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The interaction between *Trypanosoma rangeli* and the nitrophorins in the salivary glands of the triatomine *Rhodnius prolixus* (Hemiptera; Reduviidae)

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ABSTRACT

The parasite *Trypanosoma rangeli* develops in the intestinal tract of triatomines and, particularly in species of the genus *Rhodnius*, invades the hemolymph and salivary glands, where subsequent metacyclogenesis takes place. Many aspects of the interaction between *T. rangeli* and triatomines are still unclear, especially concerning the development of the parasite in the salivary glands and how the parasite interacts with the saliva. In this work, we describe new findings on the process of *T. rangeli* infection of the salivary glands and the impact of infection on the saliva composition. To ensure a complete infection (intestinal tract, hemolymph and salivary glands), 3rd instar *Rhodnius prolixus* nymphs were fed on blood containing *T. rangeli* epimastigotes using an artificial feeder. After molt to the 4th instar, the nymphs were inoculated with epimastigotes in the hemolymph. The results showed that the flagellates started to invade the salivary glands by the 7th day after the injection. The percentage of trypomastigotes inside the salivary glands continuously increased until the 25th day, at which time the trypomastigotes were more than 95% of the *T. rangeli* forms present. The salivary contents from *T. rangeli*-infected insects showed a pH that was significantly more acidic (<6.0) and had a lower total protein and hemeprotein contents compared with non-infected insects. However, the ratio of hemeprotein to total protein was similar in both control and infected insects. qPCR demonstrated that the expression levels of three housekeeping genes (18S rRNA, β-actin and α-tubulin) and nitrophorins 1–4 were not altered in the salivary glands after an infection with *T. rangeli*. In addition, the four major nitrophorins (NPs 1–4) were knocked down using RNAi and their suppression impacted *T. rangeli* survival in the salivary glands to the point that the parasite burden inside the *R. prolixus* salivary glands was reduced by more than 3-fold. These results indicated that these parasites most likely non-specifically incorporated the proteins that were present in *R. prolixus* saliva as nutrients, without impairing the biosynthesis of the antihemostatic molecules.

1. Introduction

Triatomines (Hemiptera; Reduviidae) are obligate hematophagous insects that have medical importance because they transmit *Trypanosoma cruzi,* the etiologic agent of Chagas disease in the Americas. In addition, they are also vectors of the protozoan parasite *Trypanosoma rangeli*, which commonly develops in triatomines of the genus *Rhodnius* and a variety of mammalian species, including humans and domestic and wild animals in Central and South America (Grisard et al., 2010). Although *T. rangeli* infection is harmless for the vertebrate hosts, this parasite can be pathogenic to its insect vectors by promoting defective or lethal effects in both molting and feeding processes (Brecher and Wigglesworth, 1944; Lake and Friend, 1968; D'Alessandro-Bacigalupo and Saraiva, 1992; Ferreira et al., 2010). In addition, the coexistence of *T. rangeli* and *T. cruzi* in the same area and sharing the same vertebrate and invertebrate hosts can compromise the correct diagnosis of *T. cruzi* infections due to the occurrence of crossed serological reactions (Guhl and Vallejo, 2003).

Unlike *T. cruzi*, which develops specifically within the intestinal tract of the insect vector, the *T. rangeli* life cycle has several distinct features. *T. rangeli* flagellates also develop in the intestinal tract of *Rhodnius* triatomines as epimastigotes; however, they are able to

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invade the insect hemolymph and then penetrate the salivary glands where metacyclogenesis occurs. Here, the parasite assumes the trypomastigote form, which is transmitted to the vertebrate host during the bite.

The saliva of *Rhodnius prolixus*, as with other blood-feeding arthropods, has proven to be a source of a wide variety of anti-hemostatic molecules that play an important role during hematophagy (Ribeiro et al., 1990; Champagne, 2005). Among these salivary molecules, the most abundant are hemeproteins known as nitrophorins (NPs). Seven different NPs have been described (NPs 1–7) (Moreira et al., 2003; Champagne, 2005; Knipp et al., 2007), comprising approximately 50% of the salivary protein content. Among them, the most studied are NPs 1–4. These nitrophorins are multifunctional molecules that are responsible for the reddish color of the *R. prolixus* salivary glands due to the presence of a heme group in the nitrophorin molecule, which carries nitric oxide (NO) (Champagne et al., 1995). Most of the biological activities of NPs are related to NO, which is released into the host tissue and can act as both a vasodilator and an inhibitor of platelet aggregation (Andersen et al., 2005). In vertebrate host skin, the nitrophorins can also bind to the histamine that is released by mast cells, which prevents swelling and pain at the bite and can generate a defensive response by the host (Andersen et al., 2005). In addition, nitrophorin 2 (NP2) is a powerful anticoagulant that acts by binding directly to Factor Xa of the blood coagulation cascade (Ribeiro et al., 1995). Araujo et al. (2009b) demonstrated that *R. prolixus* that have low levels of salivary NPs have poorer blood-ingestion rates when feeding from vessels that have small diameters such as venules and arterioles.

Many aspects of the life cycle of *T. rangeli* in triatomine insects are still unclear, especially the processes of invasion and metacyclogenesis of the parasite in the salivary glands, as well as how the presence of the parasite interferes with the physiology and behavior of the insect vector. It has been suggested that *T. rangeli* might induce changes in the biosynthesis of salivary compounds and thereby affect the antihemostatic properties of *R. prolixus* saliva (Garcia et al., 1994). In this work, we describe new findings on the process of *T. rangeli* colonization of the salivary glands and on the impact of a *T. rangeli* salivary gland infection on the saliva composition. We also evaluated a possible role of these abundant molecules in the development and survival of the parasites into *R. prolixus* salivary glands using RNAi to knockdown NPs expression levels.

