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Vitellogenin knockdown strongly affects cotton boll weevil egg viability but not the number of eggs laid by females

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ABSTRACT

Vitellogenin (Vg), a yolk protein precursor, is the primary egg nutrient source involved in insect reproduction and embryo development. The Cotton Boll weevil (CBW) *Anthonomus grandis* Boheman, the most important cotton pest in Americas, accumulates large amounts of Vg during reproduction. However, the precise role of this protein during embryo development in this insect remains unknown. Herein, we investigated the effects of *vitellogenin* (*AgraVg*) knockdown on the egg-laying and egg viability in *A. grandis* females, and also characterized morphologically the unviable eggs. *AgraVg* transcripts were found during all developmental stages of *A. grandis*, with highest abundance in females. Silencing of *AgraVg* culminated in a significant reduction in transcript amount, around 90%. Despite this transcriptional reduction, egg-laying was not affected in dsRNA-treated females but almost 100% of the eggs lost their viability. Eggs from dsRNA-treated females showed aberrant embryos phenotype suggesting interference at different stages of embryonic development. Unlike for other insects, the *AgraVg* knockdown did not affect the egg-laying ability of *A. grandis,* but hampered *A. grandis* reproduction by perturbing embryo development. We concluded that the Vg protein is essential for *A. grandis* reproduction and a good candidate to bioengineer the resistance against this devastating cotton pest.

1. Introduction

Cotton boll weevil (CBW), *Anthonomus grandis* Boheman (Coleoptera: Curculionidae), is one of the most harmful cotton pests in the Americas and object of major control efforts. This coleopteran has an endophytic larval phase that lives and feed inside cotton bolls or flower buds. The adult doesn't cause damage to cotton crops, however each female is responsible for laying about 300 eggs in cotton plants during its life (Monnerat et al., 2002). Feeding behavior of the larvae is responsible for the destruction of cotton fibers causing significant losses. Until now, the main tool to control CBW is through pesticide application in cotton fields. However, a better CBW control is urgently needed because of the high costs, human poisoning and environmental contamination (Luttrell et al., 1994; Ramalho, 1994; Ramalho and Wanderley, 1996; de Lima et al., 2013).

Biotechnological tools have a great potential to improve strategies of integrated pest management (IPM), including RNA interference (RNAi). RNAi is a process that results in decreased gene expression in eukaryotes, and its application can be mediated by double-stranded RNA (dsRNA) that induces gene-specific silencing (Fire et al., 1998; Younis et al., 2014). Applying the RNAi strategy using transgenic plants, for example, has therefore a great potential for insect control (Hannon, 2002; Hutvágner and Zamore, 2002; Price and Gatehouse, 2008; Zhao et al., 2011; Whyard, 2015; Zhang et al., 2015), and also for CBW, especially because CBW is able to process dsRNA in the RNAi pathway and could be susceptible to gene silencing using this technique, as suggested by Firmino et al. (2013).

Vitellogenin (Vg) is a phospholipoglycoprotein involved in yolk formation providing energy to oviparous vertebrates and invertebrates. In many insect species, Vg is synthesized in fat bodies, secreted into the hemolymph and transported by the circulatory system to the ovary. There, the protein is internalized into competent oocytes by receptor-

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mediated endocytosis (Raikhel and Dhadialla, 1992) and stored into the yolk. Because yolk is the major nutrient source for the embryo, vitellogenin biosynthesis and uptake are key processes for insect reproductive success (Tufail et al., 2005; Tufail and Takeda, 2008). However, the role of Vg in insects extends beyond embryo nutrition, involving also foraging, social behavior, hormonal dynamics, immune response and oxidative stress resistance (Amdam et al., 2003a; Amdam et al., 2004; Guidugli et al., 2005; Seehuus et al., 2006; Parthasarathy and Palli, 2011; Havukainen et al., 2013; Singh et al., 2013; Zhang et al., 2014). The importance of Vg in many insect species, especially in social insects, has been illustrated in several publications, mainly in foraging behavior (Wang et al., 2012; Havukainen et al., 2013; Nunes et al., 2013; Singh et al., 2013; Tokar et al., 2014; Lu et al., 2015; Salmela et al., 2015). For non-social insects, Vg knock-down via RNAi highlight its importance on egg formation in Hemiptera, Coleoptera, Lepidoptera, Orthoptera and Neuroptera (Lu et al., 2009; Tufail and Takeda, 2009; Parthasarathy and Palli, 2011; Shu et al., 2011; Tokar et al., 2014; Veerana et al., 2014; Liu et al., 2015; Lu et al., 2015; Tetlak et al., 2015). Despite the available data, the importance of Vg proteins on the refined processes of weevils reproduction is limited.

