Silencing of Two Vitellogenin Genes Inhibits Oviposition in the Chagas Disease Vector *Triatoma infestans* (Hemiptera: Reduviidae)

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Abstract. RNA interference caused by exogenous double-stranded RNA (dsRNA) is used to downregulate crucial genes to control insects. The reproductive success of all oviparous species depends on vitellogenin (Vg) biosynthesis and its accumulation in the developing oocytes. Adult females of *Triatoma infestans* were independently injected with two *Vg* dsRNAs (*Vg1* dsRNA or *Vg2* dsRNA) or nuclease-free water (control) 24 hours before feeding, and a group of adult females not injected was also analyzed (control). *Vg1* and *Vg2* messenger RNAs silencing was verified by reverse transcription quantitative polymerase chain reaction. The transcript levels of the *Vg1* and *Vg2* genes were significantly reduced after dsRNA treatment in fat body and ovary of *T. infestans* in relation to those detected in individuals injected with nuclease-free water and not injected (controls). Moreover, the present study demonstrated that the silencing of the *Vg1* or *Vg2* genes inhibits oviposition in the Chagas disease vector *T. infestans*. These findings may have important implications for the development of novel vector control strategies.

INTRODUCTION

Triatoma infestans (Hemiptera: Reduviidae) known as "vinchuca" is the main vector of Chagas disease in the southern Cone of Latin America between latitudes 10°S and 46°S, where it is primarily restricted to domestic and peridomestic environments. Pyrethroid insecticides have been the main means of controlling vector. However, pyrethroid resistance in the vector insect has been reported as one of the main explanations of the unsatisfactory control observed.[1]

RNA interference (RNAi) has been developed as an effective gene-silencing tool in a wide variety of organisms.[2] Double-stranded RNA (dsRNA)–mediated RNAi has emerged as one of the most powerful strategies for the rapid analysis of gene function and has considerable potential for the development of applications for the control of insect vectors.[3–5] For the last purpose, this posttranscriptional gene silencing technique requires as its target a gene involved in a vital process for the vector.

The reproductive success of all oviparous species depends on vitellogenin (Vg) biosynthesis and its accumulation in the developing oocytes. Recently, we have identified two *Vg* genes in *T. infestans* (*Vg1* and *Vg2*, GeneBank accession numbers KF915268 and KF915267, respectively) and we have analyzed the expression of these genes at transcriptional and translational levels in fat body and ovaries of adult females at different times after ecdysis (previtellogenic phase) and after blood feeding and mating of the females (vitellogenic phase).[6,7] Here we examine the role of the *Vg* genes in the oviposition of *T. infestans* using RNAi.

MATERIALS AND METHODS

Triatoma infestans was reared at 28 ± 1°C at a relative humidity of 60–70% with a 6-hour light/18-hour dark cycle and fed once every 2 weeks after molt on restrained chickens. Adult females and males were maintained segregated after emergence until they were able to have a blood meal (day 7 postecdysis).

Total RNA was isolated from pools of insect tissues using MasterPure RNA Purification Kit (Epicentre, Madison, WI). Synthesis of complementary DNA (cDNA) was performed from total RNA using Oligo-dT$_{20}$ and SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA).

A fragment of 650 base pairs (bp) of the *Vg1* gene and of 500 bp of the *Vg2* gene were amplified by polymerase chain reaction (PCR) from fat body cDNA of females using specific primers containing T7 promoter sequences (Table 1). After the PCR products were electrophoresed, the bands corresponding to the expected sizes were excised from the agarose gel and purified using QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). Then the PCR products were sequenced in an ABI 3130XL automated DNA sequencer (Applied Biosystems, Foster City, CA). Once the sequences of the *Vg* genes were verified, the products from the PCR reactions were used as templates for dsRNA synthesis using the T7 RiboMax Express RNAi System (Promega, Madison, WI) according to the manufacturer's instruction. The dsRNA was isopropanol precipitated, resuspended in nuclease-free water, and quantified spectrophotometrically at 260 nm.

