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Dynamics of expression of two vitellogenin genes in the Chagas' disease vector *Triatoma infestans*: Analysis throughout pre-vitellogenesis and vitellogenesis

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ABSTRACT

The reproductive success of all oviparous species depends on vitellogenin (Vg) biosynthesis and its accumulation in the developing oocytes. The expression levels of two *Vg* genes (*Vg1* and *Vg2*) were analyzed by qPCR and western blot in fat body and ovaries of adult females, at different times after ecdysis (pre-vitellogenic phase) and after blood feeding of females (vitellogenic phase). *Vg* genes were also evaluated in fat bodies of adult males as well as in female fifth instar nymphs. No trace of *Vg* mRNA was detected in adult males or in nymphs. *Vg1* and *Vg2* were expressed in the fat bodies and ovaries of adult females. The *Vg* genes start to be expressed slightly in both tissues of adult females during pre-vitellogenesis. 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. During this period however, the distribution patterns of *Vg1* and *Vg2* transcripts showed two peaks around early and advanced vitellogenesis (days 4 and 12 post-feeding, respectively). In the ovaries, levels of mRNAs increased from the day 10 post-blood feeding onwards. In addition, the immunofluorescence assays showed a strong signal for vitellin in the yolk bodies of terminal follicles of vitellogenic females. The involvement of fat body and ovary in the synthesis of Vg suggests different roles of Vgs in supporting the growth of oocytes.

1. Introduction

Chagas' disease, also known as American trypanosomiasis, is caused by the parasite *Trypanosoma cruzi*, which is transmitted to humans by vectors of the subfamily Triatominae (Hemiptera: Reduviidae). The disease and its vectors are extensively distributed from Southern United States of America to Southern Argentina and Chile (latitude 42°N to latitude 46°S). Currently, Chagas' disease is the fourth cause of economic losses through illness in Latin America, where about 8 million people are estimated to be infected with *T. cruzi* and more than 25 million people are at risk of contracting the infection (Rassi et al., 2010; World Health Organization, 2014).

All postembryonic stages of triatomine insects are hematophagous and at least one full blood meal is necessary for each molt. Therefore, these species may acquire the infection from their first blood meal (Lent and Wygodzinsky, 1979).

Triatoma infestans, one of the most important and widespread vectors of Chagas' disease in South America, has been the target of control programs as part of the Southern Cone Initiative (Moncayo and Silveira, 2009). However, the goals of current vector control policies are compromised by several factors including the abundance of other vector species and the extension of endemic areas, which hampers regularity in entomological surveillance (Tarleton et al., 2014). In addition, resistance to pyrethroid insecticides has been reported as a fact that renders difficult vector control strategies (Picollo et al., 2005; Lardeux et al., 2010; Gurevitz et al., 2012).

In insects, the vitellogenesis process is fundamental for egg development and therefore is a central event for their reproduction (Raikhel, 2005). During vitellogenesis, yolk protein

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precursors are produced in large amounts by extraovarian tissues, being vitellogenin (Vg) the main precursor protein of egg yolk. The fat body, a tissue hormonally regulated and functionally analogous to the vertebrate liver and adipose tissue, is the exclusive site of Vg synthesis in the majority of the insects, while in others, ovaries also synthesize Vgs (Melo et al., 2000; Giorgi et al., 2005; Bellés, 1998, 2005). The number of genes encoding insect Vgs varies from one to several in different species but in most insects, Vgs derive from a single *Vg* gene transcript of 6–7 kb (Tufail et al., 2014). Insect Vgs are large phospholipoglycoproteins of ~200 kDa with a remarkable degree of conservation in amino acid composition (Hughes, 2010). Vg synthesized in the fat body is secreted into the hemolymph and it is taken up by the developing oocytes by receptor-mediated endocytosis (Snigirevskaya and Raikhel, 2005; Tufail and Takeda, 2009). Following uptake by oocytes, Vgs are stored in specialized organelles or yolk bodies as vitellin (Vn). Vns provide raw materials for development of the embryos (Kunkel and Nordin, 1985). Although Vg is generally considered a female-specific protein, some male insects synthesize small amounts of Vg (Engelmann, 1979; Trenczek and Engels, 1986; Valle, 1993; Piulachs et al., 2003).

Hematophagous insects require a blood meal to activate numerous genes essential for digestion of the blood, synthesis of yolk protein precursors and ultimately, production of eggs (Raikhel, 2005). In the case of the vectors of Chagas' disease, the requirement of a blood meal is a key event that triggers the action of juvenile hormone for the initiation of vitellogenesis (Davey, 1997). Therefore, in these species, each gonotrophic cycle and egg development is coupled with the intake of blood (Friend et al., 1965; Stoka et al., 1987; Aguirre et al., 2008). However, autogeny, the capacity of an unfed adult female to develop eggs using blood ingested in the last nymphal stage, has been observed in some triatomines such as *Rhodnius prolixus* and *T. infestans* (Stoka et al., 1987; Noriega, 1992).