Since CBW is considered a major threat to cotton plants in which adult insects colonize host plants during early flowering, it is of great importance to block CBW attack on a large scale in the field. Therefore we have studied here *A. grandis vitellogenin*, named *AgraVg*, as a target gene and its potential to control this pest. In CBW, most studies have been concentrated on hormonal regulation of vitellogenesis (Trewitt et al., 1992; Sappington and Spurgeon, 2000; Mitchell et al., 2007; Shu et al., 2011). Trewitt et al. (1992) have reported the *AgraVg* nucleotide sequence and structure. Although Vg synthesis and uptake in boll weevil (Sappington and Spurgeon, 2000) have been previously illustrated, there is no experimental evidence until now of its relevance in this insect reproduction, especially in egg-laying and egg viability using gene knockdown by RNAi. It is known that CBW has two yolk protein subunits in the egg, which are designated as YP47 and YP160, with apparent sizes of 47 and 160 kDa, respectively (Trewitt et al., 1992). Only one gene coding for the Vg protein has been found in CBW females with a mRNA sequence of 10,017 bp (GenBank, accession number: M72980.1).

Other insect species like *Solenopsis invicta* (Hymenoptera: Formicidae) and *Spodoptera litura* (Lepidoptera: Noctuidae) have been shown to block Vg accumulation by receptor knockdown resulting in a significant decrease in egg number (Lu et al., 2009; Shu et al., 2011). Since Vg accumulation into the oocyte is mediated by the vitellogenin receptor, knocking down the receptor prevents Vg internalization, causing a comparable effect to Vg knockdown. Despite all these data, acquiring more knowledge on the precise role of Vg in insects still remains of great interest to entomologists and farmers. Considering *Vg* as a gene target, and to better evaluate the effects of its absence during CBW development, further studies are needed to allow the design of rational tools for pest control.

Here we describe the cloning of a 400 bp fragment of the CBW *Vg* mRNA, its engineering and application via RNAi. Results obtained were generated by analyzing transcription expression patterns, and the effects of transcript knockdown were monitored during female reproduction and egg development. The presented data suggest that *AgraVg* can be considered a promising target gene for the bioengineering of cotton boll weevil resistance.

2. Materials and methods

2.1. Insect rearing

The eggs, larvae and adults of CBW were obtained from the Laboratory of Bio-ecology and Insect Semiochemicals from Embrapa Genetic Resources and Biotechnology in Brasilia, Brazil. The colony was maintained on artificial diet at 28 ± 2 °C, relative humidity of 60 ± 10% and photoperiod of 12 h. The insects were reared as described by Monnerat et al. (2002). The sex determination of adults was performed by observing the external sexual organs as described by Sappington and Spurgeon (2000).

2.2. RNA isolation and qPCR

To quantify the transcript pattern of *AgraVg*, cDNA was prepared from total RNA that had been isolated from eggs, 1st, 2nd and 3rd larval instars, pupae and adults (males and females, mixed and separated) with TRIzol® reagent (Invitrogen™) according to the manufacturer's instructions. *AgraVg* expression levels were also detected by using RNA from CBW adult females from different ages (two, three and four days after emerging). Genomic DNA was removed by treating with 2 U of RNase-free DNase I (Ambion®). The extracted RNAs were subjected to RNeasy Micro Kit (Qiagen) column, and RNA integrity was confirmed by 1.5% agarose gel electrophoresis. RNA quantities were assessed with a Qubit fluorometer using a Quant-iT RNA assay (Invitrogen™), and 500 ng of total RNA was used in the steps that followed. The absence of amplicons after performing PCR with Taq-polymerase (Invitrogen™) and 1 μL of RNA solution confirmed the absence of DNA contamination in the samples. cDNA synthesis was performed by reverse-transcriptase (Superscript III™ First-Strand Synthesis SuperMix for RT-qPCR) (Invitrogen™) according to the manufacturer's instructions.

The DNA sequence of *AgraVg* (accession number: M72980.1) was retrieved from GenBank (www.ncbi.nlm.nih.gov/genbank). OligoPerfect™ Designer (Invitrogen™) was used to design quantitative real-time (RT-) PCR primers. The corresponding primer sequences are displayed in Table 1. The primers were validated with a performance dissociation protocol to detect the presence of primer dimers and the production of a single PCR product. Only a single melting peak was found for all the transcripts. Reverse transcription-qPCR assays were set up by using a 7500 Fast (Applied Biosystems) thermocycler and specific primers for each gene. Each reaction was performed in 10 μL (total volume) containing 2.5 μL of SYBR Green ROX Plus PCR Mix (LGC Biotecnologia), 2 μL of cDNA (diluted 1:40), 4.7 μL of distilled water and 0.2 μM of each primer (forward and reverse). The reaction was performed under the following sequential conditions: incubation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. After 40 cycles, a dissociation curve for each amplicon was created. Two housekeeping genes were chosen: GAPDH (GABY01019565) and beta-tubulin (GABY01011644) based on stability analysis performed by Firmino et al. (unpublished data). The transcript expression levels were determined as the number of cycles required to reach a fixed threshold in the exponential phase of PCR. The number of cycles was referred to as the Cq value (Quantification Cycle), in accordance with the RDML standards (Lefever et al., 2009). The efficiency of each primer for each reaction and Cq value were calculated individually by using the qPCR miner software (www.miner.ewindup.info) (Zhao and Fernald, 2005). The relative expression of *Vitellogenin* was calculated by using the Pfaffl method (Pfaffl, 2001), which corrects primer efficiency by using qBase plus software (Biogazelle, Belgium). Statistical differences of transcript expression were calculated using the iteration test using the Rest 2009 (Qiagen) software.

