Colonization of *Rhodnius prolixus* gut by *Trypanosoma cruzi* involves an extensive parasite killing

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SUMMARY

Trypanosoma cruzi, the etiological agent of Chagas disease, is ingested by triatomines during their bloodmeal on an infected mammal. Aiming to investigate the development and differentiation of *T. cruzi* inside the intestinal tract of *Rhodnius prolixus* at the beginning of infection we fed insects with cultured epimastigotes and blood trypomastigotes from infected mice to determine the amount of recovered parasites after ingestion. Approximately 20% of the ingested parasites was found in the insect anterior midgut (AM) 3 h after feeding. Interestingly, a significant reduction (80%) in the numbers of trypomastigotes was observed after 24 h of infection suggesting that parasites were killed in the AM. Moreover, few parasites were found in that intestinal portion after 96 h of infection. The evaluation of the numbers of parasites in the posterior midgut (PM) at the same periods showed a reduced parasite load, indicating that parasites were not moving from the AM. Additionally, incubation of blood trypomastigotes with extracts from *R. prolixus* AMs revealed that components of this tissue could induce significant death of *T. cruzi*. Finally, we observed that differentiation from trypomastigotes to epimastigotes is not completed in the AM; instead we suggest that trypomastigotes change to intermediary forms before their migration to the PM, where differentiation to epimastigotes takes place. The present work clarifies controversial points concerning *T. cruzi* development in insect vector, showing that parasite suffers a drastic decrease in population size before epimastigonesis accomplishment in PM.

Key words: *Trypanosoma cruzi*, *Rhodnius prolixus*, epimastigogenesis, trypanosome, Chagas disease.

INTRODUCTION

Chagas disease, caused by the protozoan parasite *Trypanosoma cruzi*, is the main neglected vector borne illness in terms of public health burden in the Latin American region (Hotez *et al*. 2008). Moreover, it has become an emerging threat in many countries worldwide, where infection by blood transfusion might happen in regions where screening for the disease is not routinely performed in blood banks. In recent times, the massive increase in human migration patterns makes it extremely dangerous to overlook this mode of Chagas disease transmission (Bonney, 2014). The kissing bug *Rhodnius prolixus* (Hemiptera: Reduviidae) is an important vector of Chagas disease in Northern South America (Schofield and Galvão, 2009; Hashimoto and Schofield, 2012). All developmental stages of this triatomine bug are blood-feeders and several vertebrate species can serve as their food sources. If they feed on mammals infected with *T. cruzi*, they may become vectors for this parasite (Lent and Wygodzinsky, 1979).

Bug infection begins when *T. cruzi* trypomastigotes are ingested by a triatomine during a blood meal on an infected mammal. After entering the triatomine gut, these forms are transformed into epimastigote forms, which colonize the entire intestinal tract and later differentiate into metacyclic trypomastigotes in the rectum (Garcia *et al*. 2010). *Trypanosoma cruzi* trypomastigotes must experience substantial physiological changes upon arrival to the anterior midgut (AM), the place inside the insect in which parasite–vector interactions develop. Trypomastigotes will be subjected to environmental stresses including osmolarity changes, lower temperature, as well as the presence of digestive enzymes (Kollien and Schaub, 2000). Thus, parasites need to react to these new conditions, adapt swiftly or probably be eliminated. The transformations that parasites need to go through inside

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triatomine gut might define infection success. Therefore, studying the initial phase of gut colonization becomes relevant to understand parasite population dynamics which will influence future successful transmission events. Regardless of the importance of this initial phase for the infection process, few studies about it have been developed (Dias, 1934; Eichler and Schaub, 2002).

The main aspects of the *T. cruzi* life cycle were described in 1909 (Chagas, 1909). Since then, several aspects of the interaction of *T. cruzi* and its invertebrate hosts have been studied (see review in Kollien and Schaub, 2000; Garcia *et al.* 2010). Dias (1934) described the development of *T. cruzi* in the invertebrate host, suggesting a reduction in the initial population of parasites before its establishment in the gut. In the same work, the author also mentioned that trypomastigotes differentiated to epimastigotes in the posterior midgut (PM). Nevertheless, posterior studies have assumed that trypomastigotes differentiate to the replicative forms, i.e. the epimastigotes, and to amastigotes/spheromastigotes in the AM of the insect (Azambuja *et al.* 2005; Garcia *et al.* 2010). Aiming to clarify some controversial points concerning *T. cruzi* development in the insect, we evaluated *T. cruzi* mortality and differentiation in the AM and PM of infected *R. prolixus* nymphs between 0 and 96 h post-infection (pi)-feeding.

MATERIALS AND METHODS

Organisms

Rhodnius prolixus used in this study were obtained from a laboratory colony derived from insects collected in Honduras around 1990. The colony was maintained by the Vector Behavior and Pathogen Interaction Group from CPqRR-FIOCRUZ. Triatomines were reared at 25 ± 1 °C, 60 ± 10% relative humidity and natural illumination. Insects were fed on chicken and mice anesthetized with intraperitoneal injections of ketamine (150 mg kg^{-1}; Cristália, Brazil) and xylazine (10 mg kg^{-1}; Bayer, Brazil) mixture. Fifth instar nymphs, starved for 30 days after ecdysis, were used in the assays.

