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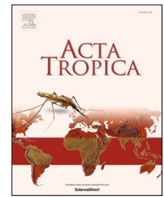

Trypanosoma cruzi infection in the wild Chagas disease vector, *Mepraia spinolai*: Parasitic load, discrete typing units, and blood meal sources

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A R T I C L E I N F O

Keywords:
Chagas disease
Trypanosoma cruzi
DTUs
Triatominae
Parasite load
Kissing bug

A B S T R A C T

Background: Mepraia spinolai, a wild vector of *Trypanosoma cruzi* in Chile, is an abundant triatomine species that is frequently infected by the parasite that causes Chagas disease. The aim of this study was to determine if the parasitic load of *T. cruzi* in *M. spinolai* is related to its blood meal source and the infecting DTUs of *T. cruzi.*
Methods: The vector was captured in rural areas. In the laboratory, DNA was extracted from its abdomen and *T. cruzi* was quantified using qPCR. Real time PCR assays for four *T. cruzi* DTUs were performed. Blood meal sources were identified by real-time PCR amplification of vertebrate cytochrome b gene sequences coupled with high resolution melting (HRM).
Results: Trypanosoma cruzi was detected in 735 *M. spinolai*; in 484 we identified one blood meal source, corresponding to human, sylvatic, and domestic species. From these, in 224 we were able to discriminate the infecting DTU. When comparing the parasitic loads between the unique blood meal sources, no significant differences were found, but infections with more than one DTU showed higher parasitic loads than single infections. DTU TcI was detected in a high proportion of the samples.
Conclusions: Higher parasitic loads are related to a greater number of *T. cruzi* DTUs infecting *M. spinolai*, and this triatomine seems to have a wide span of vertebrate species in its diet.

1. Introduction

Trypanosoma cruzi (Chagas, 1909) (Trypanosomatida: Trypanosomatidae) is the causal agent of Chagas disease, one of the most important zoonoses in the American continent, with six to seven million people infected in the world, mainly in endemic areas of Latin America (WHO, 2021). The main transmission mechanism of this protozoan is by the contact of contaminated feces or urine of hematophagous insect vectors of the Triatominae subfamily (Hemiptera: Reduviidae) with mucous membranes or wounds of mammals; additionally, blood transfusions, organ transplants, congenital, oral and laboratory accidents constitute other ways of transmission of *T. cruzi* (Rassi et al., 2012). *Trypanosoma cruzi* has high genetic diversity, with seven discrete typing units (DTUs hereafter) recognized: TcI, TcII, TcIII, TcIV, TcV, TcVI and Tcbat (Marcili et al., 2009; Zingales et al., 2009). These DTUs coexist in vectors and mammalian hosts, as mixed infections (Jansen et al., 2020).

In triatomines, the five nymphal instars and adults are hematophagous, and since hatching they can acquire *T. cruzi* through consumption of infected mammalian blood, cannibalism, and coprophagy (Noireau et al., 2009). Once ingested, *T. cruzi* multiplies and colonizes the intestine of the vectors (Garcia et al., 1995). Molecular techniques have allowed the detection and quantification of *T. cruzi* from different

Abbreviations: DTUs, Discrete typing units.

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biological samples, such as feces (Mc Cabe et al., 2019; Saavedra et al., 2016) and the digestive tract of triatomines (Dias et al., 2015; Valença-Barbosa et al., 2021), which contribute to infer the vectorial capacity: the ability of a vector species to transmit a pathogen in a given locality at a certain time, encompassing the host-vector interaction as the expected number of human blood meals the vector performs in its life, the survival probability of the infectious vector, and its expected progeny (Brady et al., 2016; Moreira et al., 2017). Vectorial transmission is influenced by multiple variables, such as host detection, feeding and defecation by the vector, its *T. cruzi* parasitic load and genotypes, and host susceptibility (Verly et al., 2020). Therefore, any information regarding *T. cruzi* genotypes and parasitic load, along with triatomine blood meal sources may raise important contributions to understand natural transmission cycles and the risk posed by insect vectors in Chagas disease transmission.