2.3. Synthesis of AgraVg dsRNA

To obtain a construct for dsRNA synthesis specific for the CBW Vg sequence, primers (Table 1) containing attB1 and attB2 sites were designed for cloning into vectors by using the Gateway® method (Invitrogen™). A selected 400 bp fragment of *Vg* was cloned from CBW cDNA into pDONR vector™ 221 using BP clonase (PCR Cloning System with Gateway® Technology Kit, Invitrogen™). Plasmids were *Eco*RV digested and sequenced. The *Vg* fragment was transferred to

Table 1
Primers used for qPCR and cloning in this study.

Target gene	Primer	Sequence (5′–3′)	Fragment size (bp)	Goal
Vitellogenin	AgraVg_qPCR_F	TCATCAAATCTATATGGCTGGTTATGAC	221	qPCR
	AgraVg_qPCR_R	GCTACAGGACTAATTGCCATAACATCAC		
GAPDH	AgraGAPDH_qPCR_F	AGATCGTCGAGGGTCTGATG	166	qPCR
	AgraGAPDH_qPCR_R	AAGGCGGGAATGACTTTACC		
β-Tubulin	AgraBtub_qPCR_F	GGTTGCGACTGTTTACAAGG	156	qPCR
	AgraBtub_qPCR_R	GCACCACCGAGTAAGTGTTC		
Vitellogenin	AgraVg_F	attB1[a] — TCATCAAATCTATATGGC	401	Cloning
	AgraVg_R	attB2[a]— ATTTGTCCCATAATTGTT		
GUS	GUS_F	attB1[a] — GAACTGAACTGGCAGACTATC	393	Cloning
	GUS_R	attB2[a] — CGGGTAGATATCACACTCTGT		

[a] attB cloning sites necessary for using the Gateway® cloning system (Life Technologies™). attB1 — GGGGACAAGTTTGTACAAAAAAGCAGGCTGG; attB2 — GGGGACCACTTTGTACAAGA AAGCTGGGTG.

the L4440gtwy vector (a gift from Guy Caldwell, Addgene plasmid # 11344) by recombination using LR clonase enzyme. The L4440gtwy vector is a modified form of the L4440 plasmid (Timmons et al., 2001), which was used for in vitro transcription.

Double-stranded RNA was synthesized by PCR and in vitro transcription as described in Clemens et al. (2000). PCR was performed by using primers with terminal 5′ T7 promoter sites, and the AgraVg_L4440gtwy construct was used as template. The PCR product was used for *in vitro* transcription in MEGAscript® T7 Transcription kit (Invitrogen™). A 393 bp fragment of β-glucoronidase (GUS) was used as a control. Primer sequences used to generate fragments for in vitro transcription are presented in Table 1. Transcription was performed according to the kit instructions, except that the reaction was allowed to proceed overnight. dsRNAs were treated with DNase, precipitated with isopropyl alcohol and eluted in DEPC-treated water.

2.4. RNAi experiments

To evaluate the effects caused by a possible knockdown of *AgraVg*, newly emerging CBW adult females (≤24 h old) were injected in the abdominal dorsal region with either 1 μL of DEPC water (mock control) or 1 μL of DEPC water containing 500 ng of dsRNA from GUS (as a non-specific dsRNA control) or 500 ng of *AgraVg* dsRNA (target-specific dsRNA). Microinjections were performed using a 10 μL Hamilton syringe. For each treatment, 42 females were injected, from which 30 were used for biological analysis and 12 collected for RNA extraction.

At 24, 48 and 72 h after microinjection, total RNA was extracted from four females for each treatment and time point. Samples were DNase-treated and subjected to qPCR to quantify *AgraVg* transcript levels as described in Section 2.2. The list of primers is given in Table 1. Three independent qPCR reactions were performed for each sample.

For biological analysis, treated females were placed with untreated males in groups of five couples per cage to allow mating for 15 days, in a total of 20 females per treatment. An artificial diet, as described by Monnerat et al. (2002), was provided as food source. The number of eggs laid by each group and the egg hatching rate were monitored daily over a period of 15 days. All the resulting data were statistically analyzed by SPSS (SPSS Inc., USA) using one-way analysis of variance (ANOVA) and means were compared with the Tukey's test ($P < 0.05$). All the experiments were independently repeated twice.

2.5. Histological evaluation of eggs laid by dsAgraVg-treated CBW females

CBW females treated with *dsAgraVg*, dsGUS and DEPC water were placed in cages together with males to allow mating and egg production. The resulting eggs were collected 72 h after oviposition, and their external morphology was analyzed by stereomicroscopy (Olympus MVX10). Images were acquired with a digital Olympus DP71 camera.

A detailed histological evaluation of eggs with at 96 h after oviposition were processed as described by Vieira et al. (2013). Briefly, the samples were fixed in 2.5% glutaraldehyde, gradually dehydrated in ethanol, and embedded in plastic resin (Technovit®, Heraeus Kulzer). Tissue sections of 5 μm were subsequently made stained with 0.05% toluidine blue and mounted in DPX (Sigma-Aldrich). Microscopic analyses were performed using bright-field optics and images were acquired with a digital camera (Axiocam, Zeiss).