At present, great progress has been made in elucidating the nutritional control of vitellogenesis in anautogenous mosquitoes, particularly in *Aedes aegypti* (Attardo et al., 2005). On the contrary, in triatomines, the studies that focused on molecular mechanisms of blood meal regulation in the expression of essential genes for vitellogenesis are still scarce. Understanding the relationship between a blood requirement for the activation of *Vg* genes and egg development is of critical relevance in the biology of reproduction of triatomines. It is also an important way in the search of new tools for insect vector control. Recently, we have identified two Vg genes, *Vg1* and *Vg2*, in females of *T. infestans* (Blariza et al., 2014). The *Vg1* and *Vg2* mRNAs were found in fat bodies and ovaries of adult females. Expression levels of *Vg2* in the heads and thoracic muscles were low, while the *Vg1* transcript could not be detected in these two organs.

In order to better understand the molecular basis of blood regulation on vitellogenesis in Chagas' disease vectors, we have analyzed the expression of *Vg1* and *Vg2* genes at transcriptional and translational level in fat bodies and ovaries of anautogenous female adults of *T. infestans* at different times of pre-vitellogenic phase (unfed period post-ecdysis) as well as during the vitellogenic phase triggered after blood feeding. Additionally, transcription levels of Vgs were investigated in fat bodies of fifth-instar female nymphs and in adult males.

2. Materials and methods

2.1. Insects and tissue sampling

T. infestans was reared at 28 ± 1 °C at a relative humidity of 60–70% with a 6-h light/18-h dark cycle and fed once every two weeks on restrained chickens. Anautogenous females were obtained under our parameters of rearing. Fifth instar male and female nymphs were sexed by the differences described by Espinola (1966) and grouped separately. Seven days after ecdysis, the insects were fed and females were placed together with males in individual containers (each couple in one container). Mating was controlled by the deposited spermatophores.

Under our standardized laboratory rearing conditions, the period between eclosion to the adult stage and the first adult blood meal (pre-vitellogenic phase) is for *T. infestans* of approximately 7 days, the oviposition takes place between days 13–15 after blood feeding and is extended approximately until the days 22–23 post-blood meal (vitellogenic phase). The onset of the post-vitellogenic period was detected with the interruption of the oviposition. Afterwards, like in other triatomines, for a second batch of eggs a new intake of blood meal was needed (Stoka et al., 1987; Aguirre et al., 2011).

The expression analysis of two *Vg* genes previously identified, *Vg1* (GeneBank accession number KF915268) and *Vg2* (GeneBank accession number KF915267) (Blariza et al., 2014), was carried out in fat bodies of adult females at days 4 and 6 after ecdysis (unfed period) and at days 1–14, 17, 20, and 24 after blood feeding. Aditionally, ovaries from adult females during pre-vitellogenic (days 4 and 6) and vitellogenic (days 1–14 and 17) phases were also sampled. At the time of advanced vitellogenesis, fully developed oocytes free of outer sheaths were removed from terminal follicles before tissue processing. Studies were also performed in fat bodies sampled from fifth-instar females sampled from the day 5 to 15 after blood intake as well as from adult males throughout post-ecdysis (days 4 and 6) and post-feeding (days 1–14, 17, 20, and 24) periods.

For RNA extraction each sample was a pool of tissues from three adult specimens and five fifth instar nymphs. Fat bodies and ovaries were dissected under aseptic conditions and stored in liquid nitrogen until used for RNA extraction. For western blot assays, fat bodies and ovaries from five adult females were dissected under cold phosphate buffered saline (PBS, 6.6 mM Na_2HPO_4/KH_2PO_4, 150 mM NaCl, pH 7.4), pooled and homogenized as described elsewhere (Aguirre et al., 2008). Protein concentration was determined according to Bradford (1976), using bovine serum albumin (BSA) as standard.

2.2. Isolation of total RNA and cDNA synthesis

Total RNA was isolated from pools of insect tissues using MasterPure RNA Purification Kit (Epicentre, Madison, WI, USA) following the manufacturer's protocol and eluted in 33 μl of nuclease-free water. This kit includes a DNase treatment in order to eliminate potential genomic DNA contamination. The RNA concentration was determined by absorption at 260 nm.

First-strand cDNA synthesis was performed with 1 μl of Oligo-dT_{20} (50 μM) (Invitrogen, Carlsbad, CA, USA), 1 μg of total RNA, and 400 U of SuperScript III RT (reverse transcriptase, Invitrogen) in a 20 μl reaction volume incubated at 55 °C for 1 h.

2.3. Quantitative PCR (qPCR)

In order to determine the patterns of yolk protein gene expression, the transcript levels of *Vg1* and *Vg2* genes in different tissues, sexes, and development stages of *T. infestans* were measured by qPCR. Gene specific primers (Table 1) and Taqman probes were designed according to the corresponding cDNAs using the Primer Express program (Applied Biosystems, Foster City, CA, USA). Reverse transcription polymerase chain reaction (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 (71 bp). The PCR products corresponding to *Vg1*, *Vg2*, and β-*actin* were cloned into the pCR4-TOPO TA cloning vector (Invitrogen) and

Table 1
Sequences of the primers used for real time PCR.