Females were injected with 5 µL of *Vg1* dsRNA (0.5 µg, *N* = 27) or 5 µL of *Vg2* dsRNA (0.5 µg, *N* = 27) or 5 µL of nuclease-free water (control, *N* = 22) 24 hours before feeding. Injections were made ventrally between the fifth and sixth abdominal segment with a Hamilton microsyringe (Hamilton, Reno, NV). To assess the effect of injection, a group of adult females in the same conditions that had not been injected were also analyzed (control, *N* = 22). After feeding, females were placed together with males in individual containers (each couple in one container). Successful mating was verified by observation of the spermatophore.

Vg1 and *Vg2* messenger RNAs (mRNAs) silencing was verified by reverse transcription quantitative PCR (RT-qPCR). Total RNA was isolated from pools of fat bodies and of ovaries extracted of three adult females of each experimental group, 4

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P6	5′-TAATACGACTCACTATAGGGGGTAAAGTTAAGGTCAATGGC-3′	*Vg1*-Forward
PARA	5′-TAAACCAAGCGTTTGCCGGT-3′	*Vg1*-Reverse
P8	5′-TAATACGACTCACTATAGGTAAACCAAGCGTTTGCCGGT-3′	*Vg1*-Reverse
PARNAiF1	5′-GGTAAAGTTAAGGTCAATGGC-3′	*Vg1*-Forward
P9	5′-TAATACGACTCACTATAGGGGTCTTGAAGCTTTTACTCCCG-3′	*Vg2*-Forward
PBRNAiR	5′-GGTACACAGTGAAACTCTACTCGC-3′	*Vg2*-Reverse
P10	5′-TAATACGACTCACTATAGGGGTACACAGTGAAACTCTACTCGC-3′	*Vg2*-Reverse
PBRNAiF	5′-GGTCTTGAAGCTTTTACTCCCG-3′	*Vg2*-Forward

dsRNA = double-stranded RNA; PCR = polymerase chain reaction.

and 5 days after feeding, respectively. The transcript levels of *Vg1* and *Vg2* genes were measured by qPCR, using β-*actin* mRNA to normalize the expression levels.[6,7] Gene-specific primers and Taqman probes were designed according to the corresponding cDNAs using Primer Express program (Applied Biosystems). RT-PCR analysis was used to verify that the PCR product, obtained with each specific primer pair, showed a single band of the expected size. The PCR products corresponding to *Vg1*, *Vg2*, and β-*actin* were cloned into the pCR4-TOPO TA cloning vector (Invitrogen) and sequenced to confirm the identity of the amplified fragments. Quantitative PCR was carried out using Mx3005P qPCR System with Brilliant qPCR Core Reagent Kit (STRATAGENE, La Jolla, CA). The reaction conditions were of 10 minutes at 95°C, and 40 cycles of 15 seconds at 95°C and 60 seconds at 58°C. The relative copy number of *Vg* mRNA was calculated according to $2^{-\triangle\triangle CT}$.[8] The threshold cycle value difference $\triangle$CT between *Vg* mRNA and β-*actin* mRNA of each reaction was used to normalize the level of total RNA. Two independent experiments were performed and data for each point were registered by triplicate to account for intraexperimental variation. Graphs and statistical tests were performed using GraphPad Prism, version 5.00 for Windows (GraphPad software, San Diego, CA). One-way analysis of variance with Bonferroni posttest were used for comparisons. The results were presented as mean ± standard deviation SD and a *P* value < 0.05 was considered statistically significant. On the other hand, to examine whether silencing of *Vg1* and *Vg2* genes influenced the oviposition, females of each experimental group were separated from males and placed in different containers and eggs deposited were counted daily.

RESULTS AND DISCUSSION

The use of RNAi for the management of insects holds considerable promise, as a system which can be used to reduce the fitness, and/or the fecundity as well as limit their capacity as vectors of diseases. However, the use of RNAi approaches to control vector insects is still in its infancy. Due to the limited number of transgenic insect species, the production of a systemic RNAi response in insects often requires delivery of dsRNA to cells and tissues via micro-injection, which is used in most of these experiments to achieve high titer of dsRNA and expose more tissues to silencing factor.