3. Results

3.1. Vitellogenin is highly transcribed in CBW females

To determine whether CBW regulates *AgraVg* expression during different developmental stages, transcript amounts were quantified by qPCR for different CBW life stages. *AgraVg* expression was approximately 140 times higher in adults (mixed males and females) than in eggs, 3rd instar larvae and pupae (Fig. 1A). Interestingly, the *AgraVg* transcript expression of 1st and 2nd instars were statistically different from eggs, 3rd instar and pupae, being twice more expressed (Fig. 1A). When comparing *AgraVg* transcript expression in males and females a 900 times higher expression level was observed in females (Fig. 1B).

To precisely assess at which age *AgraVg* mRNA levels increase in females, transcript levels were also monitored from day one to three after emergence. Our qPCR data (Fig. 1C) illustrate the gradual increase from the 1st to the 3rd day after emergence with a major increase on day 3 reaching levels around 30 times higher than those found for the 1st day after emergence.

3.2. Effect of AgraVg knockdown on the number and viability of eggs

To analyze gene knockdown by qPCR, newly emerged CBW adult females were injected with *dsAgraVg* and DEPC water as a control and monitored 24, 48 and 72 h after RNAi treatment. qPCR results showed that the *AgraVg* expression levels decreased around 90% in CBW females at 24 and 48 h after *dsAgraVg* injection in comparison to the control (Fig. 2). Seventy-two hours after *dsAgraVg* treatment, *AgraVg* levels further reduced to approximately 97% when compared with controls of similar age (Fig. 2).

To assess the effects of *AgraVg* knockdown after dsRNA injection during CBW reproduction, we investigated the following three biological parameters: 1) The pre-oviposition period, to assess a possible delay in egg laying; 2) the number of eggs laid by female groups treated with *dsAgraVg* compared to controls; and 3) the viability rate of the eggs laid by these females.

The four-day period between the injection and the oviposition of the first egg did not change between the different treatments (Fig. S1). The average number of eggs laid for each group consisting of five couples over 15 days was approximately 130 for *dsAgraVg*, 150 for water and 160 for dsGUS. Statistical analysis using one-way ANOVA ($F = 0.67$; $p = 0.535$) revealed that the *AgraVg* knockdown did not affect egg production in CBW (Fig. 3A). However, viability of eggs laid by females injected with *dsAgraVg* was dramatically affected and females were not

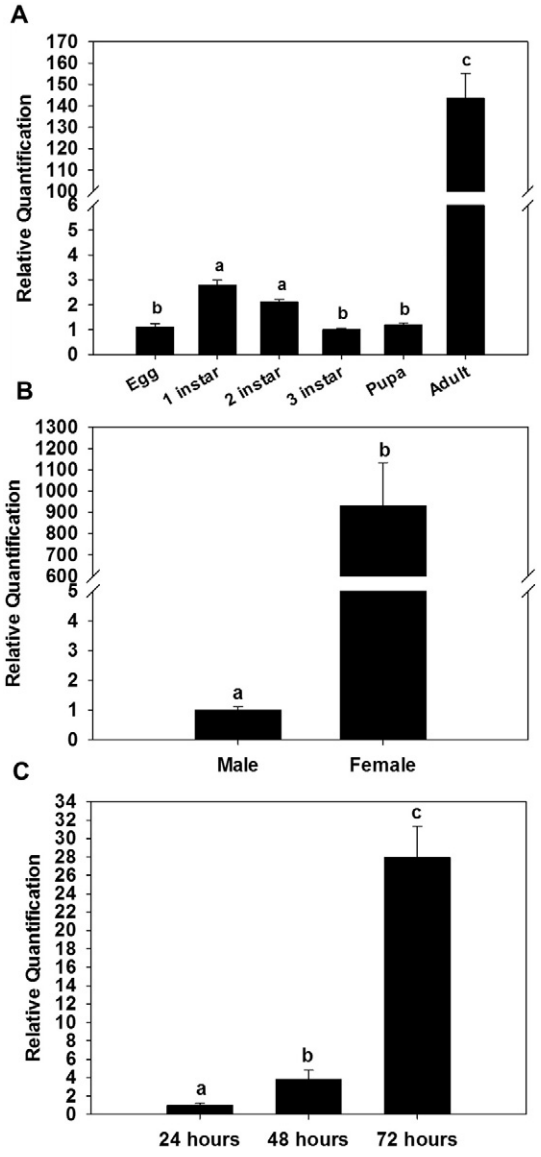

Fig. 1. Relative quantification of *Anthonomus grandis vitellogenin* (*vg*) transcripts. (A) Relative quantification of *vg* in eggs, 1st, 2nd and 3rd larval instars, pupae and adults (males + females) of *A. grandis*. (B) Relative quantification of *Vg* in males and females of *A. grandis*. (C) Relative quantification of *vg* in *A. grandis* females 1, 2 and 3 days-old. Bars are means ± SE (standard error) of two biological replicates. Each of these replicates was assayed in three times. Different letter means statistical difference. The analysis was performed using iteration test (REST 2009 Software).