2. Materials and methods

2.1. Insects and parasites

R. prolixus were reared under controlled conditions of temperature (26 ± 2 °C) and humidity (65 ± 5.0%) in a 12/12 h light/dark cycle and fed weekly on chickens or mice according to the FIOCRUZ guidelines on animal experimentation. The use of these insects was approved by the Ethical Committee on Animal Use (L-058/08).

T. rangeli of the CHOACHI strain isolated from naturally infected *R. prolixus* from Colombia (Schottelius, 1987) was used in the present study. The epimastigote forms were cultured at 27 °C in liver-infusion tryptose (LIT) medium supplemented with 15% fetal bovine serum, 100 μg/mL streptomycin and 100 units/mL penicillin. The strain infectivity was maintained through a cyclical transmission in mice and triatomines every two months.

For all of the assays, the epimastigotes were obtained from 10-day-old culture medium, washed and resuspended in sterile PBS (for hemolymph inoculation) or inactivated blood (for infective feeding).

2.2. Rhodnius prolixus infection

Because the parasite crossing from the intestinal lumen to the hemolymph does not occur in all infections (Ferreira et al., 2010), a complete infection (intestinal tract, hemolymph and salivary glands) was ensured by first infecting *R. prolixus* nymphs through feeding them with blood containing epimastigotes and then inoculating the same nymphs intracoelomically with the parasite. For intestinal infection, epimastigotes were added to heat-inactivated (56 °C for 30 min) rabbit blood at a concentration of 10^5 flagellates/mL. Third instar nymphs were allowed to feed on this blood through a membrane feeder (control insects were fed on non-infected blood). Seven days after the nymphs molted to the 4th instar, 1 μL of PBS (0.15 M NaCl in 0.01 M Na_3PO_4, pH 7.4) containing 100 parasites was injected directly into the insect hemolymph using a thin needle (13 × 3.30 G, ½") that was attached to a Hamilton syringe. A control group was inoculated with PBS alone. Twenty-four hours after inoculation, the nymphs were fed on anesthetized healthy mice.

2.3. Monitoring the T. rangeli invasion and differentiation within the salivary glands

The invasion process of *R. prolixus* salivary glands by *T. rangeli* epimastigotes was evaluated through the daily examination of 20 nymphs starting from the 5th day after the inoculation. The confirmation of infection was made through examination of the salivary glands in a drop of saline solution on a microscope slide. To identify the developmental forms of the parasite (epimastigotes, intermediates and trypomastigotes), the salivary glands were disrupted and examined through Giemsa-stained smears by counting at least 100 parasites in each slide. Intermediate forms were defined as those parasites that had the kinetoplast and nucleus in a transitional position within the cell.

2.4. Salivary features of T. rangeli infected and non-infected insects

The salivary glands from nymphs that had been infected as 3rd and 4th instars (oral and coelomatic infections, respectively) and their respective controls were examined on the 40th day after the inoculum was given. By this time, the nymphs had molted to the 5th instar stage and a chronic infection was already established.

2.4.1. Determination of pH in salivary gland contents

The salivary gland contents of 5th instar nymphs (*T. rangeli*-infected and non-infected ones) were collected in capillary tubes that were created by stretching out microhematocrit glass tubes in a Bunsen flame. The capillaries were pre-filled with 30 μL of an aqueous solution containing pH indicator dyes at 0.05% (bromothymol blue at pH 7.5 or bromocresol purple at pH 7.0). To measure the pH of the salivary contents, the salivary glands were dissected out of the insects, transferred to a slide and washed rapidly in 0.9% saline solution. After mopping up the excess saline with filter paper, the glands were pierced using the tip of the capillaries. The color at the interface of the two liquids (saliva-dye solution) in the capillary was compared with buffered standard color solutions (0.1 M) that had been prepared with these dyes at different pH values (5.0–8.0 at intervals of 0.5 pH units) (Soares et al., 2006).

2.4.2. Estimation of total protein and hemeprotein content in the salivary glands

T. rangeli-infected and non-infected salivary glands of *R. prolixus* 5th instar nymphs were dissected and transferred to ice-cold microcentrifuge tubes containing 70 μL of PBS. The glands were mechanically disrupted and centrifuged at 12,000 g for 10 min at 4 °C to pellet the gland tissue (and the parasites in the infected

group). The supernatants that contained only the salivary contents were transferred to new 1.5 mL tubes, the volume was brought to 100 μL with PBS. Ten microliters of these samples were diluted to 1:10 with PBS and read at 280 nm and 404 nm spectrophotometer wavelength absorbances, which were used to estimate the total protein and hemeprotein content, respectively, in the salivary gland extracts (Nussenzveig et al., 1995; Araujo et al., 2009a).

2.4.3. RNA extraction from salivary glands and cDNA synthesis

RNA from pools of 8 salivary glands (4 insects) from infected and non-infected 5th instar nymphs was extracted using the Nucleospin RNA XS Kit (Macherey–Nagel) according to the manufacturer's instructions. The RNA was quantified using a NanoDrop ND-1000 (Thermo Scientific) absorbance reading at 260 nm cDNA was synthesized from 0.5 μg of RNA and 0.5 μg of random hexamers (Promega) using the M-MLV reverse transcriptase system (Promega) in a final volume of 25 μL. This procedure was used to produce cDNA for both PCR and qPCR.

