

Original article

Innate trypanolytic factors in triatomine hemolymph against *Trypanosoma rangeli* and *T. cruzi*: A comparative study in eight Chagas disease vectors

Factores tripanolíticos innatos contra *Trypanosoma rangeli* y *T. cruzi* en la hemolinfa de triatominos: un estudio comparativo en ocho especies de vectores de la enfermedad de Chagas

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Abstract

There is limited information about the innate immunity of triatomines against *Trypanosoma rangeli*, an infectious, non-pathogenic human parasite, and *T. cruzi*, the causative agent of Chagas' disease. This study aimed at addressing this gap by studying the *in vitro* trypanolytic hemolymph activity from insects not infected by *T. rangeli* or *T. cruzi*. Eight triatomine species were examined including *Rhodnius prolixus*, *R. robustus*, *R. colombiensis*, *R. pallescens*, *R. pictipes*, *Triatoma dimidiata*, *T. maculata*, and *Panstrongylus geniculatus*. The hemolymph of *R. prolixus* and *R. robustus* laboratory colonies demonstrated strong trypanolytic activity during the first 14 hours of parasite incubation with 81% lysis for the *T. rangeli* C genotype, 90% for the E genotype, 95% for *T. cruzi* discrete taxonomic unit (DTU) TcII, 94% for TcV, 96% for TcVI, 94% for Tcbat, and 90% for Tcmarinkellei. No lysis activity was detected 14 or 24 hours after parasite incubation with the hemolymph of insects from *R. pictipes*, *R. pallescens*, *P. geniculatus*, and *T. maculata* colonies. Identical results (absence of lysis) were observed using hemolymph from *R. colombiensis* and *T. dimidiata* laboratory colonies and specimens captured in sylvatic environments. The hemolymph lytic activity against recently obtained cultures (95% decrease in live parasite count) and long-term *T. cruzi* TcII ones (96% decrease) was similar. Lytic activity was similar in hemolymph from *R. prolixus* nymphs, males and females and insects fed on chicken or mouse blood. This is the first comparative study of the trypanolytic activity of hemolymph from different triatomine species against *T. rangeli* and *T. cruzi* genotypes. Although the chemical structures of such lysis factors and the mechanisms determining their expression have not been fully determined, their identification furthers our understanding of triatomines' innate immunity and their role in *Trypanosoma* transmission.

Keywords: *Trypanosoma cruzi*; *Trypanosoma rangeli*; *Rhodnius prolixus*; *Rhodnius robustus*; trypanolytic factors; vectorial ability; insect immunity

Resumen

Hay poca información sobre la inmunidad innata de los triatominos contra *Trypanosoma rangeli*, un parásito infeccioso para el humano, aunque no patógeno, y *T. cruzi*, el agente causal de la enfermedad de Chagas. En este trabajo se buscó llenar ese vacío mediante el estudio de la

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actividad tripanolítica *in vitro* de la hemolinfa de insectos no infectados con *T. rangeli* o *T. cruzi*. Se examinaron ocho especies de triatomíneos: *Rhodnius prolixus*, *R. robustus*, *R. colombiensis*, *R. pallescens*, *R. pictipes*, *Triatoma dimidiata*, *T. maculata*, y *Panstrongylus geniculatus*. La hemolinfa de las colonias de laboratorio de *R. prolixus* y *R. robustus* presentó una fuerte actividad tripanolítica durante las primeras 14 horas de incubación con los parásitos, evidenciando 81 y 90 % de lisis para los genotipos C y E de *T. rangeli* y 95 % para la unidad taxonómica discreta (DTU) de TcII, 94 % para TcV, 96% para TcVI, 94 % para Tcbat y 90 % para Tcmarinkellei. No se detectó lisis a las 14 ni a las 24 horas de incubación de los parásitos con las hemolinfas de colonias de laboratorio de *R. pictipes*, *R. pallescens*, *P. geniculatus* y *T. maculata*. Se registraron resultados idénticos (ausencia de lisis) con la hemolinfa de colonias de laboratorio de *R. colombiensis* y *T. dimidiata* y de especímenes capturados en ambientes selváticos. La actividad lítica de la hemolinfa fue similar en cultivos recientes de TcII (95 % de lisis) y en aquellos mantenidos por periodos prolongados (96 % de lisis). La actividad lítica de la hemolinfa de *R. prolixus* fue similar en ninfas, machos y hembras y en insectos alimentados con sangre de gallina o sangre de ratón. Este es el primer estudio comparativo de la actividad tripanolítica en la hemolinfa de diferentes especies de triatomíneos contra genotipos de *T. rangeli* y *T. cruzi*. Aunque la estructura química de los factores líticos, así como los mecanismos que determinan su expresión, aún no se han establecido completamente, su identificación permitirá comprender mejor la inmunidad innata de los triatomíneos y su papel en la transmisión de los tripanosomas.

Palabras clave: *Trypanosoma cruzi*; *Trypanosoma rangeli*; *Rhodnius prolixus*; *Rhodnius robustus*; factores tripanolíticos; Capacidad vectorial; Inmunidad en insectos.

Introduction

Trypanosoma rangeli, a non-pathogenic hemoflagellate capable of infecting a variety of mammalian species, including humans, is transmitted via triatomine insects belonging to the genus *Rhodnius* (Hoare, 1972; Guhl & Vallejo, 2003). The interaction between the parasite and *Rhodnius prolixus* begins with the ingestion of blood containing trypomastigotes. Following ingestion, the parasites develop into epimastigotes in the intestine of the vector, where they come into contact with the products of blood digestion and other components of the midgut including the bacterial microbiota, hemolytic factors, lectins, the prophenoloxidase system, antimicrobial peptides, and reactive nitrogen and oxygen species (Vieira, *et al.*, 2015). While these factors can act as biochemical barriers to *T. rangeli* infection, the parasite can still cause immunosuppression in *R. prolixus* by inhibiting processes such as phagocytosis, microaggregation of hemocytes, prophenoloxidase activation, and eicosanoid synthesis (Figueiredo, *et al.*, 2008; García, *et al.*, 2004a, 2004b). Five *T. rangeli* genotypes (A-E) have been identified (Maia da Silva, *et al.*, 2007; Vallejo, *et al.*, 2015); however, we do not know whether all genotypes are capable of crossing the intestinal cell wall in *R. prolixus*, where they multiply in the hemocoel, migrate to the salivary glands, and produce infectious metacyclic trypomastigotes to complete their life cycle.