CL strain *T. cruzi*, originally isolated from naturally infected *Triatoma infestans* (Brener and Chiari, 1963) was used. Epimastigote forms were cultured by two weekly passages in liver-infusion tryptose supplemented with 15% fetal bovine serum, 100 mg mL^{-1} streptomycin and 100 units mL^{-1} penicillin (Fellet *et al.* 2014). To ensure strain infectivity, parasites were submitted to cycles of triatomine-mice infection every 6 months (Elliot *et al.* 2015).

Knockout interferon gamma (INF-γ) mice (B6·129S7) infected with *T. cruzi* were used in the assays with live hosts, in order to obtain high levels of parasites in circulating blood. All experiments using live animals were performed in accordance to FIOCRUZ guidelines and were approved by its Ethics Committee on Animal Experimentation (CEUA/FIOCRUZ) under protocol number L-058/08.

Insect infection and dissection

Insects were infected by either of two methods: (1) nymphs were fed using an artificial feeder containing citrated heat-inactivated (56 °C 30 min^{-1}) rabbit blood added with a suspension of culture epimastigotes; (2) nymphs were fed on infected mice (therefore ingesting trypomastigote forms). The blood used for insect infection had 1×10^4 epimastigotes μL^{-1} (artificial feeder) or between 1×10^3 and 4×10^4 trypomastigotes μL^{-1} (live hosts). Each insect was allowed ingesting 20–30 μL of blood. The AM and PM of fed nymphs were individually dissected and homogenized in 20 μL of phosphate buffered saline (PBS; 0·15 M NaCl at 0·01 M sodium phosphate, pH 7·4) at different times pi to determine the abundance of different parasite forms.

Estimation of the number of parasites recovered in the midgut after bug ingestion

The volume of blood ingested was estimated by weighing nymphs before and immediately after feeding. The number of parasites μL^{-1} found in the AM, PM, live hosts and artificial feeder blood was assessed by counting them in 5 mm^3 of blood as previously described by Brener (1962). In experiments using anesthetized mice as infective sources, a sample of blood was collected from the host tail. The amounts of parasites found in each portion of the insect midgut after blood ingestion (calculated as described immediately above) were determined after adjusting sample volumes according to their dilution in PBS. In the assays evaluating the amount of parasites 3 h after bug ingestion we also estimated the percentage of parasites found in the AM in relation to that present in the initial blood solution.

Approximately 10 μL of each sample were transferred to a 1·5 mL microtube containing 20 μL of sterile PBS and kept at −20 °C for subsequent DNA extraction and quantification by quantitative polymerase chain reaction (qPCR). AM and PM portions were evaluated at 3 h, 1, 2, 4, 7 and 15 d pi.

DNA extraction and qPCR

DNA extraction was performed using the Wizard Genomic DNA Purification Kit (Promega) following manufacturer's instructions for DNA extraction of blood samples. DNA samples extracted from parasites maintained in culture and from the intestinal tract of non-infected triatomines were used for positive and negative controls, respectively. The products obtained from DNA extractions were amplified by qPCR using specific primers for

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the *T. cruzi* gene TCZ (5′ GCTCTTGCCCACA MGGGTGC 3′; 5′ CCAAGCAGCGGATAGTT CAGG 3′; Cummings and Tarleton, 2003; Caldas *et al.* 2012) in an ABI Prism 7500 Sequence Detection System (Applied Biosystems). The qPCR was performed in a final volume of 25 μL containing 50 ng of DNA, 300 nM of each primer and 12·5 μL Power SYBR® Green PCR Master Mix (Applied Biosystems) under the following conditions: initial denaturation at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s, primer binding and extension at 60 °C for 1 min. Standard curves were generated from five serial dilutions in water (1:10) of a mixture of DNA extracted from blood and cultured parasites following previously described methodology (Caldas *et al.* 2012). Blood from non-infected INF-γ knockout mouse was used, and the first point of the curve contained DNA amounts equivalent to 1×10^5 parasites 0·1 mL^{-1} of blood.

In vitro *evaluation of mortality for* T. cruzi *blood trypomastigotes*

To evaluate if compounds originated from *R. prolixus* tissues could kill parasites, 100 μL of blood from a *T. cruzi* infected mouse were transferred to 1·5 mL microtubes containing 100 μL Roswell Park Memorial Institute (RPMI) medium without serum (Chuenkova and Pereira, 2000; Roffê *et al.* 2011) plus:

(a) one AM from a unfed nymph;

(b) one AM from a nymph recently fed on an uninfected mouse;

(c) one salivary gland;

(d) only RPMI medium.

The AMs and salivary glands used, respectively, in (a), (b) and (c) were dissected and had their lumens exposed. The number of parasites was quantified by fresh counting (Brener, 1962) at 0 and 24 h of incubation. Cell viability was assessed by existence of flagellar movements and using the vital dye Erythrosin B (Sigma-Aldrich, St. Louis, MO, USA). Five replicates were tested for each treatment.