Most triatomines can feed on blood of a wide variety of vertebrates, both wild and domestic, allowing triatomines to use different habitats (Gorchakov et al., 2016). However, the preference for a specific blood source can influence the dynamics of Chagas disease and the interaction with *T. cruzi* (De Fuentes-Vicente et al., 2018). The parasitemia of *T. cruzi* in mammalian hosts is an important factor in the parasitic infection of vectors (Jansen et al., 2020). Regarding triatomine fitness, *Rhodnius prolixus* (Stal 1859) laboratory colonies fed on human and rabbit blood achieve faster development and higher female fecundity than those fed on chicken, sheep and horse blood (Gomes et al., 1990), so the blood meal is relevant for triatomine populations.

There are 155 species of triatomines, distributed mainly in the Americas (Alevi et al., 2020). In Chile, there are four species: *Triatoma infestans* (Klug, 1834)*, Mepraia spinolai* (Porter, 1934)*, M. gajardoi* (Frías, Henry & Gonzalez, 1998) and *M. parapatrica* (Frías-Lasserre, 2010). *Mepraia spinolai* has been found in domestic, peridomestic and mainly wild habitats (Frías-Lasserre et al., 2017). Seasonal invasion of wild triatomines into dwellings has been reported, with *M. spinolai* comprising almost a third of the invading specimens, with infection rates exceeding 26% (ISPCH, 2018), constituting a risk of vectorial transmission in Chile (Canals et al., 2017; Frías-Lasserre et al., 2017).

It has been reported that *M. spinolai* does not have a preference for a particular host species, but would prefer these according to their relative abundance (Botto-Mahan et al., 2005). The feeding profile of *M. spinolai* has been evaluated by different serological techniques, maintaining a diet based on wild rodents, rabbits, goats, dogs, cats, and humans (Canals et al., 2001; Chacón et al., 2016; Molina et al., 2004). The frequency of *T. cruzi* infection in *M. spinolai* detected by conventional PCR is between 14% and 76% (Botto-Mahan et al., 2020; Coronado et al., 2009). Regarding the DTUs, TcI is most frequently reported circulating in *M. spinolai* populations; TcII, TcV and TcVI have also been detected (Coronado et al., 2009; Ihle-Soto et al., 2019).

Therefore, information on the DTUs and parasitic load of *T. cruzi*, and the blood meal sources of this vector, can contribute to the understanding of the vectorial capacity and risk that *M. spinolai* may pose in domestic environments. The aim of this study was to determine whether the parasitic load of *T. cruzi* is related to the blood meal sources of *M. spinolai* and the infecting DTUs of *T. cruzi.*

2. Materials and methods

2.1. Triatomine trapping and classification

The triatomines were captured using fermenting yeast traps placed adjacent to rock piles, rocky outcrops and terrestrial bromeliads in seven rural localities during the Austral summer of 2014–2015 in the Coquimbo Region, located in the North-Central zone of Chile (Fig. 1). The climate is semiarid-Mediterranean, with most rainfall concentrated in winter (May-August). The landscape is dominated by transversal river valleys, with the main rivers Elqui, Limarí and Choapa defining the three administrative Provinces of the Region (Novoa et al., 1989). The sampling sites in the Elqui Province were located at an average altitude of 1311 m.a.s.l. (SD ± 462.3), in the Limarí Province at 656.7 m.a.s.l (SD ±