Trypanosoma cruzi, the etiologic agent of Chagas disease, is transmitted by more than 140 species of triatomines (Galvao & Justi, 2015). Blood trypomastigotes are ingested by the vector in the intestine and then they progress to the amastigote, epimastigote, and spheromastigote developmental stages before they are finally eliminated in the feces as infectious metacyclic trypomastigotes (Vallejo, *et al.*, 1988). All *T. cruzi* strains, which are classified into six discrete-typing units (DTU) (TcI–TcVI) (Zingales, *et al.*, 2009, 2012), as well as the more recently identified Tcbat (Marcili, *et al.*, 2009) and *T. cruzi marinkellei* strains (Barker, *et al.*, 1978), remain exclusively within the intestine of the insect vector where they interact with the intestinal microbiota and counteract the effects of host defense factors such as defensins (López, *et al.*, 2003; Vieira, *et al.*, 2016), prolixicins (Ursic-Bedoya, *et al.*, 2011), lysozymes (Buarque, *et al.*, 2013; Ursic-Bedoya, *et al.*, 2008), lectins (Pereira, *et al.*, 1981; Mello, *et al.*, 1996), components of the phenoloxidase cascade (Castro, *et al.*, 2012; De Fuentes-Vicente, *et al.*, 2016), and nitric oxide (Whitten, *et al.*, 2007).

In a previous study, **Pulido, *et al.*** identified differential lytic activity against *T. rangeli* strains in hemolymph from *R. prolixus* colonies fed on chicken blood and maintained for 10 years in the laboratory (**Pulido, *et al.*, 2008**). Similarly, **Mello, *et al.*** showed differential trypanolytic activity against different *T. cruzi* strains in hemolymph from a 20-year-old colony of *R. prolixus* fed on citrated rabbit blood (**Mello, *et al.*, 1996**). These results indicate that *R. prolixus* can generate trypanolytic factors in hemolymph without prior exposure to *T. rangeli* or *T. cruzi*, which may have some effect on parasite development. While the factors responsible for this lytic activity have yet to be fully characterized, they are likely to be related to a systemic immune response of the vector to other microorganisms of the intestinal microbiota.

To understand the dynamics of parasite transmission, a tripartite approach focusing on triatomine-trypanosome-microbiome interactions has been proposed, which requires the integration of microbiological, genomic, ecological, bioinformatics, biochemical, and biological techniques to explain these interactions and how they may affect the transmission of trypanosomes (**Salcedo-Porras & Lowenberger, 2019**). To achieve this, it is necessary to use both wild and established triatomine colonies with different microbiota from geographically distinct locations together with *Trypanosoma* strains with different genotypes and DTUs to study innate immunity and links with the biology of trypanosomes that could be exploited to reduce parasite transmission (**Salcedo-Porras & Lowenberger, 2019**).

Our search of current literature revealed little information on the presence of innate trypanolytic factors in the hemolymph of triatomines other than *R. prolixus*. Therefore, we aimed to examine laboratory populations of five *Rhodnius* species, *Triatoma dimidiata*, *T. maculata*, and *Panstrongylus geniculatus*, as well as wild populations of *R. pallidus* and *T. dimidiata*, for the presence of innate trypanolytic factors against a range of *T. rangeli* and *T. cruzi* strains belonging to different genotypes and discrete taxonomic units (DTUs). Additionally, we studied whether the trypanolytic activity was related to the sex and stage of the insect, the time parasites are kept in culture and the time of the colonies in the laboratory. This is the first comparative study on the innate trypanolytic activity of hemolymph among different triatomine species. The results of this study provide a basis for further studies aimed at characterizing the intestinal microbiota of triatomines, which may impact the innate immunity of the vector species, and at identifying the factors responsible for the lytic activity of the hemolymph against *T. rangeli* and *T. cruzi* and their possible role in the transmission of parasites.

Materials and methods

Trypanosoma rangeli and *Trypanosoma cruzi* strains

Trypanosoma rangeli strains belonging to genotypes B, D, and E and *T. cruzi* strains belonging to reference DTUs TcII–TcVI were provided by Dr. Martha G. Teixeira from the Institute of Biomedical Sciences at the University of São Paulo. These strains were removed from liquid nitrogen storage and thawed. Once they arrived at the Tropical Parasitology Research Laboratory (LIPT) at the University of Tolima, they were refrozen in liquid nitrogen. Aliquots were thawed and cultured as needed. The *T. rangeli* genotype A strain (Choachi) was maintained by mouse-culture-*R. prolixus* cyclic passes every 3 months, while the genotype C strain (R. col 0226) was maintained by mouse-culture-*Rhodnius colombiensis* cyclic passes every 3 months (**Vallejo, *et al.*, 1986**). Clones of both domestic and sylvatic TcI, as well as Tcbat and *T. cruzi marinkellei* strains isolated from bats in Colombia and maintained in liquid nitrogen, were also used in this study. Additionally, TcI (MHOM/CO/03/CG) and TcII (Y) strains were maintained in ICR mice. *T. cruzi* DTUs and *T. rangeli* genotypes were confirmed using previously described molecular markers (**Souto & Zingales, 1993; Souto, *et al.*, 1996; Brisse, *et al.*, 2001; Villa, *et al.*, 2013; Vallejo, *et al.*, 2002; Maia da Silva, 2007**). For assays involving *T. cruzi*, we used 36 representative strains belonging to DTUs TcI–TcVI and Tcbat, one domestic *T. cruzi* TcI clone, and

one sylvatic *T. cruzi* TcI clone. For the assays involving *T. rangeli*, we used 23 strains representing genotypes A–E along with one *Trypanosoma leeuwenhoekii* strain (Table 1). *T. cruzi* strains were cultured at 28°C in biphasic Novy-McNeal-Nicolle/liver infusion tryptose medium supplemented with 10% fetal bovine serum while *T. rangeli* strains were cultured in biphasic Bacto™ beef medium supplemented with 10% fetal bovine serum.

Table 1. Strains of *T. rangeli* genotypes and *T. cruzi* DTUs used in this study

