* mRNA with both the doses of *brahma* dsRNA after 2 d ($F = 50.66$; df = 3,8; $P < 0.0001$), and 4 d ($F = 17.66$; df = 3,8; $P < 0.00$; Fig. 5A). In addition, we also observed significant knockdown of *brahma* in the eggs laid by the treated bed bugs ($F = 4.78$; df = 3,7; $P < 0.05$; Fig. 5B).

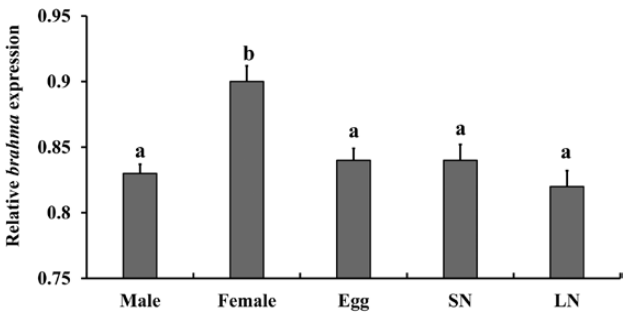


**Fig. 1.** Relative gene expression of *brahma* in male, female, egg and small nymph (SN: instars 1st–2rd and large nymph (LN: instars 3rd–5th). The relative mRNA levels were shown as a ratio in comparison with the levels of *rpL8* mRNA. Means were compared with Tukey Kramer.

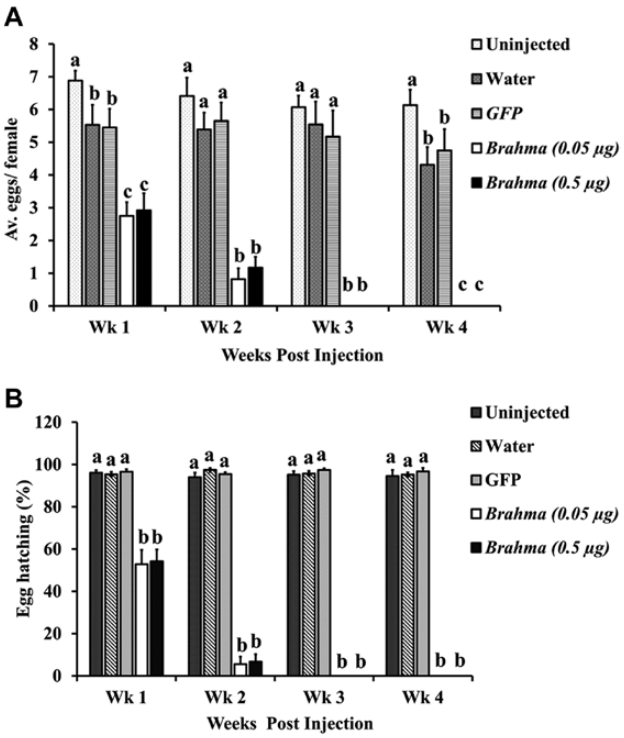


**Fig. 2.** Effect of *brahma* RNAi in bed bug oviposition and hatching. (A) Average eggs laid per female per week. (B) Percentage of eggs hatched every week. Simple effect analysis compared treatment means for each week separately. Means with different letters are significantly different.