2.4.4. Relative expression of NPs 1–4 transcripts in T. rangeli-infected and non-infected salivary glands using qPCR

The expression levels of NPs 1–4 and housekeeping genes (18S rRNA, α-tubulin and β-actin) were measured in cDNA samples of *T. rangeli*-infected and non-infected salivary glands from 5th instar nymphs. The qPCR reactions were conducted using an ABIPRISM 7500 Sequence Detection System (Applied Biosystems). Each reaction was run in triplicate and contained 10 ng of cDNA, 300 nM of each primer and 12.5 μL of Power SYBR Green PCR Master Mix (Applied Biosystems) in a final volume of 25 μL. The cDNA was amplified at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. To ensure that only a single product was amplified, a melting curve analysis was performed. A reverse transcription negative control (without reverse transcriptase) and a non-template negative control were included for each primer set to confirm the absence of genomic DNA and to check for primer-dimer or contamination in the reactions, respectively. The relative amount of gene product in each sample was determined using the established $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) using the three housekeeping genes as loading controls according to Paim et al. (2012). The primers that were used for qPCR are shown in Table 1, and the NP 1–4 primers were previously used by Araujo et al. (2009b).

2.5. Evaluation of parasite survival in the presence of salivary contents in vitro

T. rangeli epimastigotes from 10-day-old culture medium were removed from LIT medium and washed 3 times with PBS (with centrifugations at 2000 g at 4 °C). The parasites were resuspended in PBS and aliquoted into microcentrifuge tubes at a concentration of 6.0×10^6 parasites/mL in a final volume of 25 μL. These parasites were subjected to three different treatments. In the first treatment, the parasites were maintained in only PBS (control group). In the other two treatments, the salivary contents that had been obtained from either 1 or 10 salivary glands of *R. prolixus* 4th instar nymphs were added. The preparations (7 replicates in each group) were incubated at 27 °C for 24 h, and then an aliquot of each sample was directly used to count the number of parasite cells in a Neubauer chamber. The viability of the live parasites was verified by flagellar movement during cell counting.

Table 1
Primers sequences used for relative qPCR.

Target	Forward 5′/ 3′	Reverse 5′/ 3′	Amplicon (bp)
NP1	tttgctgcagtgggtgtaag	agttgcccgacgttacatct	334
NP2	catatgtttgcgggaaggat	atcgtactggcacccaagat	163
NP3	aacccagtgcaacggttaaa	ctccaccaattggcatttttt	185
NP4	ttaaaagcgccgtatctgct	agaacgtcaaatgtctctttacct	248
α-tubulin	tttcctcgatcactgcttcc	cggaaataactggggcataa	129
β-actin	aatcaagatcattgctccaccag	ttagaagcatttgcggtggac	151
18S rRNA	tccttcgtgctaggaattgg	gtacaaagggcagggacgta	105

2.6. Impact of NPs 1–4 RNAi knockdown in T. rangeli infection of R. prolixus salivary glands

2.6.1. NPs 1–4 double-stranded RNA synthesis and delivery

RNA extraction and cDNA synthesis from a pool of non-infected salivary glands from 3rd instar nymphs were performed as described in section 2.4.3. One microliter of the cDNA was amplified by PCR using specific primers that were conjugated with 23 bases of the T7 RNA polymerase promoter at the 5′ end (5′-taatacgactcactatagggaga-3′). PCR was carried out for 35 cycles (94°C for 30 s, 60 °C for 30 s, 72 °C for 45 s) in the presence of 200 nM primer, 200 mM deoxyribonucleotide triphosphates (dNTPs) and 1 unit of Taq polymerase (Phoneutria) in a final volume of 20 μL. The PCR products were used as templates to synthesize double-stranded RNA (dsRNA) that complemented NPs 1–4 according to the method described by Araujo et al. (2009b). Each third instar nymph in the NPs 1-4-knockdown group was starved for 8–10 days after molting and then injected with 1.25 μg of dsRNA that was specific for each NP (NP1, NP2, NP3 and NP4, 5 μg of NPs 1–4 dsRNA in total) diluted in 1 μL of saline solution (0.9% NaCl). The 3rd nymphs were injected directly in the thoracic cavity using a microinjector (Nanoinjector, Drummond). Control nymphs were injected with 1 μL of sterile saline solution. Forty-eight hours after injection, both groups were fed on an artificial feeder with a *T. rangeli* infective blood meal as previously described. Five days after molting to the 4th instar stage, all of the control and NPs 1-4-knockdown nymphs were inoculated with *T. rangeli* parasites as described in Section 2.2.

2.6.2. Quantification of parasite burden in infected salivary glands from NPs 1-4-knockdown insects

Parasite quantification inside the salivary gland pairs of each individual 4th instar nymph was performed on the 25th day after *T. rangeli* hemolymph inoculation. The salivary glands of the infected controls and NPs 1-4-knockdown insects were dissected in PBS and washed 3 times in PBS to remove the epimastigotes outside of the gland. The salivary glands were disrupted and homogenized in 100 μL of PBS to release the parasites. Of this dilution, 10 μL were used to estimate the parasite number in each salivary gland pair by parasite cell counting in a Neubauer chamber, and the remaining 90 μL were used to extract parasite DNA with the Nucleospin Tissue XS Kit (Macherey–Nagel) according to the manufacturer's instructions to estimate the parasite burden using qPCR.