<i>T. rangeli</i> genotype or <i>T. cruzi</i> DTU	Strain	Host	Geographical origin	Lysis caused by <i>R. prolixus</i> hemolymph
<i>T. rangeli</i> A	Duran	<i>Homo sapiens</i>	Cundinamarca (Colombia)	-
<i>T. rangeli</i> A	Choachi	<i>R. prolixus</i> (domestic)	Cundinamarca (Colombia)	-
<i>T. rangeli</i> A	TrD P19	<i>R. prolixus</i> (domestic)	Norte Santander (Colombia)	-
<i>T. rangeli</i> A	TrP 500	<i>R. prolixus</i> (sylvatic)	Casanare (Colombia)	-
<i>T. rangeli</i> B	Saimiri	<i>Saimiri sciureus</i>	Manaus (Brazil)	-
<i>T. rangeli</i> C*	Ort Q 09-1	<i>Artibeus lituratus</i>	Ortega (Colombia)	+
<i>T. rangeli</i> C	Ort Q 09-2	<i>A. lituratus</i>	Ortega (Colombia)	+
<i>T. rangeli</i> C	R. col 089	<i>R. colombiensis</i>	Coyaima (Colombia)	+
<i>T. rangeli</i> C	R. col 0201	<i>R. colombiensis</i>	Coyaima (Colombia)	+
<i>T. rangeli</i> D	SC-58	<i>Echimys dasythrix</i>	Santa Catarina (Brazil)	-
<i>T. rangeli</i> E	643	<i>Platyrrinus lineatus</i>	Miranda (Brazil)	+
<i>T. leeuwenhoekii</i>	Tas 250	<i>Bradypus variegatus</i>	Pacific Region (Colombia)	+
<i>T. cruzi</i> I **	Ort 1-5	<i>R. colombiensis</i>	Ortega (Colombia)	-
<i>T. cruzi</i> I	Dm28	<i>Didelphis marsupialis</i>	Coyaima (Colombia)	-
<i>T. cruzi</i> I	833	<i>H. sapiens</i>	Pará (Brazil)	-
<i>T. cruzi</i> I	D1	<i>D. marsupialis</i>	Coyaima (Colombia)	-
<i>T. cruzi</i> I domestic clone	MHOM/CO/03/CG	<i>H. sapiens</i>	Caquetá (Colombia)	-
<i>T. cruzi</i> Id sylvatic clone	Rcol/02	<i>R. colombiensis</i>	Coyaima (Colombia)	-
<i>T. cruzi</i> II	Y	<i>H. sapiens</i>	Sao Paulo (Brazil)	+
<i>T. cruzi</i> II	139	<i>D. aurita</i>	Sao Paulo (Brazil)	+
<i>T. cruzi</i> III	MT 3663	<i>P. geniculatus</i>	Amazonas (Brazil)	-
<i>T. cruzi</i> IV	José Julio	<i>H. sapiens</i>	Amazonas (Brazil)	-
<i>T. cruzi</i> V	967	<i>H. sapiens</i>	Bolivia	+
<i>T. cruzi</i> VI	CL Brener	<i>T. infestans</i>	Rio Grande do Sul (Brazil)	+
<i>T. cruzi</i> bat	Ortq07-1	<i>Artibeus lituratus</i>	Ortega (Colombia)	+
<i>T. cruzi</i> bat	Ortq07-2	<i>A. lituratus</i>	Ortega (Colombia)	+
<i>T. cruzi</i> bat	Ortq07-3	<i>A. lituratus</i>	Ortega (Colombia)	+
<i>T. cruzi</i> bat	Ortq08-1	<i>Carollia perspicilata</i>	Ortega (Colombia)	+
<i>T. cruzi</i> bat	Cha Q 11-2	<i>A. lituratus</i>	Chaparral (Colombia)	+
<i>T. cruzi</i> bat	Cha Q 12-1	<i>C. perspicilata</i>	Chaparral (Colombia)	+
<i>T. cruzi marinkellei</i>	Cha Q 08-1	<i>Phyllostomus hastatus</i>	Chaparral (Colombia)	+
<i>T. cruzi marinkellei</i>	Cha Q 08-2	<i>P. hastatus</i>	Chaparral (Colombia)	+

* In addition to the four strains of *T. rangeli* C described, 13 strains of the same genotype that presented lysis caused by the hemolymph of *R. prolixus* were also tested.

** In addition to the six strains of *T. cruzi* I described in the table, 14 strains of this DTU that also presented resistance to lysis caused by the hemolymph of *R. prolixus* were tested.

+: Presence of lysis

Triatomines

In this study, we used fifth-instar nymphs and adults belonging to Colombian triatomine species *R. colombiensis*, *R. pallezensis*, *R. pictipes*, *R. prolixus* (domestic), *R. prolixus* (sylvatic), *R. robustus*, *T. dimidiata*, *T. maculata*, and *P. geniculatus*, which were maintained in an insectary. The insects were fed weekly on chickens and housed in incubators with a 12-h light/12-h dark photoperiod at 28°C and 80% relative humidity. Fifth-instar nymphs and adults belonging to the species *R. colombiensis* and *T. dimidiata* were also collected from sylvatic environments and used to obtain hemolymph for the analysis of their trypanolytic activity.

Detection of trypanolytic activity against T. rangeli and T. cruzi in the hemolymph of triatomines

Insects were fed on chickens 8 days before the assays. Hemolymph was collected by making a cut in the femur of the third hind leg and applying very slight abdominal pressure. Hemolymph samples from 20 individuals of each species were pooled and then centrifuged at $14,000 \times g$ for 5 min. The resulting cell-free supernatant was used in trypanolytic activity assays performed as described previously by Pulido, *et al.* (2008). Briefly, to prevent melanization, 2 μ l of 50 mM of phenylthiourea were added to each 100- μ l volume of hemolymph. Cultures of *T. cruzi* or *T. rangeli* were washed three times with 0.9% (w/v) saline solution, centrifuged at $7000 \times g$ for 5 min, and then resuspended in 10% (v/v) liver infusion tryptose medium. Ten microliters of hemolymph were then added to 10- μ l aliquots of parasite suspension at a final concentration of 2.5×10^7 parasites/ml. To assess the lytic activity, live parasites were counted in a Neubauer chamber after 0 and 14 h of incubation. A negative control consisting of hemolymph inactivated with 10 μ l of pepsin solution (15 mg/ml in 1M HCl) per 100 μ l of hemolymph and incubated at 37°C for 4 h was included in all experiments. *T. rangeli* genotype C (Rcol 0226) and *T. cruzi* TcII (Y) strains were used as positive controls with both strains always lysed when incubated with hemolymph from *R. prolixus*. *T. rangeli* genotype A (Choachi) and *T. cruzi* TcI (Dm28) strains were used as negative control strains, which were not lysed when incubated with hemolymph from *R. prolixus*. For both the negative and positive controls, the parasites were resuspended at a final concentration of 2.5×10^7 parasites/ml. All assays were carried out in triplicate.

An underlying question of this study was whether keeping the triatomine colonies in the laboratory could affect the innate lytic factors, whereby lytic activity could be lost after several generations. To address this question, we collected *R. colombiensis* and *T. dimidiata* specimens from the field and used pooled hemolymph from each species in trypanolytic activity assays with the *T. cruzi* TcII (Y) strain. All controls described above were included in these assays. The results were compared with those obtained using colonies of the same species that had been maintained in the laboratory for 5 and 20 years.

We also investigated whether the long-term maintenance of cultured epimastigotes could affect their resistance to lysis. In these assays, *R. prolixus* hemolymph was incubated with *T. cruzi* TcII strain Y obtained from recent hemoculture of mouse blood or with *T. cruzi* TcII strain Y that had been kept in culture with weekly subcultures for 4 years. We also compared the lytic activity of hemolymph from vectors fed with chicken blood or mouse blood, and from *R. prolixus* nymphs, males, and females.