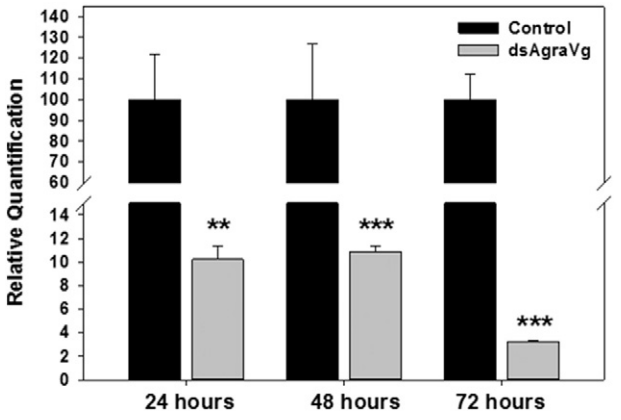

Fig. 2. Relative quantification of *Anthonomus grandis vitellogenin* (*vg*) transcripts in females 24, 48 and 72 h after microinjection. Bars are means ± SE (standard error) of two biological replicates. Each of these replicates was assayed three times. Control: treatment (microinjection with water); *dsAgraVg*: microinjection of 500 ng of dsVg. Statistical analysis was performed using iteration test (REST 2009 Software). ** ($p \leq 0.01$), *** ($p \leq 0.001$).

able to generate viable progeny (Fig. 3B). Egg viability rate in controls using water and dsGUS was between 63% and 65%, respectively. In contrast, the percentage of hatched eggs during *dsAgraVg* treatment was <1% ($F = 94.91$; $p < 0.0001$) (Fig. 3B).

3.3. Eggs laid by dsAgraVg-treated CBW females show arrests at different developmental stages

Since, *AgraVg* knockdown affected egg viability, we evaluated the egg morphology by performing a microscopic histological analysis of the embryo development. External morphological features were analyzed by sampling around ten eggs randomly chosen for each treatment. The images revealed that eggs resulting from control treatments showed normal embryonic development 72 h post-oviposition (Fig. 4A and B). Markers like normal germ-band broadening and curvature toward the dorsal side indicate normal development. At 72 h post-oviposition, 100% of eggs resulting from the *dsAgraVg* treatment appeared different from control eggs (Fig. 4C and D). We observed a reduction in the amount of tissue within the egg and the appearance of transparent zones devoid of cells. In contrast, control eggs were filled with larval tissue showing a clear segmentation pattern.

Due to insect oviposition and fertilization pattern, it is normal to find unfertilized eggs in nature. In our experiments, 96 h after oviposition all viable eggs had already hatched, and the unfertilized eggs were collected and analyzed. Twenty eggs were imaged for the control treatments (H_2O and dsGUS-treated). These eggs were filled with a fatty compound, probably vitellin, without showing any sign of a developing embryo (Fig. 5A). For the *dsAgraVg*-treated females we imaged 30 eggs. All of them were unviable and showed different phenotypes that were sorted in three groups. The first group (40% of total eggs) was characterized by a blockage in embryo development at early stages, severe tissue disorganization, and cell clustering in the central part of the egg (Fig. 5B). The second group (14% of the total eggs) includes embryos blocked in a more advanced developmental stage where the developing germ band can be observed (Fig. 5C). In the third group (33% of total eggs), more evolved structures such as mouthparts were already clearly visible (Fig. 5D and E). The embryo illustrated in Fig. 5E shows an irregular segmentation primarily at the anterior end of the body. Thirteen percent of the total imaged eggs were non-fertilized.

4. Discussion

Vitellogenin is known to be an important protein for embryonic development for a wide range of insect species (Nation, 2008; Singh et al., 2013; Amsalem et al., 2014; Ren and Hughes, 2014; Zhang et al., 2014), but Vg is also involved in other aspects of reproduction such as terminal follicular growth, stimulation of follicular epithelium differentiation and competent oocyte development via protein internalization (Raikhel and Dhadialla, 1992). Here, we present for the first time data that support the potential use of *AgraVg* gene knockdown as a tool for *A. grandis* control, demonstrating the molecular and histological effects when decreasing *vitellogenin* transcripts in this insect pest and its progeny.