Differentiation of trypomastigotes into epimastigotes in the intestinal tract of R. prolixus

Midgut samples were isolated at different times pi to evaluate the differentiation of trypomastigotes into epimastigote forms. Thin smears of AMs and PMs obtained at different times pi (3, 24, 48, 96 h and 7 days for AM and 24, 48, 96 h and 15 days pi, for PM) were stained by the Giemsa method. The numbers of parasites in 50 microscopic fields were counted under a light microscope (1000× magnification; n = 10–15 AM and PM samples for each experimental point). The distinction between epimastigote and trypomastigote forms was performed according to the classification for Trypanosomatidae (Hoare and Wallace, 1966), considering the relative position of both the kinetoplast and the flagellum in the parasite cell body. The forms in which it was not possible to determine the position of the kinetoplast were called intermediate forms. The round-shaped parasites found on triatomine guts were regarded as amastigote-like forms (Tyler and Engman, 2001; reviewed by Contreras *et al.* 2006). The proportion of each form was calculated on the total of cells evaluated for each slide.

Statistical analysis

Data normality was tested using the Kolmogorov–Smirnov test. Data showing a normal distribution were analysed by means of t tests or analysis of variance (ANOVA). In the case of ANOVA, pairwise comparisons were performed by means of Tukey *post hoc* tests. Non-parametric data were analysed by Mann–Whitney or Kruskal–Wallis tests. In the case of Kruskal–Wallis tests, pairwise comparisons were performed by means of Dunn's tests. The correlation between mice parasitaemia and number of parasites recovered in AMs was performed by means of Spearman correlation. The results from the *in vitro* experiment were analysed by means of an ANOVA, followed by a subsequent Dunnett's test for *post hoc* comparisons between treatments and the control group. In all cases, differences were considered significant when $P < 0·05$.

RESULTS

Parasite population profile following infection

Parasite numbers were counted in the insects' AM 3 h after feeding. Interestingly, only 23% of the estimated ingested epimastigotes and 25% of the trypomastigotes were found. The percentage of *T. cruzi* recovered from the AMs of insects fed on the artificial feeder was not significantly different from that of bugs fed on live hosts (t test, $P = 0·37$; $n = 20$ AMs for each group).

Regardless of which parasite form started infection, parasite number massively decreased over the first 3 days after infection (Fig. 1; Kruskal–Wallis; $P = 0·0003$ for epimastigotes, $P < 0·0001$ for trypomastigotes). However, the dynamics of parasite establishment was different depending on whether trypomastigote or epimastigote forms were used for infection. Insects fed on blood containing epimastigote forms suffered a significant reduction until 72 h pi when no parasites were found anymore (Fig. 1A; Dunn, $P < 0·05$). On the other

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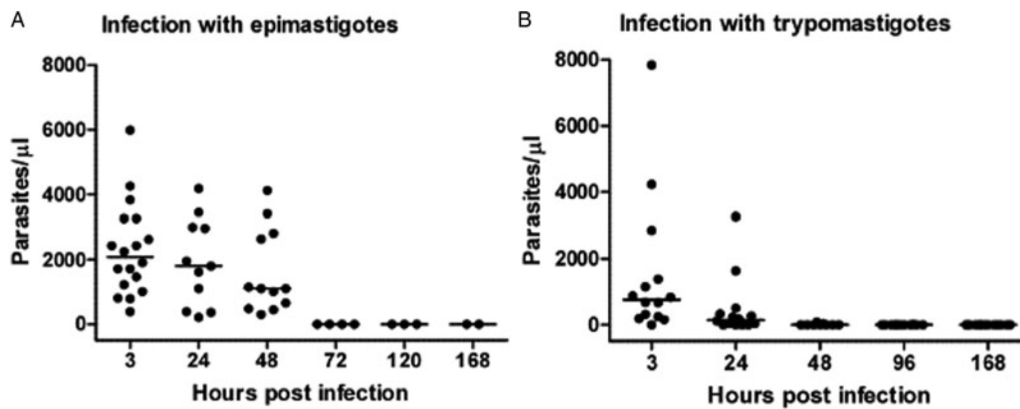

Fig. 1. Parasites are absent from the AM 72 h after infection. Temporal profile of the number of parasites μL^{-1} found in AMs after insects were fed on (A) blood containing *Trypanosoma cruzi* epimastigotes and (B) *T. cruzi* infected mice (trypomastigotes forms). The number of parasites μL^{-1} was estimated by counting parasites in 5 mm^3 of blood (Brener, 1962). Points represent the number of parasites μL^{-1} determined for each AM, while horizontal lines represent the median of each group (numbers of samples varied between 2 and 18). Abbreviation: AM, anterior midgut.