The qPCR reactions with the DNA that had been isolated from the salivary contents of the saline controls and NPs 1-4-knockdown-infected insects were conducted using the same conditions as described in section 2.4.4. However, instead of performing a relative quantification using housekeeping genes, a standard curve was constructed using serial dilutions of a plasmid that contained a 137-bp sequence that is related to the sno-RNA-Cl1 *T. rangeli*-specific multicopy gene, which has previously been used for *T. rangeli* detection (Morales et al., 2002; Pavia et al., 2007) (GenBank: AY028385.2, forward: 5′-gaaagcgcaagagagagat-3′ and reverse: 5′-tgagatggctatcacgcaag-3′). The plasmid was obtained by transforming *E. coli* DH5α cells with the plasmid bound to the fragment of interest using the pGEM-T Easy vector (Promega). This plasmid DNA was diluted to a range of 10^7 to 10^0 molecules, and the standard curve was used to estimate the number of parasite DNA copies in each sample.

2.7. Statistical analysis

Statistical analysis was done using the Graph-Pad Prism® 5.0 software. The Kolmogorov–Smirnov test was used to identify variables with normal distribution which were then compared by *t*-test or ANOVA with post hoc comparisons made using Tukey's test. The non-parametric Mann–Whitney test was used for variables with non-normal distribution. Fisher's exact test was used to compare proportions of nominal variables. The accepted significance level was $p < 0.05$.

3. Results

3.1. Monitoring the T. rangeli invasion and differentiation within the salivary glands

The colonization and differentiation of *T. rangeli* epimastigote forms inside *R. prolixus* salivary glands were monitored over time after parasite inoculation into the insect hemolymph. The salivary gland infection began on the 7th day after *T. rangeli* inoculation. On the 11th day, more than half of the insects presented parasites inside their salivary glands, and by the 25th day, this number had increased to 94% (Fig. 1A).

The proportion of *T. rangeli* trypomastigotes inside positive salivary glands progressively increased between the 9th and the 25th days after inoculation, while the epimastigote proportion was gradually reduced (Fig. 1B). On the 9th day, when half of the insects presented parasites in their salivary glands, approximately 20% of the flagellates were trypomastigotes, while on the 25th day, the percentage of this form had increased to 95% (Fig. 1B). No epimastigote was observed in process of cell division in the salivary contents that were evaluated.

3.2. Comparison of salivary features between T. rangeli-infected and non-infected salivary glands

Infected 5th instar nymphs with chronic *T. rangeli* infection presented several alterations in their salivary gland contents. At this phase, the infected salivary glands normally changed their typical reddish color to an unusual orange color (Fig. 2A).

The salivary contents from *T. rangeli*-infected insects were significantly more acidic compared with non-infected insects (Fisher's exact test, $p = 0.0046$). In the non-infected group, the pH of the salivary contents was 6.0 in 100% of the evaluated insects ($n = 9$). In the infected group, the pH of the salivary contents was below 6.0 (between 5.5 and 6.0) in approximately 2/3 of the insects and ≥6.0 in the remaining 1/3.

The estimation of protein and hemeprotein content was significantly higher in the salivary glands from the non-infected group (*t*-test, $p < 0.001$ for both) (Fig. 2B and C), but the ratio of hemeprotein to total protein was similar between the groups (*t*-test, n.s.) (Fig. 2D). The ratio close to 1 in the both compared groups (*T. rangeli*-infected and non-infected) does not indicate equal amounts of hemeprotein and total protein, given that the hemeproteins comprise about 50% of the salivary gland protein content (Champagne et al., 1995; Araujo et al., 2009b), but indicates a similar proportion of them and suggests a non-specific reduction in the amount of proteins in highly infected glands.

Despite this decrease in the protein content, the evaluation of the mRNA levels (qPCR) showed that the presence of *T. rangeli* did not affect the expression levels of the four major nitrophorins (NP1, NP2, NP3 and NP4), nor did it affect the expression levels of the housekeeping genes 18S rRNA, α-tubulin or β-actin, which were similar in infected and non-infected salivary glands (*t*-test, n.s. for all of the evaluated genes, Table 2).