Transcription analysis of different developmental stages in *A. grandis* demonstrated a clear *AgraVg* expression mostly in females (Fig. 1B). This finding is not unexpected since females are responsible for yolk transfer to developing oocytes (Tufail and Takeda, 2008). A gradual increase in *AgraVg* expression could be observed in CBW females during the first four days after emergence (Fig. 1C). This phase corresponds to the beginning of CBW vitellogenesis, as the insect presented a pre-oviposition period of four days (Fig. S1). Similar results have been

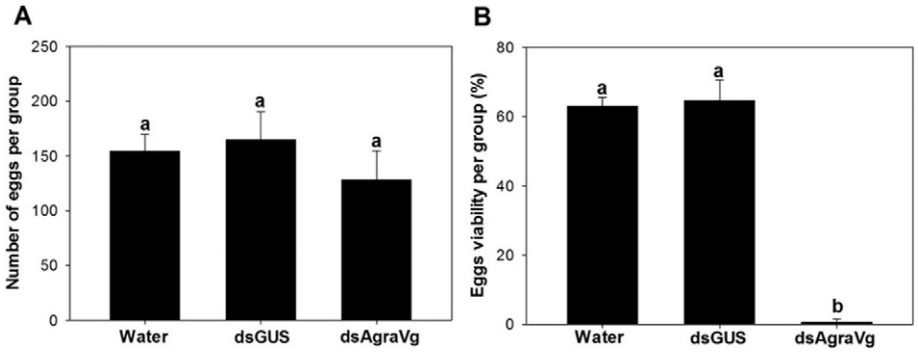

Fig. 3. Effect of *vitellogenin* knockdown on *Anthonomus grandis* reproduction. (A) Number of eggs per group of five *A. grandis* couples (four groups per treatment) during 15 days. (B) Percentage of eggs viability per group of five *A. grandis* couples for 15 days. Mean ± SE of three experiments are shown. Different letters represent statistical difference with Analysis of Variance (ANOVA) and 5% probability by Tukey test.

reported for *Apis mellifera* (Hymenoptera) (Seehuus et al., 2007) and *Nilaparvata lugens* (Hemiptera) (Tufail et al., 2010). Surprisingly, low levels of *AgraVg* transcripts were detected in eggs, larval and pupal stages. Other work did not mention the presence of either *AgraVg* transcripts or proteins in other stages than adult female in *A. grandis* (Taub-Montemayor et al., 1997a; Taub-Montemayor et al., 1997b; Taub-Montemayor and Rankin, 1997). Only very low transcript amounts were detected in comparison to adult females, representing maybe just basal transcription (Fig. 1A). Until now, no role for vitellogenin at non-reproductive stages was reported for non-social insects.

The specific knockdown of *AgraVg* as a consequence of dsRNA treatment was confirmed by qPCR, revealing a remarkable decrease in *AgraVg* expression in CBW females (Fig. 2). This finding suggests that the normal increase of AgraVg after 72 h is blocked by *dsAgraVg* treatment. The first *vitellogenin* RNAi study in insects was performed in honeybees, but knockdown was only confirmed by Western and Northern blots (Amdam et al., 2003b). Antonio et al. (2008) describing the successful knockdown of the *vitellogenin* gene in honeybees with a 25-fold reduction of target gene transcripts compared to controls. The *AgraVg* knockdown results described in this manuscript showed similar efficiency.

Moreover, we noticed that *AgraVg* knockdown also resulted in a striking reduction in egg viability, without influencing the pre-oviposition period or the amount of laid eggs (Figs. 3A and S1). Differently, in *Chrysopa septempunctata* (Neuroptera: Chrysopidae) and *Nilapavarta lugens* (Hemiptera: Delphacidae) *Vg* knockdown resulted in less eggs and a low egg-hatching ratio (Liu et al., 2015; Lu et al.,