Ethical aspects

The project was approved by the Central Research and Scientific Development Committee at the University of Tolima after verifying that the proposed protocols were in accordance with the ethical standards laid down in Resolution 8430 of 1993 by the Colombian Ministry of Health for the handling of laboratory animals. The technicians in care of the animals at the LIPT observed all aspects related to their maintenance following strict regulations for ensuring the animals' consistent care and handling.

Statistical analysis

Parasite counts at 0 and 14 h of incubation are presented as means \pm standard deviation. For strains showing lysis at 14 h, the percentage of lysed cells was calculated by comparing with the number of parasites at 0 h. *T. rangeli* and *T. cruzi* strains that were not lysed at 14 h were observed for 10 h more (24 h of incubation). We conducted a one-way analysis of variance (ANOVA) with Tukey's post hoc analysis to identify significant differences among the strains. The differences between the treatments and the controls were considered statistically significant at $p < 0.05$. Graphs were generated using the GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA).

Results

Effects of *R. prolixus* hemolymph on *T. rangeli* strains belonging to different genotypes

The effects of *R. prolixus* hemolymph on the survival of *T. rangeli* genotype A (Choachi) and genotype C (Rcol0226) strains are shown in **figure 1**. The *T. rangeli* genotype C strain showed an 86% decrease in live parasite numbers at the 14-h time point compared to those at time point 0 in the presence of native hemolymph but was not affected by incubation with the inactivated hemolymph. Instead, no changes in the live parasite counts of the *T. rangeli* genotype A strain were observed following incubation with either the inactivated or native hemolymph suggesting that it was not susceptible to the lytic effects of the hemolymph. Therefore, these two strains were used as the positive (presence of lysis) and negative (absence of lysis) controls, respectively, for all subsequent analyses of genotype B, D, and E *T. rangeli* strains.

Representative strains belonging to each of the remaining *T. rangeli* genotypes (B, D, and E) were then assessed for their susceptibility to the lytic activity of the *R. prolixus* hemolymph (**Figure 2**). No significant changes in the live parasite counts of genotype A, B, and D strains were observed after 14 h of incubation with either the inactivated or native hemolymph while 81% and 90% decreases in live parasite counts were observed for genotype C and E strains, respectively, following incubation with native hemolymph. The genotype A, B, and D strains remained resistant to lysis after 24 h of incubation (**Table 2**).

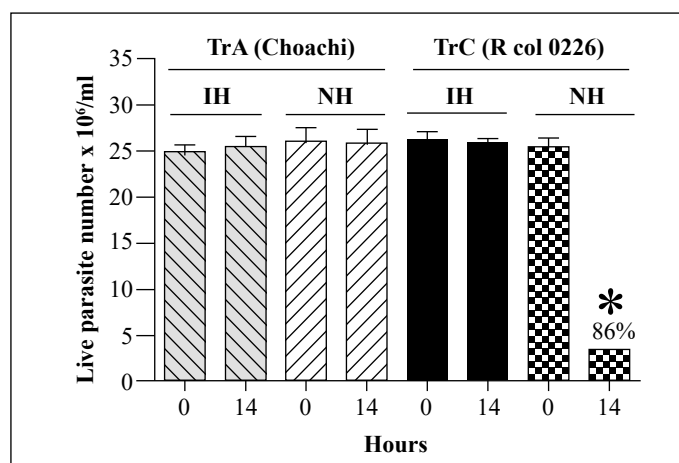


Figure 1. *In vitro* survival of *T. rangeli* genotype A (Choachi) and C (Rcol0226) strains following incubation with hemolymph from *R. prolixus*

Each bar represents the mean \pm SD of live parasite counts from three independent assays after 0 and 14 h of incubation. Each *T. rangeli* strain was incubated with inactivated hemolymph (IH) or native hemolymph (NH).

*: $p < 0.05$ compared with the live parasite counts of genotypes A and C strains incubated with NH at the same time point.

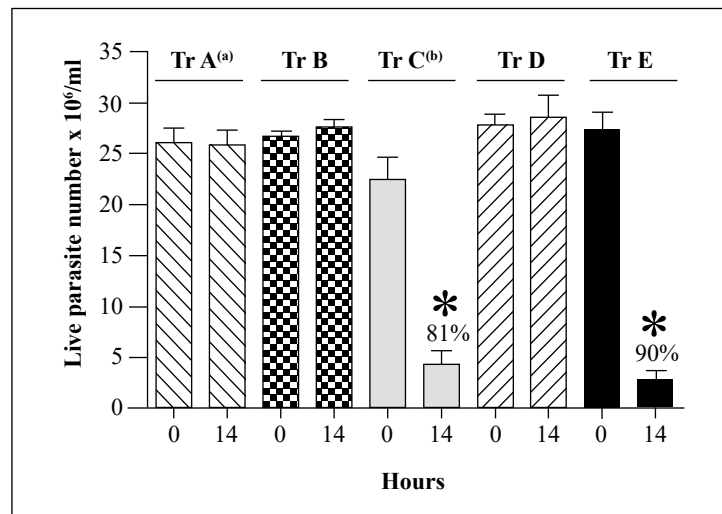


Figure 2. Summary of *in vitro* survival of genotypes A–E of *T. rangeli* strains following incubation with native hemolymph from *R. prolixus*

Each bar represents the mean \pm SD of live parasite counts from three independent assays after 0 and 14 h of incubation with native hemolymph. *T. rangeli* genotypes A (Choachí), B (Saimiri), C (Rcol0226), D (SC58), and E (643) strains were examined.

^a: Lysis resistance was observed in four genotype A *T. rangeli* strains.

^b: Lysis susceptibility was observed in 17 genotype C *T. rangeli* strains. Only one strain was tested for each of B, D, and E genotypes (Table 1). The Rcol0226 and Choachí strains were used as the positive (presence of lysis) and negative (absence of lysis) controls, respectively, for analyses of genotypes B, D, and E strains.

*: $p < 0.05$ compared with the live parasite counts of the negative control with the native hemolymph NH at the same time point.

Effects of *R. prolixus* hemolymph on *T. cruzi* strains belonging to different DTUs

The effects of *R. prolixus* hemolymph on the survival of *T. cruzi* TcI (Dm28) and TcII (Y) strains were then examined (**Figure 3**). No significant changes in the live parasite counts of the *T. cruzi* TcI strain were observed following incubation with either the inactivated or native hemolymph. In contrast, there was a 95% decrease in live parasite numbers of the *T. cruzi* TcII strain (Y) after 14 h of incubation with the native hemolymph compared with the counts at time point 0. As a result, *T. cruzi* TcI strain Dm28 and TcII strain Y were used as negative and positive controls, respectively, for all further assays.