Name	Sequences	Function
QAF5	5′-TATAATCAAGGATCCCTTAGAATTTGC-3′	*Vg-1*, forward
QAR5	5′-TTGGCTGGTCCTTCACAAGTT-3′	*Vg-1*, reverse
QAF2	5′-CGCATGAGAAGCTCTCTAACCA-3′	*Vg-2*, forward
QAR2	5′-ACGCGATGATTAGTGCATCCT-3′	*Vg-2*, reverse
QβactinaF	5′-CCCCTTTCAGTGAGGATCTTCA-3′	Internal control
QβactinaR	5′-CGCCATCCTTCGATTGGA-3′	Internal control

sequenced to confirm the identity of the amplified products. Taken into account the results of RT-PCR, relative qPCR was performed to further investigate changes in *Vg* expression. Quantitative PCR was carried out using a Mx3005P qPCR System with Brilliant qPCR Core Reagent Kit (STRATAGENE, La Jolla, CA, USA). We identified 58 °C as an annealing temperature appropriate for all primer sets, which allowed us to analyze three genes in one real-time PCR plate. The reaction conditions were 40 cycles of 10 min at 95 °C, 15 s at 95 °C, and 60 s at 58 °C. The relative copy number of *Vg* mRNA was calculated according to $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001). The threshold cycle value difference $^{\Delta}$CT between *Vg* mRNA and β-*actin* RNA of each reaction was used to normalize the level of total RNA.

2.4. Western blot

Western blot assays were carried out according to Aguirre et al. (2008), employing a rabbit polyclonal anti-vitellin antibody (gamma-globulin fraction) raised against the main apoprotein subunits of Vn (Mr ~170 and 174 kDa) from the related triatomine species *Dipetalogaster maxima*. Obtention of polyclonal antibody was achieved using purified Vn by ion exchange chromatography followed by separation of its apoprotein subunits by polyacrylamide gel electrophoresis (SDS-PAGE) (Aguirre et al., 2008). In preliminary assays the rabbit polyclonal anti-vitellin antibody from *D. maxima* cross-reacted with Vg/Vn from *T. infestans*. Proteins from fat bodies sampled at days 3, 8, and 10 after ecdysis and at days 3, 7, 10, 14, 17, 20, and 22 after blood feeding, as well as from ovaries sampled at days 3, 8, and 10 post-ecdysis and at days 3, 6, and 10 pots-feeding, were separated in 7.5% SDS-PAGE and transferred onto a nitrocellulose membrane under standard conditions (Towbin et al., 1979). After rinsing the membrane with Tris buffered saline (TBS, 10 mM Tris–HCl, 150 mM NaCl, pH 7.5), blocking steps were performed with TBS containing 0.1% Tween 20 and 5% non-fat milk under gentle agitation at room temperature. Incubations with primary and secondary antibodies were performed for 1 h each at room temperature as follows: (a) anti-vitellin antibody (anti-Vn antibody, 1:2000) in TBS containing 0.1% Tween 20 and 5% non-fat milk and (b) horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:2000) in the same medium. After three washes with TBS, the ECL detection kit (PerkinElmer, Norwalk, CT, USA) was used according to the supplier's protocol to identify immunoreactive bands by enhanced chemiluminescence.

2.5. Indirect immunofluorescence assays

The ovaries of females at pre-vitellogenesis (7 days after ecdysis) and vitellogenesis (4 days after blood meal) were fixed in 4% paraformaldehyde in PBS. Thereafter, the organs were transferred into sucrose/PBS, embedded in OCT and snap frozen in liquid nitrogen (Fruttero et al., 2011). Tissue sections of 9 μm were obtained with a Leica CM1510 cryostat (Leica Microsystems, Wetzlar, Germany) and placed onto poly-L-lysine-treated glass slides. The cryostat tissue sections were blocked by 1% BSA, permeabilized by 5% fetal bovine serum and 0.1% Triton X-100 in PBS for 60 min and incubated for 1 h with the anti-Vn antibody in PBS containing 1% BSA (1:100) followed by the anti-rabbit IgG labeled with Alexa 568 (1:500, Molecular Probes, Eugene, OR, USA) in the same medium for 1 h. All incubations were carried out in humid chamber at 37 °C and washed three times for 5 min with PBS after each step. Finally, the slides were rinsed with distilled water, air-dried, mounted in Fluorsave (Calbiochem, Darmstadt, Germany) and observed with an Eclipse TE2000-U microscope (Nikon, Tokyo, Japan), equipped with appropriate filters for red fluorescence (excitation 540–580 nm/emission 600–660 nm). Digital images were captured with a Nikon Digital Sight DS-U1 camera and processed with ACT-2U version 1.51.116.256 software (Nikon).

2.6. Statistical analyses

For the studies involving gene expression analysis by qPCR, two independent experiments were performed and data for each point were registered by triplicate to account for intra-experimental variation. Graphs and statistical tests were performed using GraphPad Prism version 5.00 for Windows (GraphPad software, San Diego, CA, USA). One and two-way ANOVA with Bonferroni post test were used for comparisons. The results were presented as mean ± Standard Deviation (SD) and a P value < 0.05 was considered statistically significant.

3. Results

3.1. Transcriptional expression of Vg1 and Vg2 genes in fat bodies and ovarian tissues

qPCR assays were performed to quantify *Vg* gene expression in females of *T. infestans* at different intervals of the pre-vitellogenic and vitellogenic phases, as well as in female fifth-instar nymphs at different days after blood meal and in adult males at different time points post-ecdysis and post-feeding. Neither *Vg1* nor *Vg2* were expressed in fifth-instar nymph or adult male fat bodies.