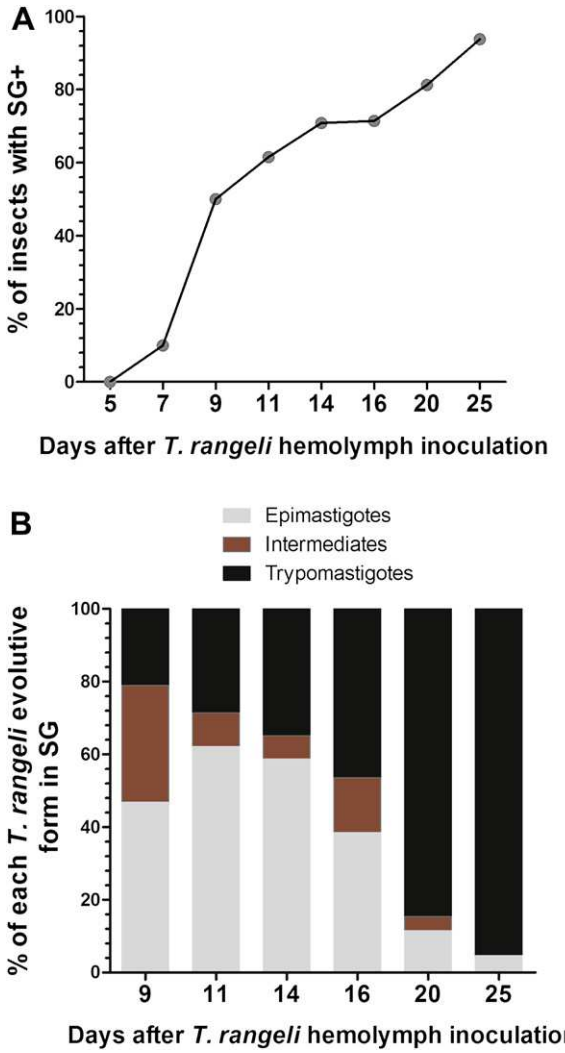

Fig. 1. Infection of salivary glands (SG) of 4th instar nymphs of *R. prolixus* after *T. rangeli* hemolymph inoculation. (A) Percentage of the evaluated insects with salivary glands infected with *T. rangeli* (SG+) until the 25th day after inoculation with 100 epimastigotes. (B) Evaluation of the metacyclogenesis process of *T. rangeli* inside the salivary gland pairs over time after parasite inoculation. Intermediate forms were defined as those parasites with the kinetoplast and nucleus in a transitional position within the cell ($n = 20$ for each evaluation time).

3.3. Survival of T. rangeli in the presence of salivary contents in vitro

The *in vitro* assay demonstrated that *T. rangeli* epimastigotes that were incubated for 24 h in PBS in the absence of other nutrient sources were able to extend their survival (Fig. 3) and maintain their motility (an indication of parasite vigor) in the presence of salivary gland extracts. The initial concentration of parasites in each sample was 6.0×10^6 parasites/mL, and the addition of the extracts that were derived from either one or 10 salivary glands significantly altered the parasite survival (ANOVA, $p < 0.0001$). After incubation in PBS alone, the parasite survival in the medium decreased to a mean of 2.3×10^6 parasites/mL, and none of the parasites showed movement. When the extract of one salivary gland was added, a significantly lower reduction was observed in the parasite survival (5.1×10^6 parasites/mL, Tukey's test, $p < 0.001$), but again, none of these parasites showed movement. However, when a 10-salivary gland extract was added, the *T. rangeli* number increased to 8.6×10^6 parasites/mL (Tukey's test, $p < 0.001$), and the majority of the parasites showed intense motility, which indicated that the

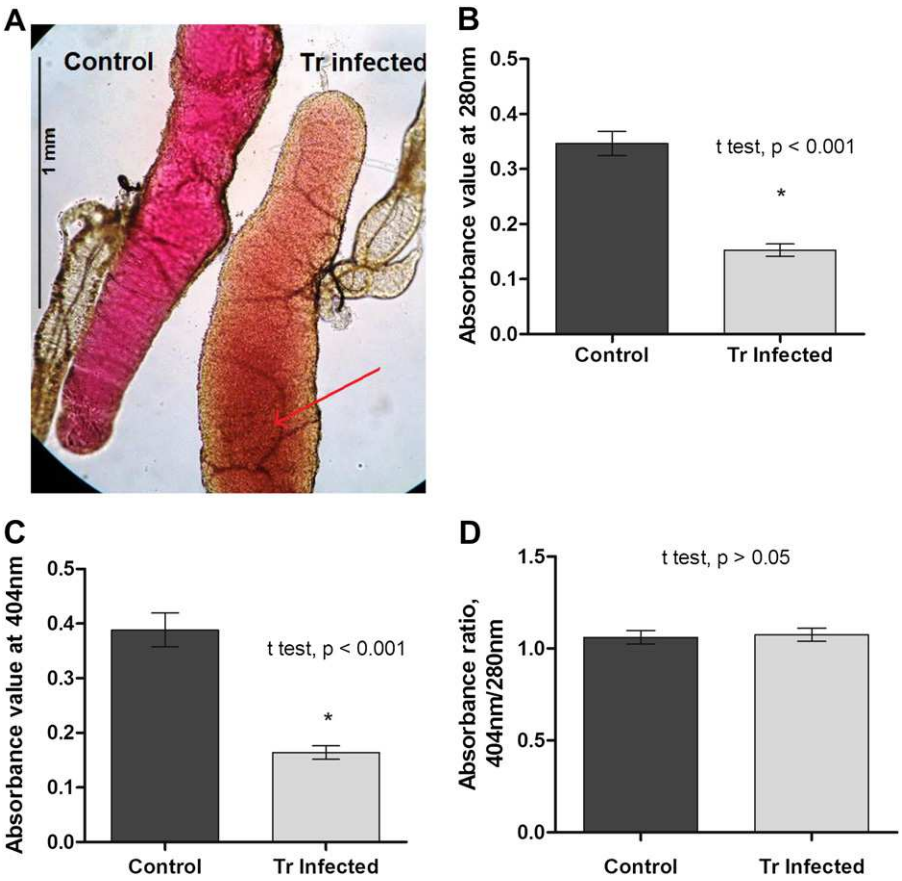

Fig. 2. Comparison of parameters between non-infected (control) and *T. rangeli*-infected (Tr-Infected) salivary gland contents of 5th instar nymphs of *R. prolixus*. (A) Salivary gland lobe aspect on optic microscopy of an uninfected insect (control) showing its typical reddish color and of a *T. rangeli*-infected insect showing the color altered to orange and the interior of the gland with a darker color due to the parasite mass (red arrow). (B) Total protein content (based on absorbance readings at 280 nm). (C) Hemeprotein content (essentially NPs, based on absorbance readings at 404 nm). (D) Ratio of hemeprotein to total protein (ratio of 404 nm/280 nm absorbance readings). The data are presented as the mean ± SE of 15 pools of the contents of 4 salivary gland pairs from *R. prolixus* fifth instar nymphs on the 40th day after parasite inoculation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

salivary nutrients acted as a source of energy to maintain parasite survival and motility depending on the amount of salivary nutrients that were available.