We examined then the susceptibility of the remaining *T. cruzi* strains (TcIII, TcV, TcVI, Tcbat, and Tcmarinkellei) to lysis by the hemolymph (**Figure 4**). Analysis of 20 TcI strains showed complete resistance to lysis in all cases. The TcIII strain showed only partial susceptibility to lysis with a 13% decrease in live parasite counts observed after 14 h of incubation with the native hemolymph compared with parasite counts at time point 0. In contrast, decreases in live parasite counts of 95%, 94%, 96%, 94%, and 90% were observed for the TcII, TcV, TcVI, Tcbat, and *T. cruzi marinkellei* strains, respectively, after 14 h of incubation with the native hemolymph. Interestingly, analysis of a TcIV strain (Jose Julio) revealed a high degree of agglutination but no evidence of lysis after 24 h of incubation with the native hemolymph (**Table 2**).

Table 2 summarises the results of the hemolymph lytic activity in eight triatomine species after 24 hours of incubation with representative strains from five *T. rangeli* genotypes, seven *T. cruzi* DTUs, and a *T. cruzi* subspecies. The *T. rangeli* C and E genotype strains and the DTU TcII, TcIII, TcV, TcVI, Tcbat, and the Tcmarinkellei strains were lysed by *R. prolixus* and *R. robustus* hemolymphs. *T. rangeli* genotype A and B strains and TcI strain were not lysed. The TcIV strain became agglutinated but not lysed. It is

Table 2. Trypanolytic activity of hemolymph of eight triatomine species on epimastigotes of *T. rangeli* genotypes and *T. cruzi* DTUs at 24 hours of incubation

Vector species	<i>T. rangeli</i> A (Choachí)	<i>T. rangeli</i> B (Saimiri)	<i>T. rangeli</i> C Rcol0226	<i>T. rangeli</i> D SC58	<i>T. rangeli</i> E (643)	<i>T. cruzi</i> I (Dm28)	<i>T. cruzi</i> II (Y)	<i>T. cruzi</i> III (MT3663)	<i>T. cruzi</i> IV José Julio	<i>T. cruzi</i> V (967)	<i>T. cruzi</i> VI (CL Brener)	<i>T. cruzi</i> bat Ort07-1	<i>T. cruzi</i> marinkellei ChaQ 08-1
<i>Rhodnius prolixus</i> domestic-Boyacá	-	-	LYSIS	-	LYSIS	-	LYSIS	LYSIS	AGGL	LYSIS	LYSIS	LYSIS	LYSIS
<i>Rhodnius prolixus</i> Sylvatic-Casanare	-	-	LYSIS	-	LYSIS	-	LYSIS	LYSIS	AGGL	LYSIS	LYSIS	LYSIS	LYSIS
<i>Rhodnius robustus</i>	-	-	LYSIS	-	LYSIS	-	LYSIS	LYSIS	AGGL	LYSIS	LYSIS	LYSIS	LYSIS
<i>Rhodnius colombiensis</i>	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Rhodnius pallescens</i>	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Rhodnius pictipes</i>	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Triatoma dimidiata</i>	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Triatoma maculata</i>	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Panstrongylus geniculatus</i>	-	-	-	-	-	-	-	-	-	-	-	-	-

AGGL = Strong agglutination; (-) = No lysis was observed

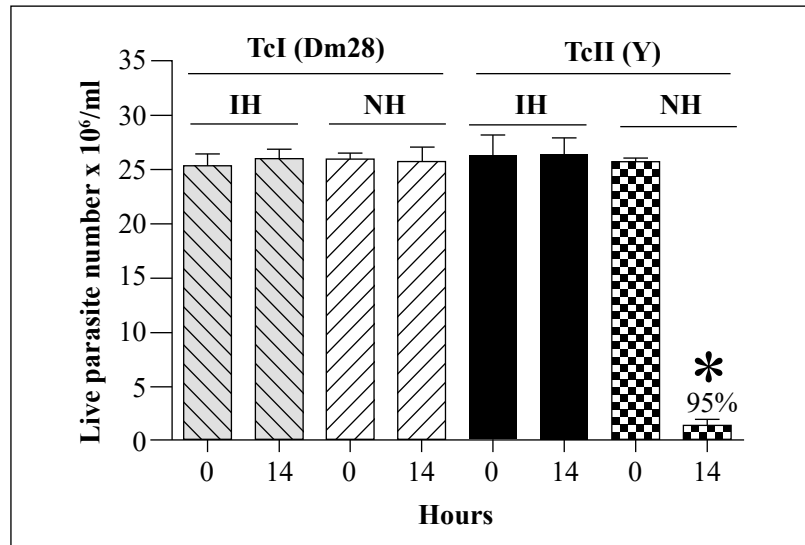


Figure 3. *In vitro* survival of *T. cruzi* TcI (Dm28) and TcII (Y) strains following incubation with hemolymph from *R. prolixus*

Each bar represents the mean ± SD of live parasite counts from three independent assays after 0 and 14 h of incubation with hemolymph. Each *T. cruzi* strain was incubated with inactivated hemolymph (IH) or native hemolymph (NH).

*: p<0.05 compared with the live parasite counts of the *T. cruzi* TcI and the TcII strains incubated with NH at the same time point

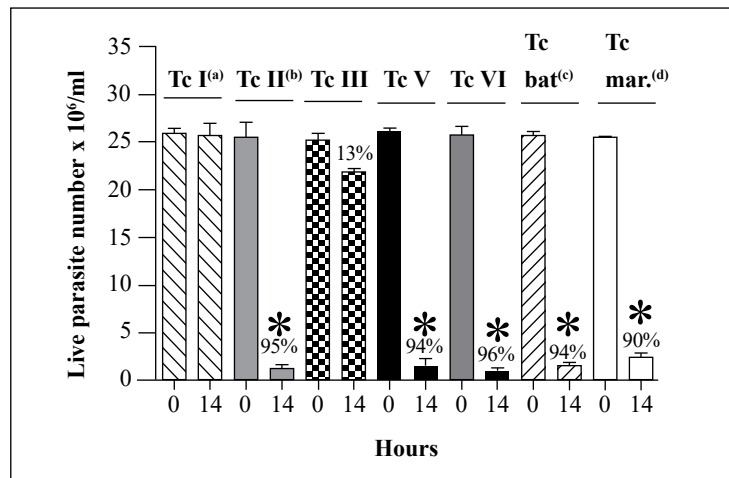


Figure 4. Summary of *in vitro* survival of *T. cruzi* strains belonging to six different DTUs, as well as one *T. cruzi* subspecies (*T. cruzi marinkellei*), following incubation with native hemolymph from *R. prolixus*.