In the fat body and ovaries of adult females of *T. infestans*, transcriptional patterns of expression of *Vg1* and *Vg2* genes differed (Figs. 1 and 2). In the fat body, the *Vg1* and *Vg2* genes were expressed at relatively low levels during pre-vitellogenesis. In this tissue however, both *Vg* transcripts were up regulated after blood feeding and their levels raised soon on the first day of vitellogenic phase (Fig. 1). Thereafter, the mRNA levels increased progressively until they peaked on day 4 and then they decreased between days 5 and 8, being the levels at day 8 within the range of those detected at the first day post-blood feeding. Then, *Vg1* and *Vg2* mRNA levels increased again until a maximum at day 12 post-feeding. After that, the transcription rate of both genes declined slowly. The *Vg1* transcript level on day 12 was statistically significantly higher than at the other days ($P < 0.001$). Significantly higher levels of *Vg1* gene expression were also registered at days 4 and 11 post-blood meal in comparison to nearly all other days ($P < 0.05$). On the other hand, the expression of *Vg2* gene was significant at days 10, 11 and 12 after feeding (Fig. 1), but only the expression on day 12 was significant with respect to all the days analyzed. The *Vg1* transcript level was significantly higher than *Vg2* at days 4 and 12 after the blood meal ($P < 0.001$) (Fig. 1).

In ovarian tissues *Vg1* and *Vg2* genes were slightly expressed during pre-vitellogenesis and the initial 9 days of vitellogenesis. Afterwards, the expression levels strongly increased, close to the first oviposition at 13–15 days after feeding (Fig. 2). The *Vg1* mRNA level was significantly high on day 12 of the vitellogenic phase compared with all the other time points. Also, on day 13 it remained elevated in comparison with most of the days analyzed. As shown in Fig. 2, *Vg2* transcript levels were high at days 11, 12, 13, and

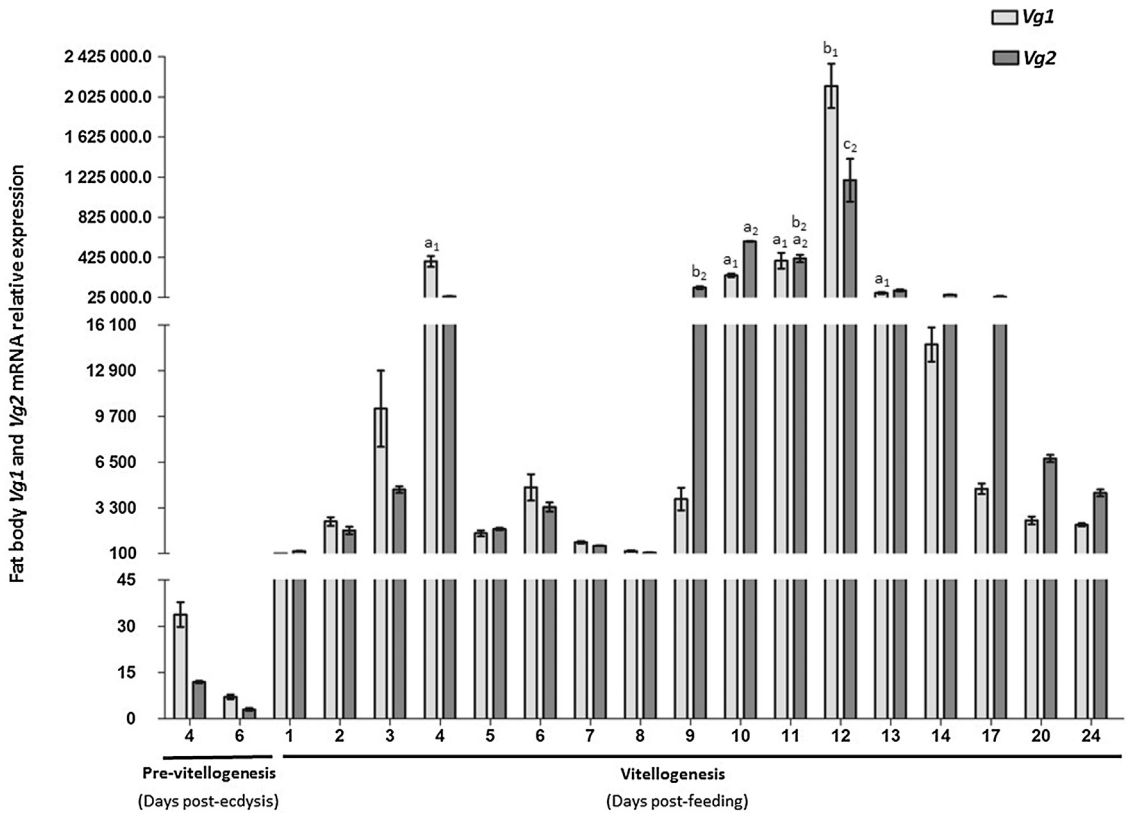

Fig. 1. Relative vitellogenin expression (mRNA) of *Vg1* and *Vg2* in the fat body of *Triatoma infestans* females at different times after ecdysis (unfed period, pre-vitellogenic phase) and during the first 24 days after a blood meal (vitellogenic phase). The error bars represent the standard deviation of the mean. The same letter and subscript indicate no significant difference. *Vg1* presented significant level of expression at day 12 (b_1) of the vitellogenic phase with respect to all the days analyzed ($P < 0.001$). The *Vg1* expression at days 4 and 11 (a_1) after a blood meal was significant relative to the days 4 and 6 post-ecdysis, and *vs.* days 1–3, 5–9, 14, 17, 20, and 24 post-feeding ($P < 0.05$). The *Vg2* mRNA level was significant at day 12 (c_2) of vitellogenic phase with regard to all the days analyzed ($P < 0.001$). The *Vg2* expression at day 10 (a_2) after blood intake was significant *vs.* days 4 and 6 post-ecdysis, and *vs.* days 1–9, 13–14, 17, 20, and 24 post-feeding ($P < 0.001$). *Vg2* mRNA level was also significant on day 11 (a_2–b_2) *vs.* days 4 and 6 post-ecdysis, and compared to the days 1–8, 17, 20, 24 ($P < 0.01$), and 13–14 ($P < 0.05$) after blood meal.