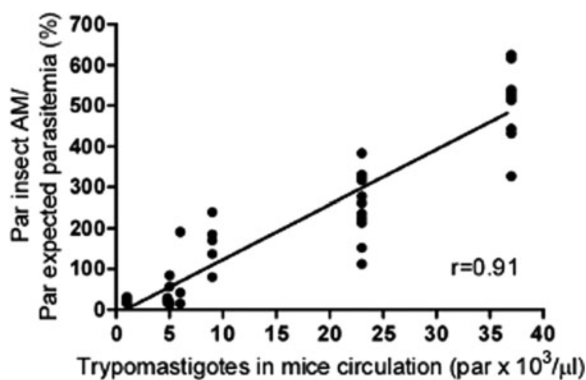

Fig. 2. Mouse parasitaemia influences the percentage of recovered parasites in insect AM. Correlation between the percentages of parasites found in the insect AM in relation to the amount expected according to the parasitaemia of the blood provided to the insect and the volume ingested and mice parasitaemia. Each point represents the percentage of parasites found in one AM (n = 42). Abbreviation: AM, anterior midgut.

hand, insects infected with trypomastigotes from infected mice showed a significant reduction in the amount of AM parasites between 3 and 48 h pi (Fig. 1B; Dunn, $P < 0{\cdot}05$). No parasite was found in the AMs in late time points (96 and 168 h pi).

Mice used to feed and infect insects showed a large variation in their parasitaemia. To check whether this variation would influence the percentage of recovered parasites in the AM of infected insects, we performed the Spearman correlation test. We found that insect population in AM samples 3 h after ingestion was directly correlated with the number of circulating parasites in corresponding mice (Fig. 2; Spearman's test, $r = 0{\cdot}9148$, $P < 0{\cdot}0001$). Parasite load in AMs varied overtime regardless of the initial number of ingested parasites (Fig. 3; Kruskal–Wallis, $P < 0{\cdot}0001$ for low and high parasitaemia). Insects fed on mice having low parasitaemia (up to 9×10^3 par mL^{-1}) showed statistically significant reductions in parasite amounts from 3 to 48 h and from 24 to 96 h pi (Fig. 3A; Dunn, $P < 0{\cdot}05$). Insects fed on mice with high parasitaemia (more than 9×10^3 par mL^{-1}) showed a reduction in the numbers of parasites from 3 to 24 h pi (Fig. 3B; Dunn, $P < 0{\cdot}05$). Nevertheless, no parasites were detected in the insect AMs after 96 h pi (Fig. 3A and B).

Since the dynamics of colonization of *T. cruzi* in *R. prolixus* AMs was similar independently of mice parasitaemia, data were thereafter evaluated in combination. In order to confirm that the decrease in parasite number observed over time was not caused by an inability to visualize parasites hindered in the guts (for instance, parasites adhered to the epithelium) we also estimated parasite loads by qPCR. AM parasite numbers varied over time regardless of which quantification method was used (ANOVA, $P = 0{\cdot}0007$ and $P = 0{\cdot}0026$ for counting parasites in fresh samples and qPCR, respectively). Parasite density in fresh samples showed a reduction of approximately 80% in the first 24 h pi (Fig. 4A; Tukey test $P < 0{\cdot}05$). On their turn, qPCR values remained similar during the first 48 h (Tukey, not significant); but a significant reduction in the amount of trypanosome DNA was observed from 48 to 96 h pi (Fig. 4B; Tukey test $P < 0{\cdot}05$). Differently from fresh samples evaluation, qPCR estimation showed a remaining amount of parasite DNA in the AMs after 96 h of infection (medians of 136·2, 109·5 and 2·38 parasite DNA at 4, 7 and 15 days, respectively).

The numbers of parasites from PMs were also measured by counting parasites on fresh samples and qPCR (Fig. 4C and D). In the fresh quantification, parasite load varied over time (Kruskal–Wallis test, $P = 0{\cdot}005$), the values obtained at 15 days pi being significantly higher than those from 2 days pi (Fig. 4C; Dunn, $P < 0{\cdot}05$). The amounts of parasite DNA measured by qPCR did not show

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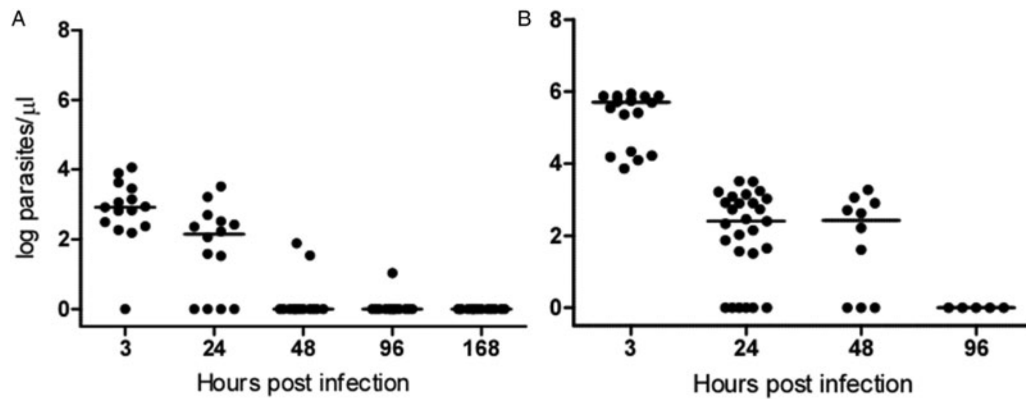

Fig. 3. Number of parasites ingested does not affect infection profile over time. Temporal variation of the number of parasites μL^{-1} in the *Rhodnius prolixus* AM after insects had fed on *Trypanosoma cruzi* infected mice with low (A) and high parasitaemia (B). The number of parasites μL^{-1} was estimated by counting parasites in 5 mm^3 of blood (Brener, 1962). Points represent the log of the amount of parasites μL^{-1} determined for each AM, while each horizontal line represents the median of each group (numbers of samples varied between 5 and 27). Abbreviation: AM, anterior midgut.