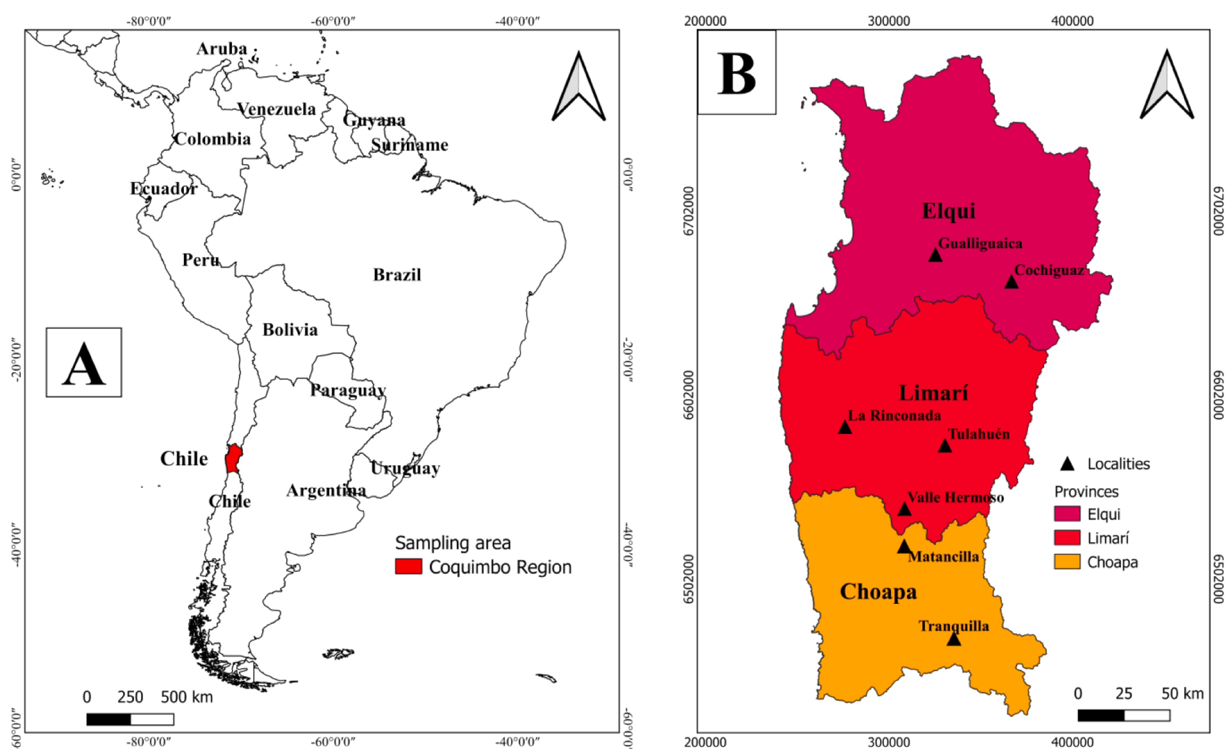

Fig. 1. Map showing the location of the sampling Region in Chile, South America. (A) Map in geographical coordinates of the Coquimbo Region within continental Chile in South America. (B) Map showing the location of the seven rural localities within the three Provinces of the Coquimbo Region: Elqui, Limarí and Choapa, in UTM Zone 19 S.

331.1) and in the Choapa Province at 942.5 m.a.s.l (SD ± 78.0). The location of the sampled triatomines was recorded by a handheld geopositioning system; maps were compiled in QGis Desktop 3.18.1, using available shapefiles from the website of Biblioteca del Congreso Nacional de Chile (https://www.bcn.cl/siit/mapas_vectoriales/index_html) and the georeferenced data (Fig. 1)

Triatomines were maintained at room temperature in individual flasks with cap perforations, provided with folded paper as refuge, and were transported to the laboratory protected from extreme environmental conditions. Species and instar determination of the captured triatomines was performed upon arrival at the laboratory, following classification keys based on morphology (Frías et al., 1987, 1998). In the laboratory, they were maintained at room temperature. After all triatomines were classified, we proceeded with the molecular analyses.

2.2. Extraction of nucleic acids

Mepraia spinolai specimens were cut with single scalpels and DNA was extracted from the whole abdomen of each insect using a commercial kit (Mo Bio, UltraClean® Tissue & Cells DNA Isolation Kit) according to the manufacturer's recommendations. The DNA was stored at -20 °C until use in the following determinations.