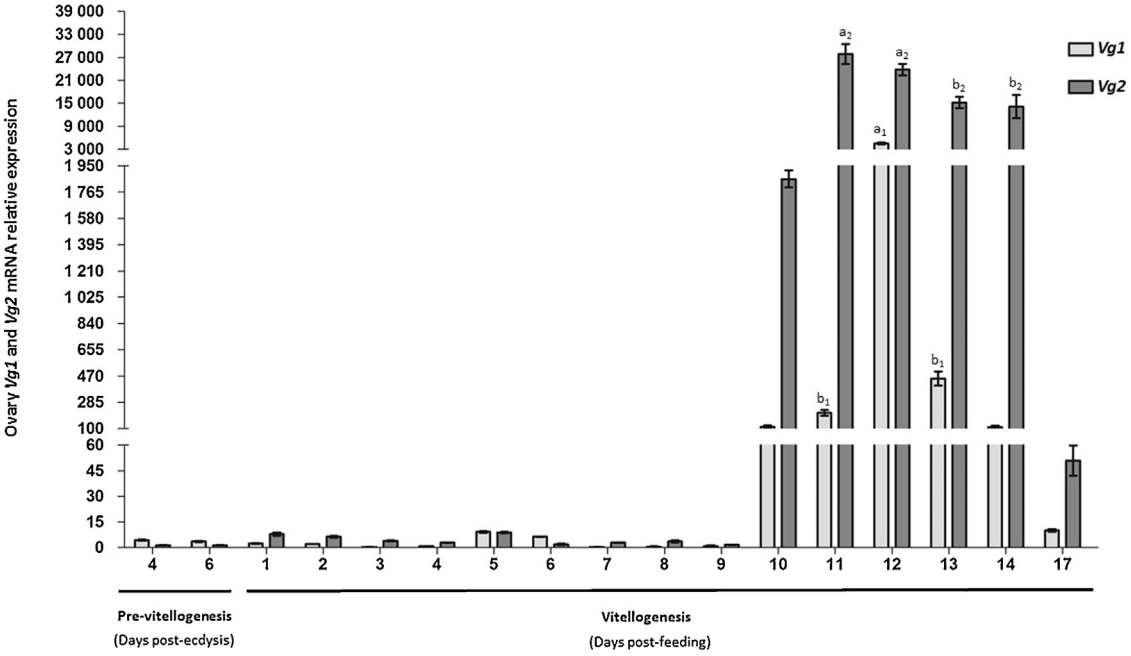

Fig. 2. Relative vitellogenin expression (mRNA) of *Vg1* and *Vg2* in the ovary of *Triatoma infestans* females at different times after ecdysis (unfed period, pre-vitellogenic phase) and during the first 17 days after a blod meal (vitellogenic phase). The error bars represent the standard deviation of the mean. The same letter and subscript indicate no significant difference. The *Vg1* mRNA level was significant on day 12 (a_1) post-blood meal compared with the rest of the days analyzed ($P < 0.001$) and on day 13 (b_1) *vs.* days 4 and 6 post-ecdysis, and *vs.* to the days 1–9, 17 ($P < 0.001$), 10 and 14 ($P < 0.05$) post-feeding. The *Vg2* transcript level was high and significant at days 11 and 12 (a_2) of the vitellogenic phase with regard to all the days analyzed ($P < 0.001$) except between themselves, and at days 13 and 14 (b_2) *vs.* days 4 and 6 post-ecdysis, and *vs.* days 1–10 and 17 after blood feeding ($P < 0.001$).

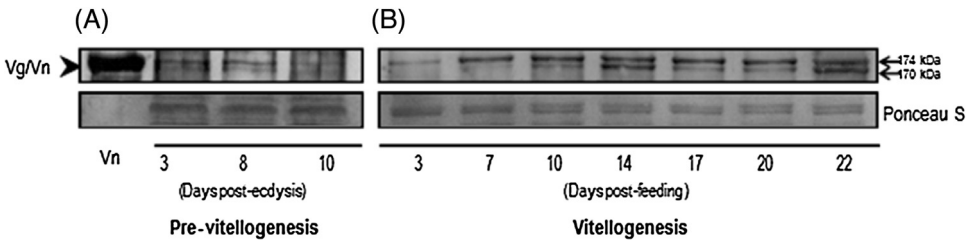

Fig. 3. Vitellogenin (Vg) expression in the fat body of *Triatoma infestans*. The expression of Vg in the fat body was analyzed by western blot, using a polyclonal anti-vitellin antibody (1:2000). The studies were performed after ecdysis (pre-vitellogenic phase) and at different times post-blood feeding (vitellogenic phase) (A and B, respectively, 15 μg/lane). Lane Vn: purified vitellin (0.2 μg/lane to avoid overexposition). Top panel: the arrows indicate the molecular mass of the main subunits of Vn/Vg. The bottom panel shows the protein loading control stained with Ponceau S. The western blot shown was one representative experiment of the three independent assays.