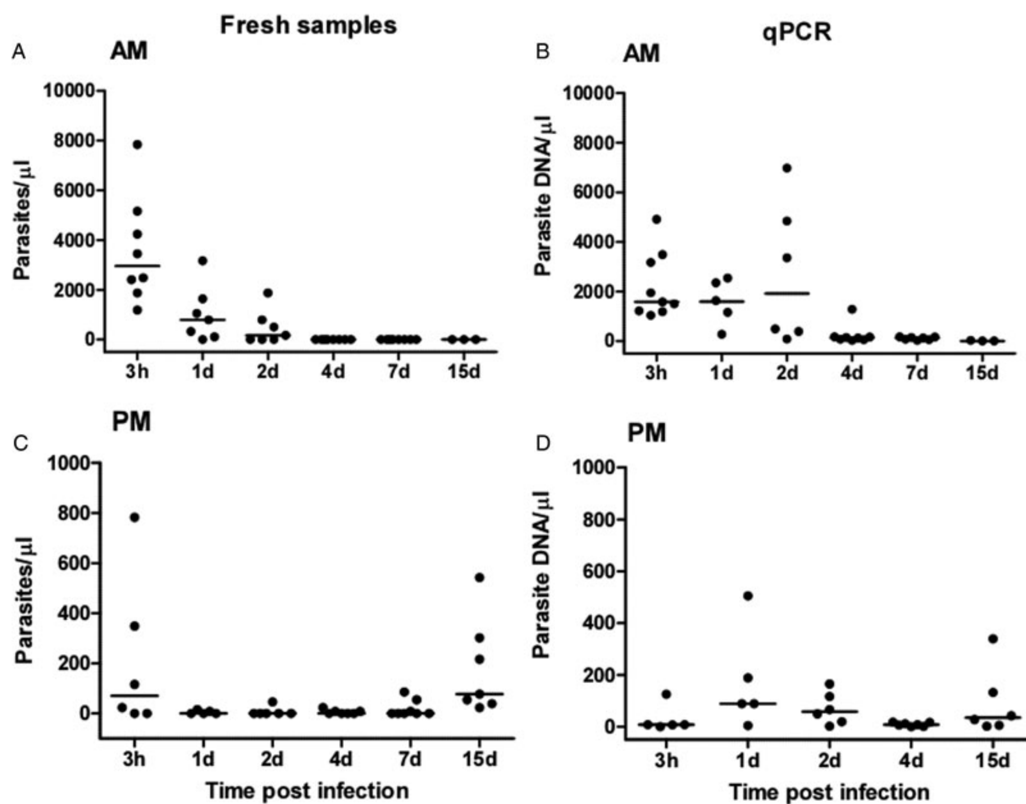

Fig. 4. Temporal profile of the number of parasites μL^{-1} and parasite DNA μL^{-1} detected in the AM (A, B) and PM (C, D) of *Rhodnius prolixus* after feeding on *Trypanosoma cruzi* infected mice. (A, C) The numbers of parasites μL^{-1} were estimated by counting parasites in 5 mm^3 of blood (Brener, 1962). (B, D) Values represented were obtained by qPCR. Each point represents the numbers of parasites μL^{-1} or parasite DNA μL^{-1} in the specific portion of the gut and each horizontal line represents the median of the group (numbers of samples varied between 3 and 8). Abbreviations: AM, anterior midgut; PM, posterior midgut; qPCR, quantitative polymerase chain reaction.

statistically significant differences along time (Fig. 4D; Kruskal–Wallis test, $P = 0{\cdot}1$).

In vitro *evaluation of mortality in* T. cruzi *blood trypomastigotes*

To evaluate whether the reduction of parasite numbers observed along the first days of infection was due to vector-produced factors, parasites were incubated for 24 h with extracts from different insect tissues. Results showed that *T. cruzi* mortality was altered by such treatments (Fig. 5; ANOVA, $P < 0{\cdot}0001$). The number of parasites kept in pure RPMI medium for 24 h showed a reduction of about 6% (Fig. 5). Lower numbers of parasites were detected after incubation with salivary glands,

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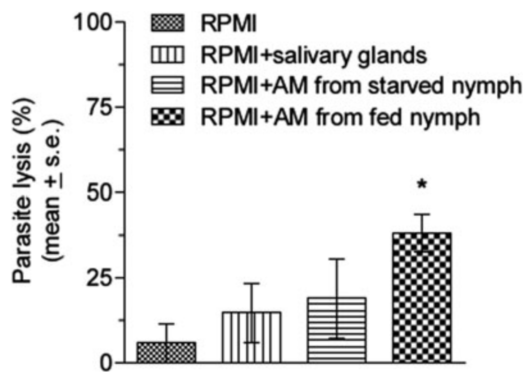

Fig. 5. Tissue extracts from *Rhodnius prolixus* kill *Trypanosoma cruzi* trypomastigotes. Reduced *T. cruzi* abundance (percentage) relative to the initial number of parasites μL^{-1} after 24 h of incubation in RPMI media added with different tissues. The bars correspond to the mean ± S.E. of 5 replicates per treatment (Dunnett, $P < 0{\cdot}05$).