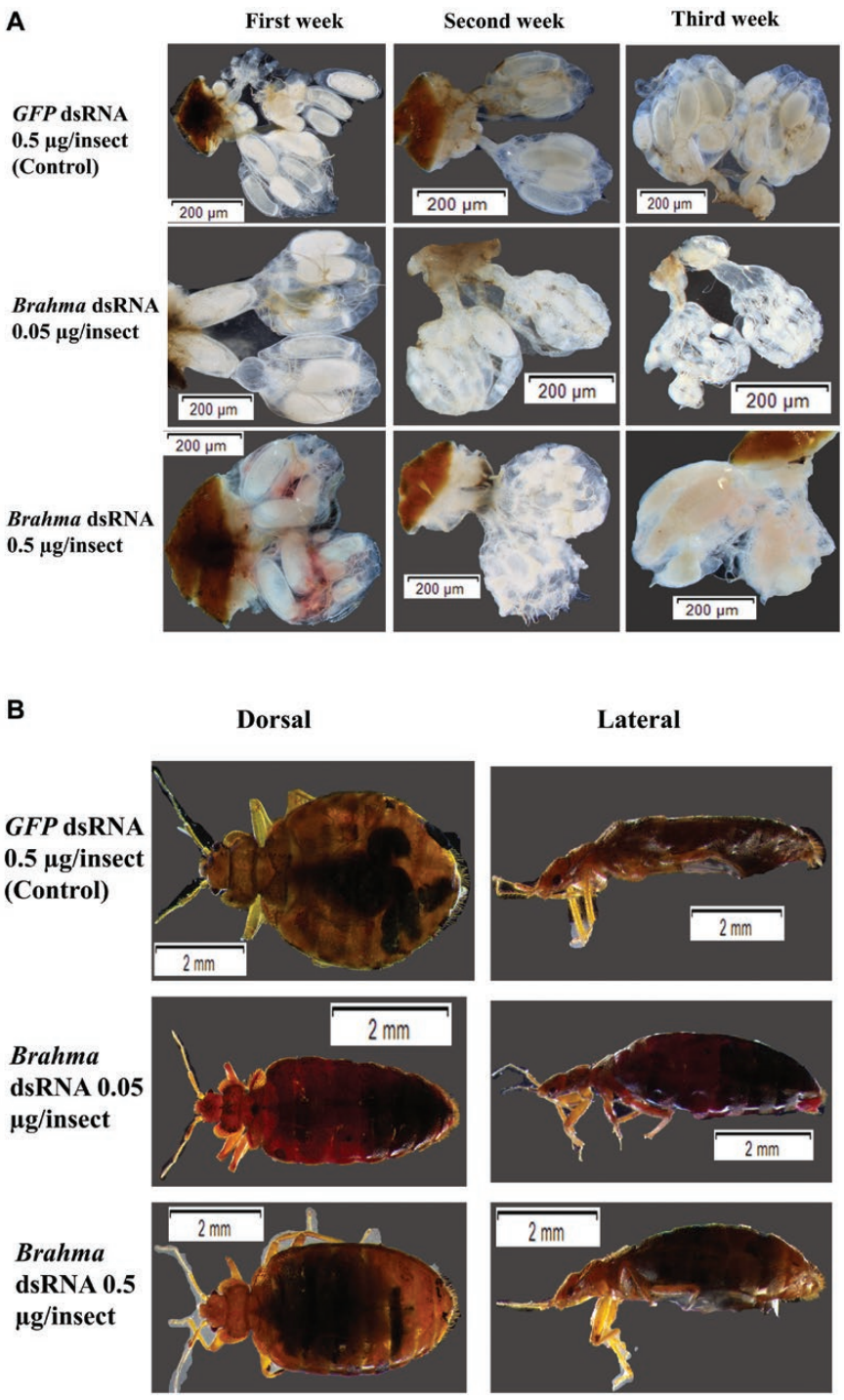


**Fig. 3.** Phenotypic response of *brahma* RNAi in female bed bugs. (A) Normal ovaries in control and deformed in treatments. (B) Normal abdomens in control and inflated in treatments in the third week.

## Discussion

The phenotypic responses of the *brahma* RNAi in bed bugs contribute towards bed bug population suppression by reducing reproductive capacity, egg viability and survival. The chromatin remodeling proteins regulate expression of multiple genes involved in homeotic and cellular functions and complete loss of *brahma* gene can be lethal to insects (Brizuela et al. 1994). The lethal phenotype observed with *brahma* RNAi elucidate the critical function of this gene in bed bugs. Lately, the *brahma* gene has been studied as a potential RNAi targets in multiple insects for population control. However, the phenotypic responses have been different in different insects. Egg laying was normal, but the hatching rate was very low in *D. virgifera virgifera* (Khajuria et al. 2015), while there was complete absence of oogenesis in *Drosophila* (Brizuela et al. 1994). In *E. heros*, Fishilevich et al. (2016) showed complete absence of egg laying and high mortality. Protein sequence analysis of bed bugs and *E. heros* showed 100% identity suggesting the important biological function of *brahma* gene in insects, predominantly in the hemipteran insects. All these studies

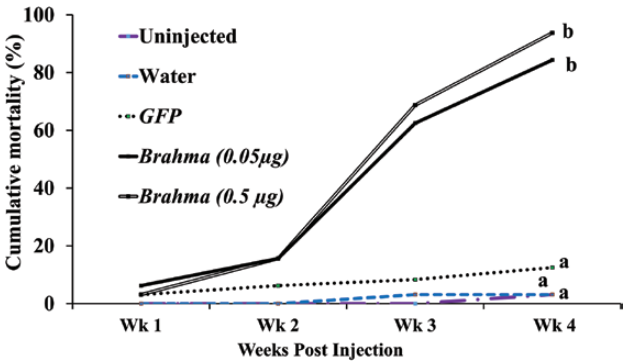


**Fig. 4.** Effect of *brahma* RNAi in bed bug survival. The accumulative mortality in the fourth week was analyzed and mean comparisons were performed using Tukey Kramer. Different letters assigned to mean cumulative mortality in the fourth week suggest significant differences.