14 post-feeding. At these four days, it was also observed that *Vg2* mRNA level was significantly higher than *Vg1* ($P < 0.001$).

3.2. Protein expression of Vg in fat bodies and ovarian tissues

Western blot analyses were carried out to confirm the effect of blood feeding on the expression of Vg protein in adult females of *T. infestans*. In the fat body, during pre-vitellogenesis, the immunoreactive band corresponding to Vg was faint at days 3 and 8 post-ecdysis, being hardly detected on day 10 post-ecdysis (Fig. 3A). By contrast, the Vg signal notoriously increased after few days of blood feeding, remaining high throughout the vitellogenic phase (Fig. 3B).

In the ovaries, western blots showed a weak immunoreactive band for Vg/Vn protein throughout pre-vitellogenesis (days 3, 8, and 10 after ecdysis), but a strong increase in the amounts of Vn at 3–10 days after feeding was found (Fig. 4A and B). In agreement with the pattern of Vg/Vn found in the ovaries, the immunofluorescence assays showed a strong signal for Vn in the yolk bodies of terminal follicles of vitellogenic females (Fig. 5B). Conversely, in pre-vitellogenic ovaries, the Vg/Vn signal was found mainly in the basal region of the follicular epithelium, although inside the oocytes, a weak fluorescent punctate pattern was also observed (Fig. 5A).

4. Discussion

The insect vectors of Chagas' disease ingest large amounts of blood in a single meal; usually this engorgement represents several times the insect's body weight (Friend et al., 1965; Atella et al., 2005). In these species, at least for the production of a second batch of eggs, blood meal is a requirement for the initiation of vitellogenesis (Stoka et al., 1987). Investigating the process of vitellogenesis in Chagas' disease vectors at a molecular level, we have recently identified two *Vg* genes in the triatomine *T. infestans*. At early days of the vitellogenic phase (3–6 days after blood feeding), we found *Vg1* and *Vg2* mRNA transcripts in fat bodies and ovarian tissues. To a much lesser extent, *Vg2* mRNA was also detected in heads and thoracic muscles (Blariza et al., 2014).

In the present study, our interest was focused on nutritional regulation of vitellogenesis and therefore, we analyzed the dynamics of the expression of *Vg1* and *Vg2* genes throughout the pre-vitellogenic and vitellogenic phases of the reproductive cycle of anautogenous female of *T. infestans*. In addition, *Vg* expression was analyzed in fat bodies from fifth-instar females and adult males. Synthesis of Vg occurs in males of some insect species, including the triatomine *R. prolixus* (Engelmann, 1979; Trenczek and Engels, 1986; Valle, 1993; Piulachs et al., 2003). In the latter species, Vg has been also detected in fifth-instar females (Valle et al., 1993). However, the physiological relevance of those findings remains elusive. In agreement with the results reported in fifth-instar nymphs analyzed only at a single time after feeding (Blariza et al., 2014), neither *Vg1* nor *Vg2* were expressed in fat bodies of fifth-instar females sampled during the time-course of 5–15 days after blood feeding (results not shown). Moreover, no *Vg* transcripts were detected in fat bodies of adult males at different time points post-ecdysis and post-feeding (results not shown).

In female adults of *T. infestans* the two *Vg* genes were expressed at transcriptional level in fat body and ovaries. However, both tissues showed different expression patterns (Figs. 1 and 2). In female fat bodies, *Vg* mRNAs were found after adult emergence and the main subunits of Vg were faintly detected by western blot during this unfed period (Fig. 3). Although of several orders of magnitude lower than in blood fed females, *Vg1* and *Vg2* genes were expressed in the fat body during the pre-vitellogenic phase. Insect Vgs have been also implicated in the transport of sugars, lipids, phosphates and hormones (Chen et al., 1997; Sappington and Raikhel, 1998). Thus, it is possible that the presence of Vg during the pre-vitellogenic phase would be related with some of these functions. On the other hand, hematophagous insects like vectors of Chagas' disease require large blood meals to activate genes involved in the synthesis of yolk protein precursors and oogenesis (Raikhel, 2005). Although autogeny was observed in *T. infestans* (Noriega, 1992), anautogenous females were obtained under standardized parameters of rearing. Therefore, it is likely that even if insufficient to promote vitellogenesis and egg production, the remmant of blood ingested by anautogenous females during the last nymphal stage would be sufficient to prompt synthesis of small levels of Vg during pre-vitellogenesis.