AM from unfed insects and AM from fed insects, where a decrease of 15 ± 8, 19 ± 11 and 38 ± 6% was found, respectively. Those parasites incubated with AMs from fed insects showed a significant increase in mortality when compared with the control treatments (Fig. 5; Dunnett, $P < 0{\cdot}05$).

Differentiation of T. cruzi *trypomastigotes into epimastigotes*

Evaluation of different parasite forms present in the AM (Fig. 6A) revealed that 3 h after being ingested, 100% of the *T. cruzi* observed therein were trypomastigotes (Figs 7A and 6B1). These forms were gradually being substituted by intermediate/amastigote-like forms (Fig. 6B2–4) which corresponded to more than 80% of the few remaining parasites by the 7th day pi (Fig. 7A). No epimastigote forms were observed in the AM during the evaluated period. It is worth mentioning that even if trypomastigote morphology is maintained, parasites could be already under differentiation, morphology *per se* not being the best signal (although the most widely used) of parasite functional phase.

The analyses of the PM (Fig. 6A) were affected by the reduced number of parasites found in this intestinal portion (only 15 ± 6 flagellates could be counted per slide). One day after infection, the dissections showed that almost all parasites were intermediate/amastigote-like forms (Figs 7B and 6B5). The proportion of epimastigote forms started to increase at the 3rd day of infection (Fig. 7B), becoming the forms most frequently observed at 15 days pi (Figs 7B and 6B6–8).

DISCUSSION

When ingested by a triatomine, *T. cruzi* and blood quickly travel through the bug foregut to reach the AM, which seems to be the first environment where a steady interaction with vector-produced factors takes place. Since several factors that can affect parasite survival are present in this intestinal portion (Pereira *et al.* 1981; Mello *et al.* 1996; Azambuja *et al.* 2005), the time spent by parasites inside the AM should have critical implications for their future establishment as a viable/infective metacyclic trypomastigote form in the vector rectum. Although some aspects of the development of triatomine infection by *T. cruzi* have already been studied, to date most reports focused on times starting at 10 days of infection (Garcia and Gilliam, 1980; Schaub and Böker, 1986; Kollien *et al.* 1998; Carvalho-Moreira *et al.* 2003).

As stated earlier, blood trypomastigotes of *T. cruzi* are the infective forms, acquired by triatomines while feeding on infected mammals. Despite few studies have used blood trypomastigotes to infect their vectors (Carvalho-Moreira *et al.* 2003; Cordero *et al.* 2008; Botto-Mahan, 2009), most published works that evaluated parasite–vector interactions used cultured epimastigotes as an infective form for triatomines (Mello *et al.* 1996; Cortez *et al.* 2002; Azambuja *et al.* 2004; Araújo *et al.* 2007; Uehara *et al.* 2012). Our analyses using trypomastigotes and epimastigotes for infection, to test whether different developmental forms could modify population dynamics and colonization of *R. prolixus* AM showed that both forms were severely reduced from the AM soon after ingestion. However, trypomastigotes remained for a shorter time in that intestinal portion. In all assays, flagellate populations decreased with time until it was not possible to detect any forms in fresh counts after 96 h of infection. This was our first indication suggesting that *T. cruzi* does not multiply in the AM.

The number of parasites ingested by triatomines showed a positive correlation with mice parasitaemia. However, independently of the number of parasites ingested by nymphs, a drastic decrease in their populations was observed in the first 24 h pi. Work done on *Glossina morsitans* infected with *Trypanosoma brucei* showed that 99% of ingested parasites were eliminated during the initial developmental stage inside the vector (Van Den Abbeele *et al.* 1999). Two hypotheses could explain the reduction observed after the first 24 h of infection in our study. The first one proposes that parasites would quickly move from the AM to the PM, where they would differentiate to epimastigotes and subsequently multiply. In this case, a relatively high number of parasites should have been found in the PM over the first hours after infection. However, our results obtained in the PM analyses did not support this hypothesis. Specifically, a small number of parasites was found in PM 24 h pi. Significant higher number of parasites was observed in the PM only after 15 days of infection, although