3.4. The impact of NPs knockdown on T. rangeli infection of R. prolixus salivary glands

Because *T. rangeli* infection reduced the amount of nitrophorin that was available inside the salivary glands, NPs 1-4-knockdown insects were produced to evaluate whether the absence of these proteins could affect parasite development.

The salivary content of infected fourth instar nymphs was evaluated 40 days after knockdown of NPs 1–4 and 25 days after parasite inoculation. Both total protein and hemeprotein were reduced in the NPs 1-4-knockdown group by 46.4% (*t*-test, $p < 0.0001$) and 91% (*t*-test, $p < 0.0001$), respectively, compared with the saline control group. The ratio of hemeprotein to total protein was also significantly reduced by 83% (*t*-test, $p < 0.0001$) in the NPs 1-4-knockdown group, which indicated that NPs expression was specifically suppressed in the salivary glands. The color of the salivary glands corroborated these results (Fig. 4) because the

Table 2
Relative quantification of housekeeping genes and normalized quantity ($2^{-\Delta Ct}$) of nitrophorin genes in salivary glands (SGs) of non-infected and *T. rangeli*-infected 5th instar nymphs using qPCR ($2^{-\Delta\Delta Ct}$ method).

		Non-infected SG	*T. rangeli*-infected SG	*p*-value (*t*-test)
Housekeeping genes	18S rRNA	0.73 ± 0.04	0.81 ± 0.16	= 0.45
	β-actin	0.76 ± 0.15	0.82 ± 0.20	= 0.69
	α-tubulin	0.75 ± 0.23	0.56 ± 0.13	= 0.30
Nitrophorin genes	NP1	0.66 ± 0.18	1.0 ± 0.54	= 0.35
	NP2	0.95 ± 0.34	0.66 ± 0.25	= 0.29
	NP3	0.73 ± 0.38	0.83 ± 0.45	= 0.79
	NP4	0.83 ± 0.21	0.87 ± 0.37	= 0.86

The data are presented as the mean ± SE of 9 pools of four salivary gland pairs.

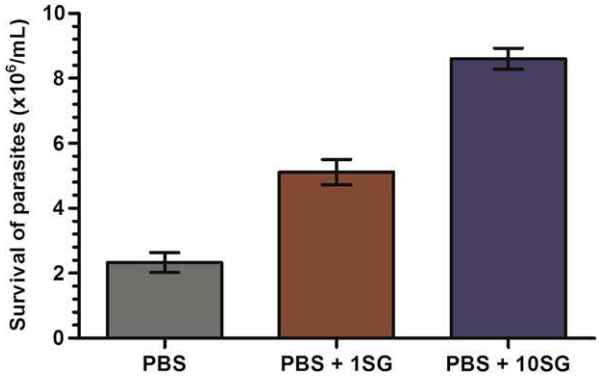

Fig. 3. Survival of *T. rangeli* epimastigotes after incubation at 26 °C for 24 h in PBS (phosphate-buffered saline) with or without salivary gland extracts (SG). The parasites were counted using a Neubauer chamber. The data are presented as the mean ± SE of 7 samples in each group.

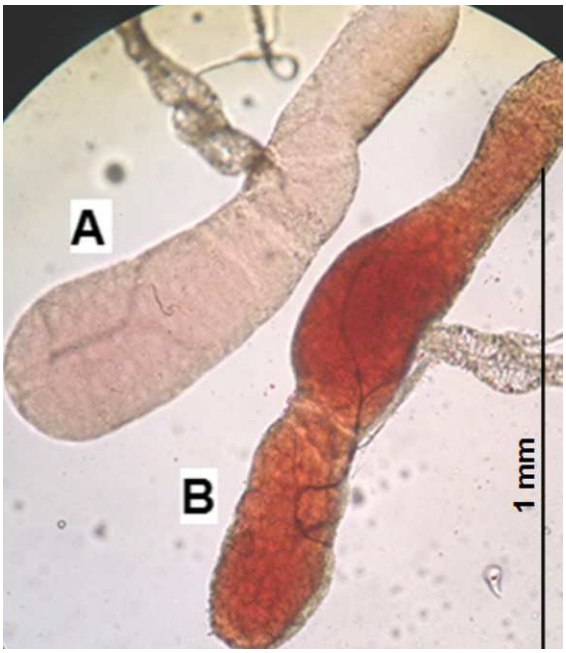

Fig. 4. Salivary gland aspects of *T. rangeli*-infected *R. prolixus* 4th instar nymphs (25th day after parasite inoculation) on an optic microscope. (A) Salivary gland lobe of an infected NPs-knockdown insect (NPs 1-4 dsRNA injection) showing the whitish color (suppression of NPs molecules). (B) Salivary gland lobe of an infected control insect (saline injection) showing its orange-colored aspect.

typical reddish color of the *R. prolixus* salivary glands was altered to a whitish color (indicating a suppression of the hemeproteins).

The suppression of NPs 1—4 did not impair the adhesion, invasion and colonization capacities of *T. rangeli* parasites in the salivary glands because these parasites were found adhered inside the salivary glands. However, by the 25th day after inoculation, the burden of *T. rangeli* parasites inside the salivary glands was reduced by more than 3 fold in NPs 1-4-knockdown nymphs. A similar reduction in proportion was observed between the two methods that were used to measure the number of parasites inside the salivary glands (Neubauer chamber counting and qPCR, Table 3).