In a previous work, the expression levels of *Vg1* and *Vg2* genes were analyzed by qPCR and western blot in fat body and ovaries of adult females throughout previtellogenesis and vitellogenesis.[7] *Vg1* and *Vg2* start to be expressed slightly in the fat bodies and ovaries of adult females during previtello-genesis. After blood feeding, *Vg1* and *Vg2* were up regulated and significant levels of *Vg* transcripts as well as protein expression were observed in fat bodies sampled throughout vitellogenesis. These findings agreed with the studies that established that in most insect species the female fat body is the major site involved in Vg synthesis.[9] However, in addition to the fat body, synthesis of Vgs has been observed in the ovaries of some insect species.[10–13] The detection of *Vg* transcripts in ovaries of *T. infestans* sampled throughout vitellogenesis was in agreement with this observation.[7] Based on the results of this study, adult females were injected after ecdysis (24 hours before feeding) with *Vg1* dsRNA or *Vg2* dsRNA or nuclease-free water (control) and a group of adult females that had not been injected were also analyzed (control). Subsequently, pools of fat bodies were extracted from adult females of each experimental group 4 days after feeding and pools of ovaries were extracted 5 days after feeding.

The relative expression levels of the *Vg1* and *Vg2* genes in fat body and ovary of adult females of *T. infestans* are shown in Figures 1 and 2. The transcript levels of the *Vg1* and *Vg2* genes were significantly reduced after dsRNA treatment in fat body (Figure 1A and B) and ovary (Figure 2A and B) in relation to those detected in individuals injected with nuclease-free water and not injected (controls). Besides, it is also important to point out that the expression levels either of *Vg1* or *Vg2* of the group of individuals injected with nuclease-free water and the group not injected did not present significant differences in both tissues and are congruent with those detected in our previous work.[7]

In insects, the vitellogenesis process is fundamental for egg development and therefore is a central event for their reproduction.[14] To examine whether silencing of *Vg1* and *Vg2* genes influenced the oviposition, females of each experimental group were subjected to an oviposition bioassay. The *Vg*-silenced *T. infestans* adult females failed to lay eggs. The *Vg1* dsRNA-treated group (N = 15) and the *Vg2* dsRNA-treated group (N = 15) did not present egg production. Contrary, the females injected with nuclease-free water (N = 10) and not injected (N = 10) showed similar level of oviposition. Therefore, this study demonstrated that the silencing of *Vg1* or *Vg2* inhibits oviposition in the Chagas disease vector *T. infestans.* These findings are potentially helpful for the development of effective vector management strategies.
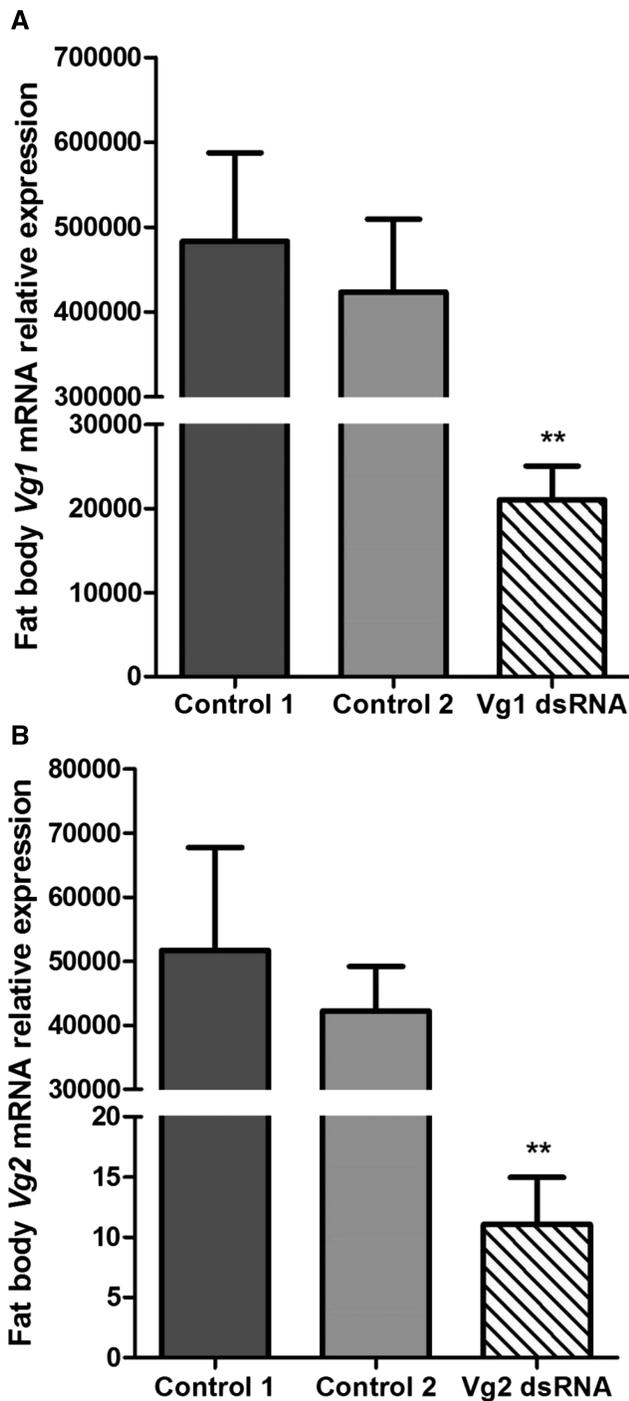
A