Each *T. cruzi marinkellei* bar represents the mean \pm SD of live parasites counts from three independent assays after 0 and 14 h of incubation with hemolymph. TcI (Dm28), TcII (Y), TcIII (MT3663), TcV (967), TcVI (CL Brener), Tcbat (Ort07-1), and *T. cruzi marinkellei* (ChaQ 08-1) strains were examined in this assay. ^a, Lysis resistance was observed in 20 TcI strains. ^b, Lysis susceptibility was observed in two TcII strains. ^c, Lysis susceptibility was observed in six Tcbat strains. ^d, Lysis susceptibility was observed in two *T. cruzi marinkellei* strains. Only one strain was examined for each of the TcIII, TcV, and TcVI DTUs (Table 1). *T. cruzi* TcI strain Dm28 and TcII strain Y were used as negative (absence of lysis) and positive (presence of lysis) controls, respectively, for assays with TcIII, TcV, TcVI, Tcbat, and *T. cruzi marinkellei* strains. *, $p < 0.05$ compared with the live parasite counts of the negative controls with the native hemolymph (NH) at the same time point.

worth noting that none of the *R. colombiensis*, *R. pallescens*, *R. pictipes*, *T. dimidiata*, *T. maculate*, and *P. geniculatus* hemolymphs had lytic activity against *T. rangeli* and *T. cruzi* strains incubated in the same experimental conditions used here indicating the absence of trypanolytic activity in their hemolymphs during the first 24 hours of incubation.

Comparison of hemolymph from triatomines from wild and laboratory colonies

Next, we examined whether the maintenance of insects in laboratory colonies had any effect on the lytic activity of hemolymph. Hemolymph was obtained from 37 *R. colombiensis* specimens captured from palm trees in Coyaima, Colombia, in September 2018. Hemolymph was also extracted from *R. colombiensis* individuals from a colony that has been maintained in the laboratory for 5 years. Assays were conducted using *T. cruzi* TcII strain Y, which is susceptible to lysis. No significant differences in live parasite counts were observed for any of the treatments (**Figure 5**). Identical results (absence of lysis) were observed when the assay was repeated using hemolymph from a laboratory colony of *T. dimidiata* and specimens captured in sylvatic environments in Boyacá, Colombia, in May 2019 (data not shown).

Effects of parasite culture time on susceptibility to the lytic activity of native hemolymph from *R. prolixus*

We then compared the susceptibility of recently obtained and long-term cultures of *T. cruzi* TcII strain Y to lysis by *R. prolixus* hemolymph. The first TcII strain Y isolate was obtained from recent hemoculture of mouse blood while the second isolate had been maintained in a 4-year culture with weekly subcultures. As shown in **Figure 6**, the lytic activity of the hemolymph against the two cultures was similar with 95% and 96% decreases in *T. cruzi* live parasite counts following incubation of native hemolymph with

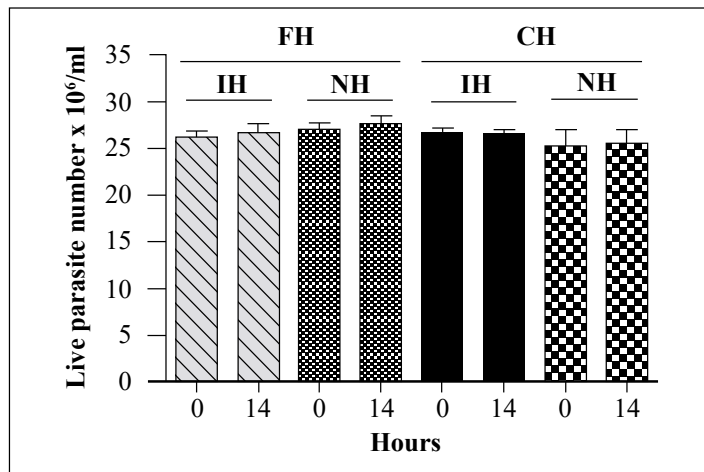


Figure 5. *In vitro* survival of a *T. cruzi* TcII strain following incubation with hemolymph from laboratory and field-collected *R. colombiensis* insects and comparison of the number of live *T. cruzi* TcII (Y) epimastigotes following incubation with the hemolymph from *R. colombiensis* specimens captured in the field (FH) and hemolymph from *R. colombiensis* individuals from a colony maintained in the laboratory for 5 years (CH). Each bar represents the mean \pm SD of live parasite counts from two independent assays after 0 and 14 h of incubation with hemolymph. The *T. cruzi* parasites were incubated with inactivated hemolymph (IH) or native hemolymph (NH) from both populations.

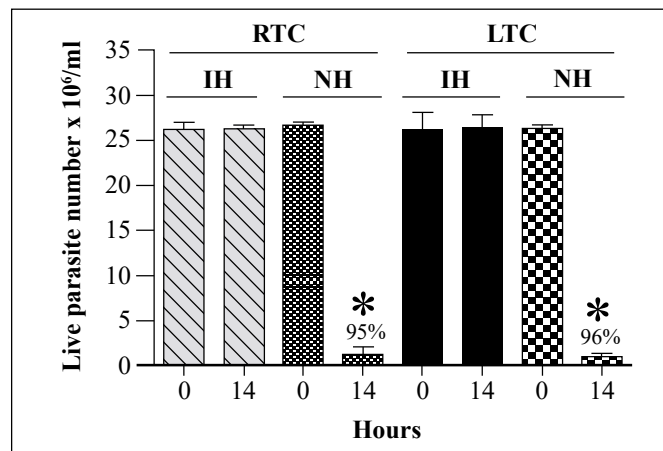


Figure 6. *In vitro* survival of *T. cruzi* TcII epimastigotes from recent or long-term cultures following incubation with native hemolymph from *R. prolixus*. *T. cruzi* TcII strain Y isolates recently cultured (recent-term cultivation, RTC) from trypomastigotes from mouse blood or cultured for 4 years (long-term cultivation, LTC) were incubated with native hemolymph from *R. prolixus*. Each bar represents the mean \pm SD of live parasite counts from three independent assays after 0 and 14 h of incubation with hemolymph. The same number of *T. cruzi* epimastigotes from the RTC and LTC cultures were incubated with inactivated hemolymph (IH) and native hemolymph (NH).
 *: $p < 0.05$ compared with live parasite counts of IH and NH cultures at the same time point

recently obtained and long-term cultures, respectively. Besides, no lysis of *T. rangeli* A strain Choachí maintained either via a cyclic culture-*R. prolixus*-mouse-culture method, which allowed us to obtain recent cultures of the parasite, or by weekly subculture for 3 years, was observed following incubation with native hemolymph from *R. prolixus* (data not shown). On the other hand, lysis of *T. rangeli* C strain Rcol0226 maintained via a cyclic culture-*R. colombiensis*-mouse-culture method or by weekly subculture for 3 years was observed following incubation with *R. prolixus* native hemolymph (data not shown).