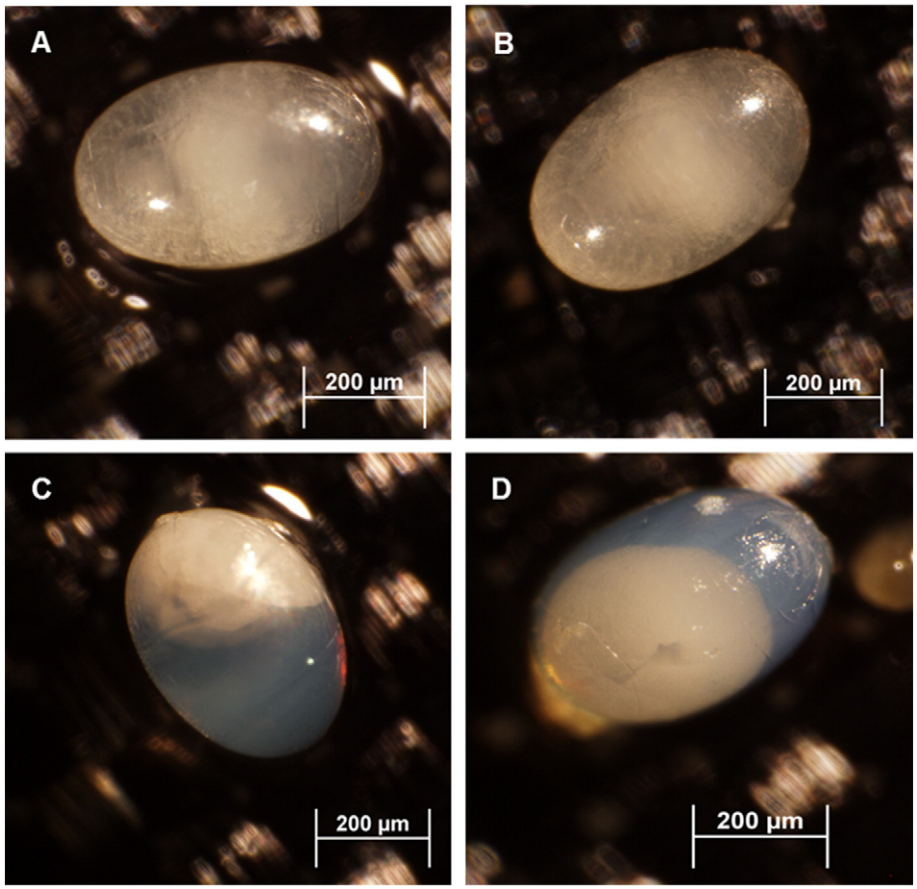

Fig. 4. External morphology of *Anthonomus grandis* eggs laid by females treated with DEPC water (A), 500 ng of dsGUS (B) and 500 ng of *dsAgraVg* (C and D).

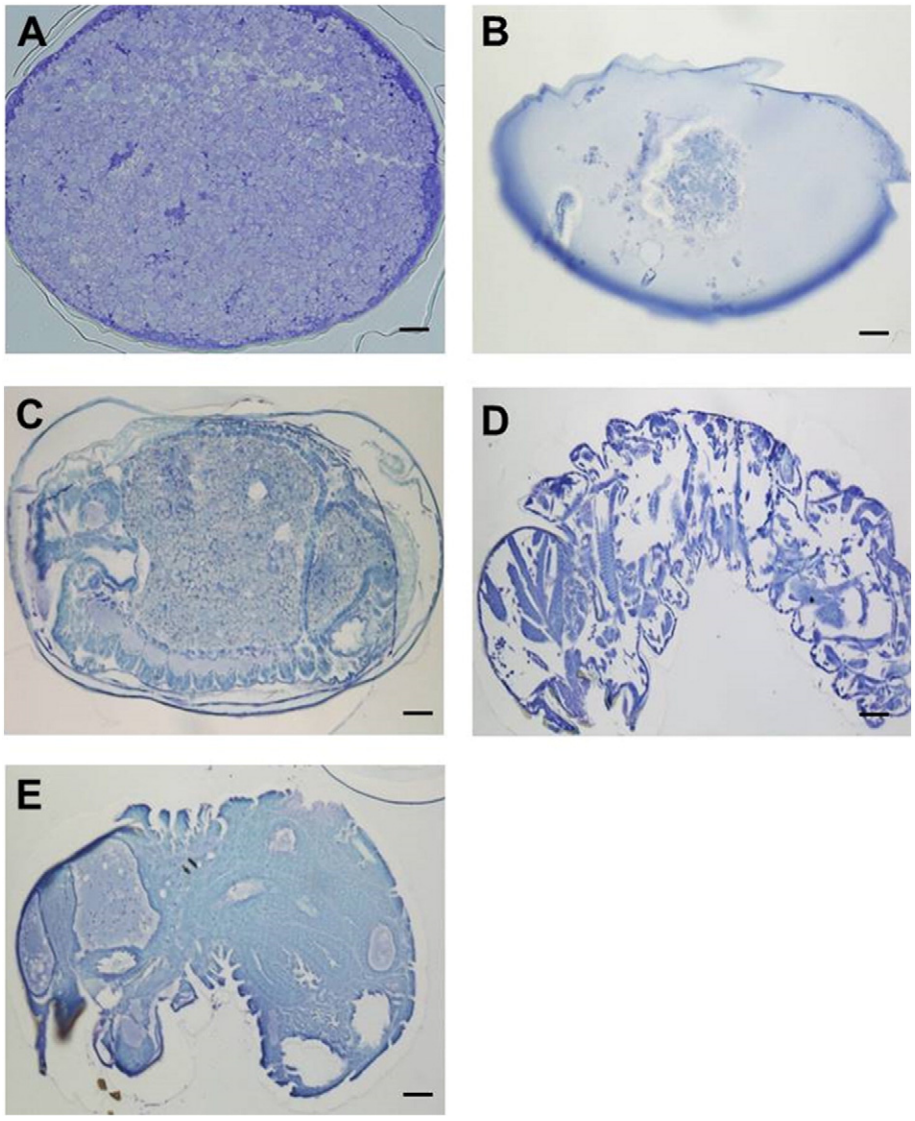

Fig. 5. *Vitellogenin* knockdown significantly affects the embryonic development in *Anthonomus grandis*. Histological analysis of 96 h-old eggs. A) Unfertilized egg laid by females from control treatment. B) Eggs from females treated with *dsAgraVg* with developing embryos blocked at early embryonic stage; C) Eggs from females treated with dsAgraVg with a more advanced embryonic development, showing a developing germ band. D–E) Eggs from females treated with *dsAgraVg* at very advanced stages showing complete larvae formation, but with malformation. Overall staining performed with toluidine blue for morphological observations. Bar: 50 μm.