B

FIGURE 1. Relative vitellogenin expression messenger RNA (mRNA) of (**A**) *Vg1* and (**B**) *Vg2* in the fat body of *Triatoma infestans* adult females after injecting *Vg1* double-stranded RNA (dsRNA) and *Vg2* dsRNA, respectively. The transcript levels of *Vg* genes were measured by quantitative polymerase chain reaction using β-*actin* mRNA to normalize the expression levels. The error bars represent the standard deviation of the mean. Two asterisks on the standard error bar indicate significant difference between the mean of the treatment with dsRNA and the mean of the controls at $P < 0.01$. Control 1: group injected with nuclease-free water; Control 2: group no injected.
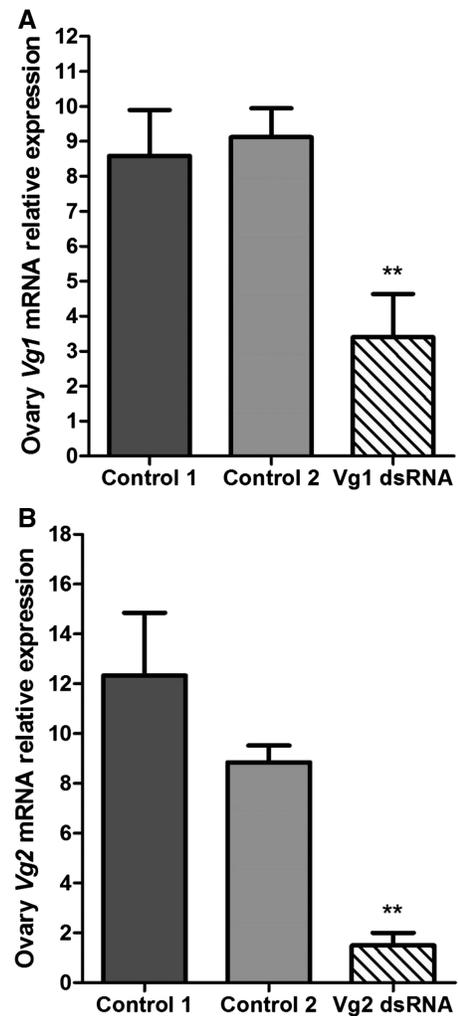
A

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FIGURE 2. Relative vitellogenin expression messenger RNA (mRNA) of (**A**) *Vg1* and (**B**) *Vg2* in the ovary of *Triatoma infestans* adult females after injecting *Vg1* double-stranded RNA (dsRNA) and *Vg2* dsRNA, respectively. The transcript levels of *Vg* genes were measured by quantitative polymerase chain reaction (qPCR) using β-*actin* mRNA to normalize the expression levels. The error bars represent the standard deviation of the mean. Two asterisks on the standard error bar indicate significant difference between the mean of the treatment with dsRNA and the mean of the controls at $P < 0.01$. Control 1: group injected with nuclease-free water; Control 2: group no injected.

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