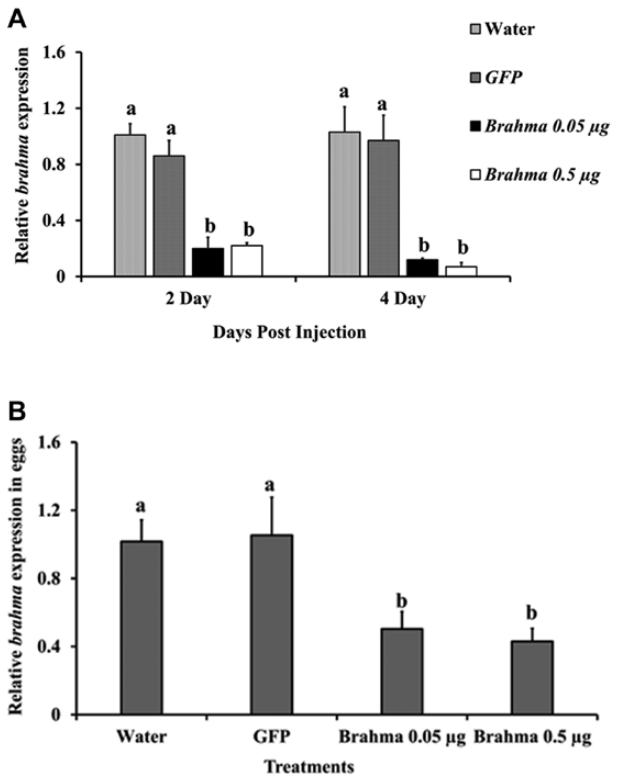


**Fig. 5.** (A) Relative *brahma* expression in female bed bugs 2 and 4 d post injection. (B) Relative expression of *brahma* in eggs laid within a week by bed bugs injected with *brahma* dsRNA (15 egg/sample). *Brahma* mRNA expression level were normalized to *rpL8*. Means comparisons were performed using Tukey Kramer method.

have shown *brahma* can be a potential RNAi target gene for insect control.

This study represents the first demonstration of robust parental RNAi (pRNAi) properties in bed bugs. From an insect management prospective, using RNAi target gene with parental properties could be more effective in population suppression because gene silencing is inherited to subsequent generation. Lack of oogenesis in the ovaries, low hatching rate and significant knockdown of *brahma* transcript in eggs confirmed *brahma* pRNAi. Studies have shown that *brahma* is expressed both maternally and zygotically; maternal *brahma* involved in oogenesis and zygotic *brahma* involved in embryo development (Tamkun et al. 1992, Brizuela et al. 1994). Significant reduction in oviposition in bed bugs injected with *brahma* dsRNA suggests the role of *brahma* in oogenesis and the data on low hatching rate suggests its role in embryogenesis. Based on our findings, it is reasonable to hypothesize that the *brahma* gene has a critical function in bed bug female reproduction. The mechanism by which pRNAi occurs is not fully understood, but researchers have proven that the RNAi silencing of embryonic gene can be heritable. Lynch and Desplan (2006) observed the pRNAi in progenies of *Nasonia vitripennis* Ashmead by injecting the dsRNA specific to embryonic patterning genes in pupae. Burton et al. (2011) also observed gene silencing after the transfer of siRNA to progeny in *C. elegans* and research by Carmell et al. (2003) also found gene knockdown from shRNAs (short hairpin RNAs) in multiple generations in mice. These studies showed that pRNAi is prevalent in a diverse group of eukaryotic organisms.

Bed bugs increased their size after a blood meal with a conspicuous reddish inflated abdomen. As the blood is digested, they regain their original shape which is dorsoventrally flattened. The bed bugs treated with *brahma* dsRNA remain inflated over time. The abdominal inflation may be caused due to improper digestion, lack of the transport of digested products or removal of waste/fluid. Blood meal is necessary protein source for oogenesis. The failure of egg development in ovaries may have caused the accumulation of nutrients. This phenotypic response was conspicuous in the third week after *brahma* dsRNA injection. RNAi of the *vitellogenin* gene also caused the abdominal inflation in bed bugs (Moriyama et al. 2016). Since the phenotypic responses of *brahma* RNAi and vitellogenin RNAi were similar, further research is necessary to elucidate the biological pathways being disrupted by *brahma* and *vitellogenin* RNAi in bed bugs.

The bed bug genome was very recently published (Benoit et al. 2016, Rosenfeld et al. 2016), which opens the possibility of the identification of novel RNAi targets for bed bug control. Our study revealed the RNAi mechanism has tremendous potential to suppress bed bug population. A major challenge of using RNAi for bed bug control in the field setting is the discovery of an effective method for dsRNA delivery. Bed bugs feed exclusively on blood and dsRNA are degraded very rapidly when mixed in the blood. Using blood product for killing bed bug is not pragmatic and therefore other delivery techniques such as spraying the dsRNA, use of the nanoparticles in the form of aerosols or engineering the endosymbionts of bed bugs to produce dsRNA lethal to bed bugs can be explored, but further research is necessary to evaluate the feasibility of these delivery techniques. Screening of the chemicals with potential to inhibit the *brahma* function in bed bug can also be explored.

In conclusion, we have shown that RNAi knockdown of *brahma*, a chromatin remodeling gene involved in homeotic gene expression, reduced fecundity in bed bugs that are serious household pest around the world. The lethal phenotype observed after the *brahma* gene knockdown is promising and this study could be a crucial step towards using *brahma* gene for RNAi mediated population suppression. Additionally, we showed the parental properties of *brahma* RNAi which further contributes to population suppression.

## Supplementary Material

Supplementary data are available at *Journal of Medical Entomology* online.

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