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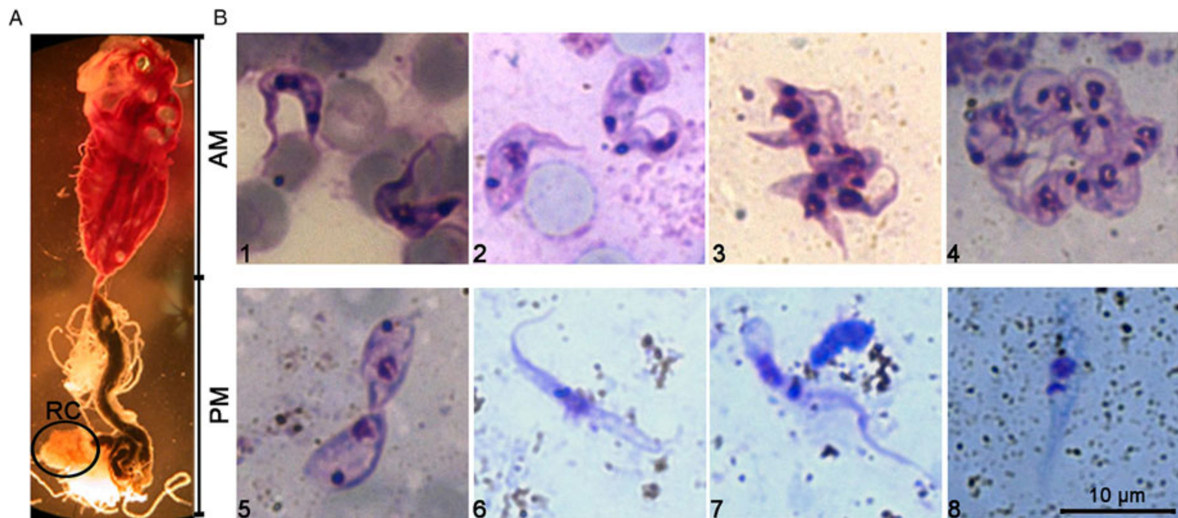

Fig. 6. *Trypanosoma cruzi* development in triatomine gut. (A) Dissected intestinal tract of fifth instar nymph 3 days after a blood meal, under a stereomicroscope, showing the AM, PM and RC. (B) Giemsa stained smears showing *T. cruzi* forms found in AM (upper row, images 1–4) and PM (lower row, images 5–8); trypomastigotes (1), intermediate forms (2, 3), amastigote-like (4, 5) and epimastigotes (6–8). Abbreviations: AM, anterior midgut; PM, posterior midgut; RC, rectum.

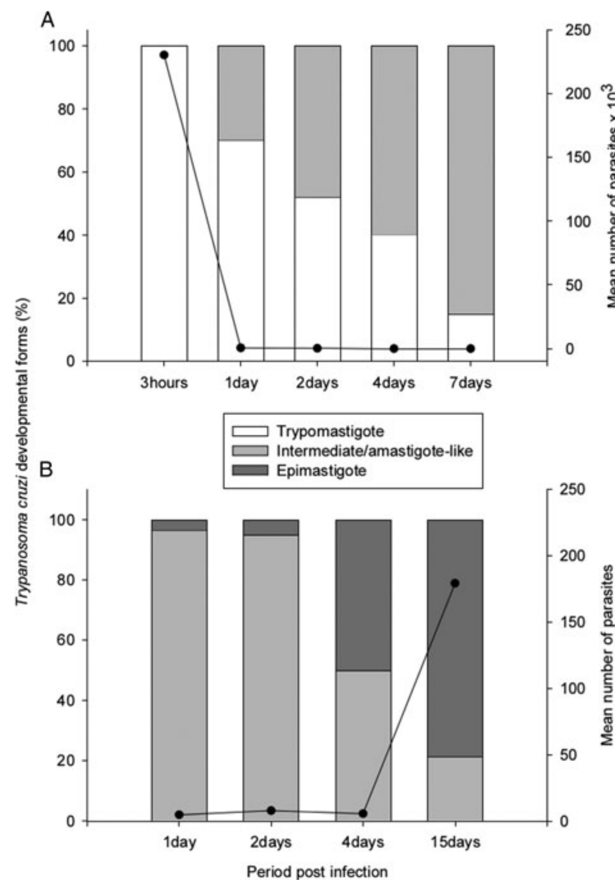

Fig. 7. Differentiation into epimastigotes does not happen in the AM. Time course showing *Trypanosoma cruzi* differentiation (%) from trypomastigotes into intermediate forms and epimastigotes. The percentage of parasite forms was calculated for AMs (A) and PMs (B) by counting 50 microscopic fields in slides stained by the Giemsa method ($n = 5$ slides evaluated for each time). The mean number of parasites found in each intestinal portion was plotted on the right axis to represent the total parasite burden (all evolutive forms) in each portion and period. Abbreviations: AM, anterior midgut; PM, posterior midgut.

still being greatly reduced in comparison with the initial infection. The evaluation by qPCR of AM and PM samples corroborated that parasites were not migrating to the PM, or staying adhered to the AM epithelium, but having their populations reduced at that portion of the digestive tract. It is worth mentioning that differently from evaluation of fresh samples, qPCR analyses were able to

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detect the presence of remaining parasite DNA in the AM after 96 h of infection. These results are in agreement with data from Dias (1934) and suggest that a residual population remains in the AM. Future studies will evaluate the role of these parasites in *T. cruzi* development.