In addition to the results presented here, we also determined that the lytic activity was similar in hemolymph from vectors fed on chicken or mouse blood and in hemolymph from *R. prolixus* nymphs, males, and females.

Discussion

The results of the study support previous reports on the existence of a rapid innate response to various *T. rangeli* genotypes and *T. cruzi* DTUs in the hemolymph of *R. prolixus*. **Sánchez, *et al.*** (2005) and **Pulido, *et al.*** (2008) showed that the epimastigotes of *T. rangeli* genotype C strains isolated from *R. colombiensis*, *R. ecuadoriensis*, and *R. pallescens* were lysed when incubated *in vitro* for 10 h with hemolymph from *R. prolixus*. In contrast, the epimastigotes of a *T. rangeli* genotype A strain were completely resistant to lysis under the same conditions. In the present work, *T. rangeli* genotype A, B, and D strains were resistant to lysis during incubation with hemolymph from *R. prolixus* for 24 h while 81% and 90% decreases in live parasite counts were observed for genotype C and E strains after 14 h of incubation with the hemolymph (**Figure 2, Table 2**).

In vivo assays where the hemocoel of *R. prolixus* was inoculated with *T. rangeli* genotype C strains showed the rapid disappearance of the flagellated parasite from the hemolymph of the vector (data not shown). This is consistent with the results of **Sánchez, *et al.*** (2005) regarding the complete clearance of *T. rangeli* genotype C cells from the hemolymph at 2 h post-inoculation. In contrast, inoculated *T. rangeli* genotype A cells were observed in the hemolymph of *T. rangeli* for several months with the production of infective metacyclic trypomastigotes (**Vallejo, *et al.***, 1986). As a result, this inoculation method is commonly used to obtain infective trypomastigotes of *T. rangeli* genotype A strains. Similar findings had already been reported by **Mello, *et al.*** (1995), who inoculated *T. rangeli* strain San Agustín (genotype A from Colombia) into the hemocoel of *R. prolixus*. The researchers observed the multiplication of the parasite in the hemolymph for several days with elevated levels of lysozyme activity in the hemolymph but no trypanolytic or antimicrobial peptide activity.

Under experimental conditions, *T. rangeli* genotype A strains are not lysed following inoculation into the *R. prolixus* hemocoel while genotype C strains are lysed. This innate lytic response would explain why after characterizing more than 100 *T. rangeli* strains isolated from the salivary glands of *R. prolixus* from Colombia, Honduras, and Venezuela (**Salazar-Anton, *et al.***, 2009), wild *Rhodnius neglectus* from the Federal District of Brazil (**Gurguel-Goncalves, *et al.***, 2004; **Urrea, *et al.***, 2005), wild *R. prolixus* from Casanare, Colombia (**Urrea, *et al.***, 2011), and *R. robustus* from Venezuela and Brazil were found to be genotype A (**Maia da Silva, *et al.***, 2007; **Vallejo, *et al.***, 2015). In these studies, none of the *R. prolixus*, *R. robustus* or *R. neglectus* vectors were infected with genotype C or E strains, which are susceptible to hemolymph lysis.

Trypanosoma rangeli genotype C strains have been isolated from wild *R. pallescens* colonies from Panamá and Colombia, from wild *R. colombiensis* colonies from central Colombia, and from domesticated *R. ecuadoriensis* in the northern region of Perú (**Urrea, *et al.***, 2005; **Vallejo, *et al.***, 2015). No trypanolytic activity was detected in the hemolymph of these vectors and no lysis was observed following experimental inoculation of a *T. rangeli* genotype A strain into the hemocoel of *R. colombiensis* and *R. pallescens*, although the parasites did not invade the salivary glands (data not shown).

The absence of lytic activity in the hemolymph of *Triatoma* and *Panstrongylus* species (*T. dimidata*, *T. maculata*, and *P. geniculatus*) in the current study (**Table 2**) coincides with the observations of **De Stefani-Marques, *et al.*** (2006), who orally infected *P. megistus*, *T. infestans*, *T. sordida*, *T. braziliensis*, and *T. vitticeps* individuals with a strain of *T. rangeli* isolated in Uberaba-Minas Gerais, Brazil, where genotype A predominates. The authors observed the parasites in the hemolymph for 10–30 days following infection without invasion of salivary glands, thus confirming the absence of lytic factors with immediate activity in these vectors.

The inability of *T. cruzi* to develop in the hemolymph of triatomine vectors was observed in *in vivo* experiments performed by **Alvarenga & Bronfen** (1982), who inoculated *T. cruzi* strains Y and CL into the hemocoel of *T. infestans* and *Dipetalogaster maxima*. They observed that the parasites could not persist in the hemolymph for more than 40 days. In comparison, **Mello, et al.** (1995) inoculated *T. cruzi* clone Dm28 into the hemocoel of *R. prolixus* and found that the parasites were completely cleared by day 5 post-inoculation suggesting that the humoral response in hemolymph from *R. prolixus* against *T. cruzi* is faster than that in hemolymph from *T. infestans* and *D. maxima*. Early and strong lytic activity was previously observed by **Mello, et al.** (1996) in *in vitro* experiments with various *T. cruzi* strains. The authors observed trypanolytic activity in the hemolymph of *R. prolixus* against *T. cruzi* strain Y but not against strains Dm28c or CL after 3 h of incubation. Thus, *T. cruzi* clearance depends on both the parasite strain and the vector species. However, the exact mechanism that causes *T. cruzi* to die in the hemocoel but thrive in the gastrointestinal tract remains unknown (**Salcedo-Porras & Lowenberger**, 2019).

Although under natural conditions *T. cruzi* does not enter the hemolymph of the vectors, we decided to use epimastigotes of *T. cruzi* belonging to various DTUs to detect the presence of trypanolytic factors related to an innate immune response in the vectors. Differences in susceptibility to lysis were observed amongst the *T. cruzi* strains belonging to different DTUs, with a TcIII strain showing a 13% decrease in live parasite numbers after 14 h of incubation, while a TcI strain remained unaffected after 24 h of incubation. We observed strong *in vitro* lytic activity during incubation of *T. cruzi* with hemolymph from *R. prolixus*, with 90%–96% decreases in live parasite counts recorded in the first 14 h of incubation (**Figure 4**). We could not determine the cause of this variability; however, susceptibility or resistance to lysis of *T. cruzi* strains belonging to different DTUs and *T. rangeli* strains belonging to different genotypes may be related to the ability of the different strains to survive the oxidative stress of the hemolymph. It is likely that the parasites that showed resistance to lysis maintain a repertoire of genes involved in oxidative stress tolerance, which would aid in survival under environmental stress conditions. The variability of the antioxidant defenses of the various *T. cruzi* and *T. rangeli* strains may reflect specific adaptations to the vector species (**Stoco, et al.**, 2014; **Beltrame-Botelho, et al.**, 2016). However, the most intriguing result of the present study was that none of the other vector species, including *R. pallezensis*, *R. colombiensis*, *R. pictipes*, *T. dimidiata*, *T. maculata*, and *P. geniculatus*, showed hemolymph lytic activity against any of the *T. cruzi* or *T. rangeli* strains after 24 h of incubation (**Table 2**).