2015). RNAi studies of other genes involving insect reproduction, such as the juvenile hormone acid *O*-methyltransferase (HJAMT), Methoprene tolerant (Met) and Kruppel (Kr-h1), resulted in a 90% reduction in egg production in *Tribolium castaneum*. However, knockdown of the ecdysone receptor (EcR) and ultraspiracle (USP) genes of *T. castaneum* provoked a delay in ovary, ovarioles and oocytes maturation as well as an arrest in egg production (Parthasarathy et al., 2010a; Parthasarathy et al., 2010b). Knockdown of the *vitellogenin receptor* (*VgRc*) in *S. litura* (Lepidoptera: Noctuidae) (Shu et al., 2011) and *Dermacentor variabilis* (Acari) (Mitchell et al., 2007) have also been reported. Similarly as we found in CBW, in *S. litura*, decreasing *VgRc* transcripts via dsRNA treatment of freshly emerged adult females did not significantly change oviposition rates (Shu et al., 2011). However, hatching was not evaluated, and therefore, reproductive success could not be assessed (Shu et al., 2011). In the Acari *D. variabilis*, dsRNA treatment resulted in no egg-laying and an absence of Vg accumulation in the ovaries (Mitchell et al., 2007). Only one single study reported a one-day delay in the pre-oviposition of *Blattella germanica* (Blattodea) when the lipophorin gene was knocked down. The lipophorin protein is involved in the transport of lipids between different tissues and also acts during vitellogenesis (Ciudad et al., 2007).

Histological analysis of CBW eggs clearly showed that Vg knockdown leads to a range of phenotypes, suggesting multiple responses to *dsAgraVg* treatment (Fig. 5). These variations could be explained by a dose effect caused by the amounts of injected *dsAgraVg* in females. Variations reported in the literature were most likely associated to the different methodologies of dsRNA delivery (feeding or microinjection) or the target species (Terenius et al., 2011).

We hypothesize four explanations for the range of phenotypes encountered in our study: (1) As shown in Fig. 2, the efficiency of the *AgraVg* knockdown increased over time, based on the suppression of *AgraVg* accumulation in four-day-old females (Figs.. 2 and 1C). Therefore, early-produced eggs may have accumulated more vitellin, being still not sufficient to allow hatching. This sequence of events could have assured a more advanced embryonic development. (2) The efficiency of the RNAi machinery in each individual female insect may be slightly different because of intra-species genetic variability. (3) Different phenotypes could have resulted from variable body weights and

consequently different amounts of hemolymph, leading to variable final dsRNA concentrations. This variation could affect the amount of protein accumulated in the oocytes, allowing some embryos to proceed embryonic development. (4) *AgraVg* knockdown might interfere with the expression of other genes involved in embryonic development. It is known that embryo development requires highly synchronized gene expression (Klowden, 2007), and variable *Vg* levels caused by the knockdown could negatively affect accurate synchronization.

Concerning the importance of yolk protein for embryo growth, Nordin et al. (1990) reported that improper Vg processing in *B. germanica* affect embryo development. They have shown that there is a functional relation between the size of the Vg polypeptide that is processed and the presence of protease activity in the egg sacs that are laid (Nordin et al., 1990). It has been proposed that Vg-programmed proteolysis during embryogenesis in insects may release maternal ecdysteroids with hormonal activity to initiate certain embryonic events (Lagueux et al., 1984; Bownes et al., 1988).

The use of RNAi to control coleopteran pest was firstly tested by Baum et al. (2007). dsRNA was offered by feeding to insects like *Diabrotica virgifera virgifera*, *Diabrotica undecimpunctata* howardii, *Leptinotarsa descemlineata* and *A. grandis*. Gene knockdown caused lethality in the first three species, but not in *A. grandis*. For the latter, larvae may only use a restricted portion of the diet as food source, or species-specific intestinal nucleases may degrade dsRNA before knockdown (Arimatsu et al., 2007; Liu et al., 2012). However, several improvements on dsRNA delivery and uptake methods have been established making this technique more generally applicable in different fields. For example, the expression of long dsRNA in plastids has been tested with success. (Jin et al., 2015; Whyard, 2015; Zhang et al., 2015). Other strategies foresee the possibility to stabilize and protect dsRNA molecules with nanoparticles (Zhang et al., 2010) to prevent dsRNA degradation until it is uptake by midgut insect cells.

In summary, this study is the first to show that altering *AgraVg* expression in CBW can be done with success using the strategy of dsRNA for gene knockdown. Our work demonstrates that *A. grandis* Vitellogenin protein is an important player in the insect's reproductive performance, since gene knockdown leads to females that lay eggs that do not hatch. A range of embryo phenotypes were observed in females subjected to *Vg* knockdown suggesting that the amount of gene silencing may differ between individuals, or that the microinjection procedure used here does not allow for the precise delivery of equal amounts of dsRNA in each insect. Clearly, other unknown experimental variables maybe involved.

Based on the presented data, we believe to provide solid evidence that the RNAi strategy applied to the *Vg* gene might be a promising and safe alternative for the genetic control of CBW. Indeed, CBW has only one *Vg* gene (Trewitt et al., 1992), which avoids possible interference due to functional redundancy. To diversify pest management, the approach proposed here should be tested on transgenic cotton plants or, as an alternative to OGMs, using nano-compounds carrying *AgraVg* dsRNA to be sprayed on cotton plants.

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