4. Discussion

The establishment of *R. prolixus* infection by *T. rangeli* involves a series of biological adaptations throughout its life cycle in the insect vector, including passing through the intestinal tract, migrating through the hemolymph and finally reaching and penetrating into the salivary glands. However, salivary gland infection rates are quite low (approximately 13%) when insects are only infected orally through the ingestion of parasites (Ferreira et al., 2010). To bypass this problem, an additional inoculation was performed directly into the hemolymph to facilitate the infection of the salivary gland. Even so, the process of salivary gland invasion is not always successfully completed. According to our experience, the proportion of infected salivary glands rarely reaches 100% in an experimental group because the parasites cannot reach and invade the gland in some individuals for unknown reasons. In addition, because parasites do not invade the salivary glands at the same time after reaching the hemolymph, the salivary parasite burden is extremely variable among the infected individuals. This variation makes it unfeasible to estimate a representative mean number of parasites for each evaluated time after inoculation. Although no more than 10% of the insects presented infected salivary glands on the 7th day after inoculation, this infection rate increased to close to 100% by the 25th day after inoculation. As the infection became chronic, the burst of parasites that was observed as a cloud through optical microscopy gradually increased and reached populations as high as 10,000 parasites/gland pair. Little is known about the mechanisms by which the parasites penetrate the salivary glands or differentiate into trypomastigote infectious forms. Our experiments also suggest that after invasion, the parasite does not multiply inside the salivary gland. In Giemsa-stained slides of salivary gland contents that were evaluated at different intervals after hemolymph inoculation of *T. rangeli*, no parasite was observed in the process of division. Dividing parasites were often observed among the stained parasites obtained from the hemolymph, in which the multiplication of epimastigotes is usually intense (data not shown). Furthermore, even after long periods post inoculation (on the 25th day), when the salivary glands were already strongly infected, less than 5% of the forms were epimastigotes and/or intermediate forms. During the entire lifespan of an infected insect, even at long periods after gland infection, a baseline burden of parasites can still be found in the hemolymph that is clearly reduced after salivary gland colonization (data not shown). This basal population could yield a continuous parasite supply into the gland that could be responsible for the replacement of parasites in the gland even after several feedings and a constant release of saliva.

The presence of *T. rangeli* in the intestinal tract of triatomines can lead to a series of deleterious effects on the vector, including a delay in nymph development (Brecher and Wigglesworth, 1944; Lake and Friend, 1968; Ferreira et al., 2010), an increase in mortality rates, disturbances in digestion and excretion (Brecher and Wigglesworth, 1944; Eichler and Schaub, 2002) and reductions in the tracheal system (Eichler and Schaub, 2002). After reaching the salivary glands, it has been shown that the parasite alters antihemostatic salivary activities and affects the efficiency of blood feeding on vertebrate hosts (Garcia et al., 1994).

The present results showed that a robust *T. rangeli* infection in the salivary gland alters its typical reddish color to an orange aspect. This new color is usually observed after several weeks after parasite inoculation, especially in chronic infections (up to 40 days post-inoculation). Because nitrophorins are the molecules that confer the reddish color on the glands, we decided to study their relationship to salivary infection by *T. rangeli*.

In addition to the color alteration, the presence of *T. rangeli* also modifies the saliva pH by slightly acidifying the *R. prolixus* salivary contents. This change in the pH of infected salivary gland could be the result of the parasite metabolism within the gland or even

Table 3
Parasite burden in the salivary gland pairs (SGs) of control (saline injection) and NPs-knockdown (NPs 1-4 dsRNA injection) 4th instar nymphs on the 25th day after *T. rangeli* inoculation.

Quantification method	Control	NPs 1—4 knockdown	Ratio of control/ NPs 1—4 knockdown	*p*-value
Neubauer chamber (number of parasites/SGs)	$8.59 \times 10^4 \pm 1.59 \times 10^{4*}$	$2.72 \times 10^4 \pm 8.13 \times 10^3$	3.16	*t*-test $p = 0.007$
qPCR (number of sno-RNA-CL1 gene copies/SGs)	$6.12 \times 10^5 \pm 2.08 \times 10^{5*}$	$1.73 \times 10^5 \pm 5.94 \times 10^4$	3.53	Mann Whitney $p = 0.026$

*Mean ± SE of 10 salivary gland pair contents.

a deregulation in the salivary gland mechanism for pH control. Unlike other triatomine genera (e.g., *Triatoma*, which has a salivary gland contents pH of approximately 7.0), *R. prolixus* has a mechanism for pH acidification in the salivary gland, which is essential to maintain the NO strongly bound to the nitrophorins until the saliva is secreted into the host's skin (Soares et al., 2006). The pH of *R. prolixus* hemolymph has been demonstrated to vary between 6.5 and 7.0 (Soares et al., 2006). A more acidic environment inside the salivary glands might also be related to the parasite differentiation process, which appears to occur as soon as the epimastigotes from the hemolymph reach the salivary gland lumen (Meirelles et al., 2005). It is thought that changes in pH levels in the rectal portion of the intestinal tract of triatomines might contribute to the metacyclogenesis process in *T. cruzi* parasites (Zeledon et al., 1977; Ucros et al., 1983; Kollien and Schaub, 1997). Additional studies should be performed to evaluate the role of pH on the metacyclogenesis of *T. rangeli.*

Our data showed a reduction of approximately 58% in the levels of both hemeproteins (which, in the salivary glands, are essentially nitrophorins) and total protein in salivary glands that were massively infected with *T. rangeli* parasites. The observed reduction in the total protein was similar to the reduction that was observed by Garcia et al. (1994), who found a reduction of approximately 60% in the total protein composition of a *T. rangeli*-infected salivary gland, and by Van Den Abbeele et al. (2010), who observed a 70% reduction in the salivary gland total protein in the fly *Glossina morsitans* after infection with *Trypanosoma brucei*.