After females were fed, *Vg1* and *Vg2* mRNA levels in the fat body were significantly increased and high rates of transcription for the two genes were observed at all the days tested during vitellogenesis. However, during this reproductive phase, the distribution patterns' of *Vg1* and *Vg2* transcripts showed a bimodal distribution, with two peaks around early and advanced vitellogenesis (days 4 and 12 post-feeding, respectively) (Fig. 1). In triatomines, the stimuli of a blood meal elicits the asynchronous development of ovarioles and the enlargement of terminal oocytes by recruitment of yolk protein precursors synthesized in the fat body, but it is at mid and advanced vitellogenesis where such a feature starts to became remarkable and terminal follicles display different degree of maturation (Huebner, 1981; Stoka et al., 1987; Fruttero et al., 2011). Thus, from a physiological point of view, high levels of transcription of *Vg* genes in the fat body at early vitellogenesis may correlate with the involvement of this tissue in the synthesis of Vg in order to facilite the growth of most terminal oocytes. At advanced vitellogenesis, the peak of *Vg1* and *Vg2* mRNAs may reflect that high rates of Vg synthesis are needed to privilege the maturation of the largest oocytes. In fact, it is important to take into account that in the fat body of *T. infestans*, the peak of both *Vg* transcripts occurred close to the first oviposition event (days 13–15 post-blood meal). On the other hand, when the expression of Vg was analyzed by western blot, Vg was clearly detected at day 3 post-feeding and as vitellogenesis proceeded, sustained high levels of the protein were observed

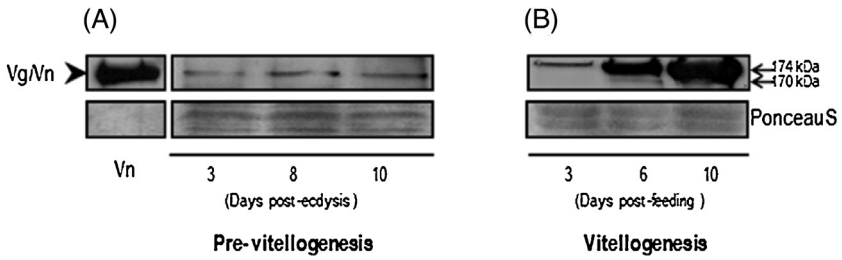

Fig. 4. Vitellogenin (Vg)/vitellin (Vn) in the ovary tissue of *Triatoma infestans*. Changes in Vg/Vn profile in the ovaries were analyzed by western blot, using a polyclonal anti-vitellin antibody (1:2000). The studies were performed after ecdysis (pre-vitellogenic phase) and at different times post-blood feeding (vitellogenic phase) (A and B, respectively, 10 μg/lane). Lane Vn: purified vitellin (0.2 μg/lane to avoid overexposition). Top panel: the arrows indicate the molecular mass of the main subunits of Vn/Vg. The bottom panel shows the protein loading control stained with Ponceau S. The western blot shown was one representative experiment of the three independent assays.

(Fig. 3). Similar results were obtained in *D. maxima* by Aguirre et al. (2008). In this related triatomine species, minimal expression of Vg protein in the fat body was detected during pre-vitellogenesis. However, expression of Vg protein in the fat body as well as the levels in the hemolymph significantly increased after a blood meal. Taken together, our findings agreed with the studies that established that in most insect species the female fat body is the major site involved in Vg synthesis (Tufail and Takeda, 2008). As it was observed in *R. prolixus* (Coles, 1965; Wang and Davey, 1993) and *Triatoma protracta* (Mundall and Engelmann, 1977), activation of *Vg* gene expression and synthesis in the fat body after blood meal is controlled by juvenile hormone secreted by the corpora allata, but the mechanisms responsible for such regulation are not completely understood. Characterization of *Vg1* and *Vg2* genes in *T. infestans* females at transcriptional and translational level would greatly contribute to unravel the molecular processes linking endocrinologycal and nutritional regulation of vitellogenesis in Chagas' disease vectors.

Vg is the precursor protein of egg yolk Vn that provides energy reserves in oviparous vertebrates and invertebrates. In female insects, the fat body synthesizes yolk protein precursors such as Vgs. During their synthesis, Vgs are subjected to post-translational modifications and proteolytic cleavage. After secretion into the hemolymph, Vgs are sequestered by competent oocytes via receptor-mediated endocytosis (Dhadialla and Raikhel, 1990; Snigirevskaya and Raikhel, 2005; Tufail and Takeda, 2009). In the oocytes, Vgs are stored in the yolk bodies as Vns. In addition to fat body, it has been observed synthesis of Vgs by the ovaries in some insect species (Melo et al., 2000; Giorgi et al., 2005; Bellés, 1998, 2005). In this work, the detection of *Vg* transcripts in *T. infestans* ovaries (Fig. 2) was in agreement with observations in females of other insect species showing the involvement of the follicular epithelium in yolk protein synthesis (Giorgi et al., 2005; Bellés, 1998, 2005). For instance, although in *R. prolixus* the fat body is the major source of Vg (Valle et al., 1993), follicular epithelium of late follicles that had ceased Vg uptaking from hemolymph also synthesize Vg. In this species synthesis of ovarian Vg reaches its peak during the late phase of oocyte growth, just before formation of the chorion. More recently, immunohistochemical studies of ovarian tissue of *D. maxima* employing an anti-Vn antibody revealed immunoreactivity in few follicular epithelial cells of the largest oocytes, suggesting that at least some follicular cells could synthesize Vg (Aguirre et al., 2008). As all triatomines, during vitellogenesis *T. infestans* ovarioles displayed simultaneously asynchronous development and oocytes with different degrees of maturation (Pratt and Davey, 1972; Huebner, 1981; Stoka et al., 1987). Therefore, it is possible that in *T. infestans* late vitellogenic follicles close to chorion deposition could account for Vg synthesis, which in turn would explain the up regulation of two *Vg* genes observed from day 10 of vitellogenic phase (Fig. 2), in coincidence with the beginning of egg formation.