A second hypothesis proposes that the majority of trypomastigotes entering the AM would be eliminated within 24 h pi, as it has been shown for tsetse flies infected by *T. brucei* blood forms (Turner *et al.* 1988). Dias (1934) suggested that the initial development of *T. cruzi* in triatomine would be essentially a regression phase, which would precede the multiplication phase in PM. To test the hypothesis of trypomastigote lysis in AM, we developed an *in vitro* assay in which blood trypomastigotes were incubated with tissues extracts from different organs of the insect. Salivary glands and the AM from fed insects were included in the assay, since triatomines ingest part of the released saliva during the feeding process (Ribeiro and Francischetti, 2003; Soares *et al.* 2006). In addition, *T. infestans* saliva has been shown to contain a pore-forming molecule (trialysin) that lyses *T. cruzi* trypomastigotes (Amino *et al.* 2002). A significant parasite killing was only observed when parasites were incubated with the AM from a recently fed insect, suggesting that factors present in the AM after feeding, such as a trialysin-like molecule, play a role in parasite elimination. Several factors have been related to the establishment of *T. cruzi* infection in the vector, such as lytic factors (Azambuja *et al.* 1983), lectins (Pereira *et al.* 1981; Mello *et al.* 1996) and haemoglobin fragments (Garcia *et al.* 1995). The induction of immune response factors (Whitten *et al.* 2007; Ursic-Bedoya *et al.* 2008), which also occurs in order to control symbiont population growth (Garcia *et al.* 2010), would also contribute to the AM becomes an inhospitable environment for *T. cruzi*. Recent studies have shown that *T. cruzi* infection can modulate microbiota growth and suggest that this modulation is necessary to guarantee parasite development (Castro *et al.* 2012; Soares *et al.* 2015). The population size of *Rhodococcus rhodnii*, a *R. prolixus* intestinal symbiont, can show an increase of almost 80 times in the anterior portions of the intestinal tract after the ingestion of a blood meal (Eichler and Schaub, 2002). Whether the decrease in parasite population size could also be related with a symbiont-insect immune response-trypanosome interaction is an interesting question for further studies.

Most of the recent studies working with triatomine–*T. cruzi* interactions assume that after a few days at the AM trypomastigotes differentiate into epimastigotes and spheromastigotes (Garcia and Azambuja, 1991; Kollien and Schaub, 2000; Azambuja *et al.* 2005; Garcia *et al.* 2010). Our data, however, showed that no *T. cruzi* epimastigotes can be found in the AM after blood trypomastigote ingestion, indicating that the differentiation into those multiplicative forms, also known as epimastigogenesis, occurs in the PM of *R. prolixus*. Classical work from Dias (1934) corroborate our data, showing that the differentiation to epimastigotes only begin in the AM, ending after the arrival of intermediate forms at PM, where epimastigotes will replicate.

Studies analysing the differentiation of cultured cell derived trypomastigotes to epimastigotes have showed that *in vitro* epimastigogenesis occurs by amastigote-like (Albesa and Eraso, 1981; De Lima *et al.* 2007; Graterol *et al.* 2013) and spheromastigote (Rondinelli *et al.* 1988) forms that, in turn, give rise to epimastigotes. Furthermore, the occurrence of these intermediary forms seems to be related to nutritional composition of culture medium (Albesa and Eraso, 1981). Our results from *in vivo* experiments suggest a similar differentiation route with the presence of non-replicative amastigote-like intermediary forms. The Giemsa-stained smear analysis suggests that trypomastigotes transform into amastigote-like and intermediate forms, but these amastigote-like forms did not seem to replicate, since cells in division were not found. Considering *in vivo* observations (Dias, 1934) and *in vitro* studies (Albesa and Eraso, 1981; Rondinelli *et al.* 1988; De Lima *et al.* 2007; Graterol *et al.* 2013) together with our results, we propose that differentiation of *T. cruzi* blood trypomastigotes into epimastigotes occurs mostly through intermediate amastigote-like forms. Nevertheless, distinctly from *in vitro* studies, *in vivo* epimastigogenesis show the uniqueness of spatial separation, since complete differentiation and epimastigotes replication occurs only in PM.

One should acknowledge that *T. cruzi* represents a very diverse group of strains composed of six discrete typing units (DTUs) (reviewed by Zingales *et al.* 2012), indicating that results obtained with specific strains may not be generalized. In our study, we have used the CL strain (group VI), which has been shown to produce similar infection profiles in *R. prolixus*, when compared with DM28c, a *T. cruzi* I (TCI) strain (Mello *et al.* 1996; Uehara *et al.* 2012). We suggest that our findings represent relevant information for understanding the development of *T. cruzi* in its invertebrate host. However, future work addressing such parasite diversity would be desirable.

In conclusion, we suggest including parasite transformations taking place during the first hours of triatomine infection in the current description of the *T. cruzi* cycle. Our current model shows that immediately after ingestion, factors present in the AM would be responsible for a significant reduction of the incoming trypomastigote population, leading to a bottleneck in parasite population. The surviving trypomastigotes transform into amastigote-like and intermediate forms that will differentiate into

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epimastigotes in the PM. Since the process leading to this extensive parasite elimination inside the triatomine AM is still largely unknown, it will be interesting to investigate whether the mechanisms promoting such events are based on immune response activation, antimicrobial factors or other biochemical/physiological processes.

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