The lytic activity of hemolymph from field-collected insects and laboratory colonies of *R. colombiensis* (**Figure 5**) and of *T. dimidiata* against TcII was not observed in any of the hemolymph samples indicating that the absence of lytic activity is independent of the maintenance of colonies in the laboratory. Similarly, lytic activity tests against TcII using *R. prolixus* colonies from six different departments in Colombia (Boyacá, Casanare, Cundinamarca, Magdalena, Santander, and Tolima) showed equivalent levels of lytic activity in hemolymph from all colonies.

We also found that there was no difference in the lytic susceptibility of *T. cruzi* TcII epimastigotes recently obtained by blood culture and those maintained in culture for 4 years with weekly subcultures (**Figure 6**), which indicates that the maintenance time of epimastigotes in culture does not affect their susceptibility to lysis caused by the native hemolymph from *R. prolixus*. There were no differences either in the trypanolytic activity of hemolymph from *R. prolixus* adults (males and females) and fifth-instar nymphs or in hemolymph from insects fed on chicken or mouse blood.

A question that is yet to be resolved is whether these innate trypanolytic factors detected in the hemolymph could also be activated in the intestine to modulate the transmission of intestinal *T. cruzi* parasites. More studies are needed to address this hypothesis, identify innate trypanolytic factors, locate production sites, and determine circulation dynamics and their effector activities in different vector organs and tissues.

Regarding the possible mechanisms related to hemolymph lytic activity, it is known that prophenoloxidase system (PPO) activation leads to melanin synthesis by indolquinone polymerization. Both melanin and the intermediary products formed during its synthesis help kill bacteria, viruses, and parasites (Walters & Ratcliffe, 1983; Söderhäll & Cerenius, 1998; Cerenius, *et al.*, 2008, Azambuja, *et al.*, 2017). PPO availability in the hemolymph of the insects is derived from hemocyte lysis (Kanost and Gorman, 2008). Hemocyte lysis mainly occurs after exposure to microorganisms (Brehélin, *et al.*, 1989), but may also occur spontaneously to maintain basal prophenoloxidase (PPO) levels in hemolymph (Kanost & Gorman, 2008). Hemolymphs from the eight triatomine species were used in all the parasite incubation experiments in our study; they were hemocyte-free and treated with phenylthiourea, which would have prevented basal PPO levels activation. In principle, the lytic activity observed in *R. prolixus* and *R. robustus* hemolymph should not have been attributed to PPO activation but related to factors other than the PPO cascade.

In a previous study, it was found that *R. prolixus* hemolymph lytic factors or their precursors were proteins (Pulido, *et al.*, 2008); here, we confirmed this finding because hemolymph lytic activity disappeared completely after pepsin incubation. It can be predicted, then, that potential lytic factors or their precursors are proteins and could be antimicrobial peptides (AMP), precursors of reactive nitrogen species (NOS) or reactive oxygen species (ROS) or nitrophorins that are NOS transporters or proteins not yet identified. Proteomic and transcriptomic studies comparing vector species having or lacking lytic activity should be carried out to identify possible candidates and subsequent tests performed to confirm their role in hemolymph lytic activity.

The strategies used by *T. rangeli* and *T. cruzi* to achieve successful vector infection depend on a tripartite interaction among the parasite, the insect's immune system, and the intestinal microbiota. Experimental infection studies using different bacterial species have shown species-specific differences in the intestinal immune response of the vectors (Vieira, *et al.*, 2014). These interactions constitute an important field of research that will open new perspectives on our understanding of parasite-vector relationships. As part of this tripartite interaction, it will be very important to characterize the microbiomes of insect populations that have a very strong innate humoral response, such as that detected in *R. prolixus* and *R. robustus*. Investigations of this tripartite interaction could lead to the identification of intestinal microbial species that naturally induce an immune response in the intestine or hemolymph of the vector to avoid parasite infection. Further research is needed to identify the components of the strong differential immune response between insects having and lacking lytic activity to verify whether such factors could be involved in *T. rangeli* genotypes and *T. cruzi* DTUs' predominance in Colombia and some Latin American regions.

Conclusions

Using *in vitro* incubations of *T. rangeli* and *T. cruzi* epimastigotes from different genotypes and DTUs with the hemolymph of eight species of triatomines, we detected strong trypanolytic activity in the hemolymph from *R. prolixus* and *R. robustus*, which was absent in that from *R. colombiensis*, *R. pallezensis*, *R. pictipes*, *T. dimidiata*, *T. maculata*, and *P. geniculatus*. The trypanolytic activity was independent of the sex and stage of the insect, the time the epimastigotes were maintenance in culture, and the type of food on which the vectors were fed (chicken or mouse blood). The absence of trypanolytic activity was observed in both laboratory colonies and field-captured insects of the species *R. colombiensis* and *T. dimidiata*. There are still gaps in our understanding of the innate immune responses of different triatomine species against the same strains of *T. cruzi* and *T. rangeli* or the same triatomine species against geographically-separated isolates of *T. cruzi* and *T. rangeli*. Interactions between triatomines and trypanosomes involve complex ecological, biochemical, and coevolutionary relationships that are just beginning to be

investigated. Understanding the factors that determine how a vector is infected and how the parasite is transmitted is key to the success of control programs targeting Chagas disease vectors; unfortunately, most of these factors are unknown. Therefore, it is vital to investigate the ecological, physiological, and immunological factors that determine how and why the vectors transmit some genotypes of the parasite and others do not.

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Author Contributions

Conceptualization: YSQ, JCCM, FGN, GAV. Data curation and Formal analysis: YSQ, HJBV, SAGG, JLOM, DZG, JCCM, GAV. Funding acquisition: YSQ, JCCM, GAV. Investigation: YSQ, HJBV, SAGG, JLOM, DZG, FGN, OCB, JCCM, GAV. Methodology: YSQ, GAV. Project administration: JCCM, GAV. Technical and biological resources: YSQ, FGN, OCB, JCCM, GAV. Supervision: JCCM, GAV. Writing—original draft: YSQ, GAV. Writing—review&editing: YSQ, HJBV, SAGG, JLOM, DZG, FGN, OCB, JCCM, GAV.

Conflicts of interest

The authors declare that there are no conflicts of interest related to the contents of this article.

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