In addition to the reduction in total protein content, Garcia et al. (1994) reported a decrease in apyrase and anticoagulant activity and a reduction in the NO-like compounds in *T. rangeli*-infected salivary glands. Based on these alterations, the authors suggested a possible parasite interference in the synthesis of specific salivary proteins with antihemostatic properties (Garcia et al., 1994). In the same way, our data also showed that *T. rangeli* infection affects the protein composition of *R. prolixus* salivary glands. We showed that the total amount of protein stored was non-specifically decreased because the proportion of hemeprotein in the total protein was similar in both infected and non-infected salivary glands. Our qPCR data analysis showed a normal level of transcription of NP mRNA and similar expression levels of housekeeping genes in infected and non-infected salivary glands. Taken together, these findings suggest that the parasite does not cause an expressive damage to the gland cells that is able to affect the salivary protein-expression machinery.

Inside the gland, the parasites most likely non-specifically absorb the salivary proteins that are available in the gland lumen as nutrients to maintain their metabolism. This activity could indirectly affect the antihemostatic proteins and consequently reduce the antihemostatic salivary activities that are essential to promote an efficient hematophagy on vertebrate hosts. The nitrophorins appear to be affected by the parasite infection in the same manner as any other proteins in the saliva, which indicates that the parasite does not manipulate specific components of the saliva. The *in vitro* assay corroborated the role of the salivary proteins in the nutrition of the parasite inside the triatomine salivary glands because the presence of salivary contents was sufficient to prolong the survival and to maintain the motility of *T. rangeli* parasites in the absence of other sources of nutrition.

The development of *T. rangeli* inside the salivary glands is still a very controversial issue in the literature. Our findings are in accordance with Meirelles et al. (2005) who reported that after reaching the gland lumen, *T. rangeli* epimastigotes remain attached by the flagellum to the microvillus of the salivary gland cells during the beginning of the metacyclogenesis process. However, Hecker et al. (1990) suggested that the parasites divide in the salivary gland lumen and then differentiate into metacyclic forms. Kitajima et al. (1998), analyzing longitudinal sections of *T. rangeli* cells in the lumen of *Rhodnius equadoriensis* salivary glands, described that most of the parasites were epimastigotes with some of them undergoing the division process. Additional studies should be developed to clarify the differentiation process of *T. rangeli* inside the salivary glands.

To examine the importance of NPs for *T. rangeli* survival, NPs 1–4 were knocked down in insects using RNAi before being infected. The number of *T. rangeli* within the gland was measured using a Neubauer chamber and qPCR. When the salivary gland is disrupted, the salivary contents form a gel-like material that precipitates and may compromise the counting of parasites in the Neubauer chamber. Therefore, this counting was combined with the quantification by qPCR, which is also a sensitive method. The results obtained by both methods showed that the suppression of the NPs impacted the *T. rangeli* survival in the salivary glands. The lower amount of *T. rangeli* parasites in the salivary glands was likely related to the significant reduction in the total protein content of NPs-knockdown nymphs, which led to a reduced availability of protein nutrients for these parasites. Additionally, the development of parasites inside the gland could have been affected by the reduction in hemeprotein. Similarly to the other kinetoplastid organisms, *T. rangeli* parasites lack a heme biosynthetic pathway and depend on the uptake of heme from their hosts (Tripodi et al., 2011). It is not clear how these organisms acquire heme and distribute this cofactor to the cells, although the trypanossomatids incorporate it into various hemeproteins and cytochromes, which are involved in essential metabolic pathways (Tripodi et al., 2011). Commonly, the *in vitro* cultivation of *Trypanosoma* parasites also requires the addition of a heme compound in the form of hemoglobin, hematin or hemin to the medium.

T. rangeli shows a complex developmental cycle in its invertebrate host, invading and colonizing diverse micro environments with different nutritional and immunological conditions. *Rhodnius* salivary glands, where metacyclogenesis takes place, are formed from a single layer of flattened epithelial gland cells enveloped by a thick basal lamina in which smooth muscle fibers, neuronal cells and tracheal cells are embedded (Meirelles et al., 2003). Because triatomines can regularly spend long periods between blood meals, salivary proteins are gradually produced and stocked inside salivary glands. *R. prolixus* salivation occurs during all phases of blood meal intake (Soares et al., 2006), and half of the salivary proteins that had been previously stocked in the salivary glands are released (Nussenzveig et al., 1995). Our results suggest that parasite colonization does not physically damage the gland cells because the expression of both constitutive and specific genes was not altered during a massive infection. Infection with *T. rangeli* can persist throughout an insect's lifespan (Pifano et al., 1948; Cuba, 1975; D'Alessandro, 1976; Hecker et al., 1990), and preliminary data have shown that the organ can be recolonized by parasites coming from the hemolymph after salivary gland emptying. Thereafter, an infection that would destroy gland cells could impair salivary protein replacement and consequently parasite development and transmission. The massive reduction in the salivary protein content after a chronic infection by *T. rangeli* together with the observed reduction in mortality of parasites maintained with salivary contents suggest that parasites could be consuming the stored proteins as a nutritional supply. Further studies should be developed to understanding of how these parasites develop inside the salivary glands using proteins as a nutritional supply as well as whether there are other molecules available for their maintenance there.

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