As in most insects, in *D. maxima* Vg and Vn share similar immunological properties (Aguirre et al., 2008). It was proved that anti-Vn antibody raised against the large subunits of Vn from *D.*

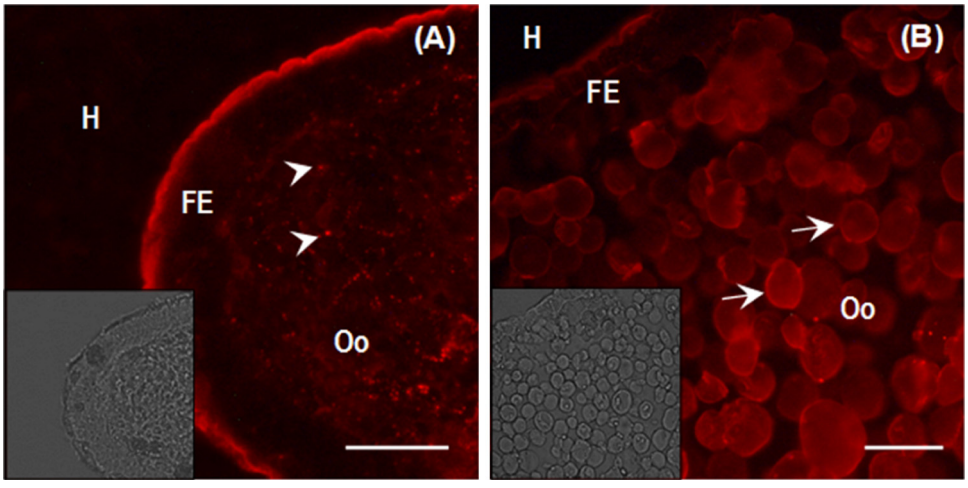

Fig. 5. Vitellogenin (Vg)/vitellin (Vn) localization in the ovarian tissue of *T. infestans* by indirect immunofluorescence. Ovaries from females at pre-vitellogenesis (7 days after ecdysis) and vitellogenesis (4 days after blood meal) were dissected and processed as stated in Materials and Methods. Cryostat sections were incubated with a rabbit anti-vitellin antibody followed by anti-rabbit antibody labeled with Alexa 568 and analyzed by fluorescence microscopy. (A) Section of a terminal follicle of a pre-vitellogenic female showing fluorescence in the basal region of the follicular epithelium and in the oocyte (arrowheads). (B) Section of a terminal follicle of a vitellogenic female showing the fluorescent signal for vitellin at the yolk bodies (arrows). The inserts are the corresponding bright field images for (A) and (B). FE, follicular epithelium; Oo, oocyte; H, hemolymph. Bars: 25 μm. Similar results were observed after examination of 4–5 ovarioles per ovary in two separate experiments.

maxima also recognized immunologically Vgs from the female fat body and the hemolymph. In the ovary of *T. infestans*, western blot assays using such an anti-Vn from *D. maxima* revealed progressive increases of Vn after blood feeding (Fig. 4). Nonetheless, this approach does not allow to distinguish between Vns that has been originated from either, fat body or ovarian tissue. The high levels of Vg in the fat body after a blood meal (Fig. 3) were consistent with the increasing amounts of Vn found in the ovarian tissue (Figs. 4 and 5). In this regard, a remarkable level of Vg endocytosis was detected in vitellogenic follicles of the triatomines *R. prolixus*, *Panstrongylus megistus*, and *D. maxima* (Oliveira et al., 1986; Frutero et al., 2011; Aguirre et al., 2011). In *T. infestans*, immunofluorescence assays revealed that at early vitellogenesis (day 4 post-blood meal), terminal ovarian follicles stored large amounts of Vn (Fig. 5B), more likely via endocytosis of Vg synthesized in the fat body. In addition, in the pre-vitellogenic ovaries the Vg/Vn signal was found mainly in the basal region of the follicular epithelium (Fig. 5A), suggesting that during pre-vitellogenesis synthesized Vg accumulates in the basal region of the follicular epithelium, to be taken up by the oocyte during vitellogenesis.

Regardless the quantitative contributions of fat body and ovary in the synthesis of Vg in the female of *T. infestans*, its seems very likely that both tissues are required to accomplish protein deposition during the highly demanding period of vitellogenesis. However, the dynamics of *Vg1* and *Vg2* expression patterns at the mRNA level observed in fat body and ovary throughout the vitellogenic phase suggest that different regulatory factors might be involved in the expression of *Vg* genes. In *Drosophila melanogaster*, yolk protein genes in fat body and in specific stages of oogenesis in follicular epithelium respond differentially to environmental triggers and require different hormonal signals to synthesize and secrete yolk (Bownes, 2005). In triatomines, juvenile hormone governs vitellogenesis but our knowledge about the molecular bases of juvenile hormone signaling are still incomplete (Davey, 1997). Methoprene-tolerant protein has been pointed out as juvenile hormone receptor candidate (Charles et al., 2011). Recently, approaches employing RNA interference (RNAi) to block methoprene-tolerant protein gene in *R. prolixus* resulted in abnormal development of ovaries and accumulation of yolk (Villalobos-Sambucaro et al., 2015). In the context of the biology of reproduction of Chagas' disease vectors, our work represents an important step for further studies of the role of specific factors on regulation of *Vg* genes during vitellogenesis.

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