2.3. Quantification of *Trypanosoma cruzi* parasitic load

Detection and quantification of *T. cruzi* was performed in duplicate by real-time PCR assays with oligonucleotides targeting the conserved satellite nuclear region (Table 1). The reaction mixture consisted of 2 µL of sample, 4 µL of 5X HOT FIREpol® EvaGreen® qPCR mix Plus (Solis BioDyne), 0.6 µL of each of the oligonucleotides and 12.8 µL of nuclease-free water, for a total of 20 µL. As an endogenous control, a conserved region of the 18S ribosomal DNA subunit of *M. spinolai* was targeted (Table 1), and each sample was run in duplicate, with the same reaction mixture as described above. Cycling conditions for both reactions were 15 min at 95 °C, followed by 40 cycles at 95 °C for 15 s, 60 °C for 20 s, and 72 °C for 20 s in a Rotor-Gene® Q (QIAGEN). After finishing the amplification cycles, a melting curve was run. Each run was carried out with a no-template control with water instead of DNA; one point of the standard curve of *T. cruzi*; and one point of the standard curve of the endogenous control, all in duplicate.

The standard curve for the absolute quantification of *T. cruzi* was made from genomic DNA of strain DM28c (TcI) and strain Y (TcII) in equal parts, due to the variability in the number of copies of the satellite region described for the different DTUs (Duffy et al., 2009), at an initial concentration of 10^6 parasite-equivalents/mL (par-eq/mL, hereafter), with 1:10 serial dilutions until reaching 0.1 par-eq/mL. The calculations were estimated considering the equivalent DNA of the parasite, since one parasite contains approximately 200 fg of DNA (Duffy et al., 2009). The standard calibration curve for the endogenous control was made with a range of 0.86 to 8.60×10^{-5} µg/mL. Both targets were used to estimate the normalized *T. cruzi* parasitic load in *M. spinolai* in parasite equivalents/gram of triatomine DNA (par-eq/g, hereafter).

2.4. Identification of blood meal sources in *Mepraia spinolai*

A 383-bp fragment from the cytochrome b gene (Cyt b, hereafter) was amplified to differentiate among species-specific genotypes of natural blood meal sources of *M. spinolai* by real-time PCR using primers specific to vertebrates (Table 1). Real-time PCR was performed at a final volume of 20 µL, containing 1 µL of each primer, 2 µL of sample, 10 µL of SensiFAST™ HRM (Bioline) and 6 µL of nuclease free water in a Rotor-Gene® Q (QIAGEN); each run included a no-template control with water instead of DNA, and DNA extracted from blood of 17 vertebrate species as references (Table 2), in duplicate. Following the run, a high resolution

Table 2
List of vertebrate species used as references in the High Resolution Melting analysis, with scientific and common name indicated.

Scientific name	Common name	Reference Tm (°C)	N	%
Abrothrix olivaceus	Olive grass mouse	81.57	40	17.8
Octodon degus	Degu	81.25	38	17.0
Oryctolagus cuniculus	Rabbit	81.44	29	12.9
Abrothrix longipilis	Long-haired grass mouse	81.18	21	9.4
Homo sapiens	Human	84.85	19	8.5
Rattus rattus	Black rat	80.98	13	5.8
Capra hircus	Goat	80.72	13	5.8
Equus caballus	Horse	80.73	10	4.5
Phyllotis darwini	Darwin's leaf-eared mouse	80.12	8	3.6
Ovis aries	Sheep	80.55	8	3.6
Gallus gallus	Chicken	85.30	7	3.1
Felis catus	Cat	82.24	6	2.7
Oligoryzomys longicaudatus	Long-tailed rice mouse	81.88	5	2.2
Lycalopex culpaeus	Culpeo fox	80.06	3	1.3
Mus musculus	House mouse	80.21	2	0.9
Canis lupus familiaris	Dog	79.73	2	0.9
Equus asinus	Donkey	83.25	-	-

Tm: Temperature of melting, in degrees Celsius; N: number of triatomines with each unique blood meal source from the 224 *T. cruzi* positive specimens that had DTU(s) determined; %: corresponding percentage.

Table 1
Primers sequences qPCR assays.

Target	Primer	Sequence (5′–3′)	Concentration
T. cruzi satellite DNA [a]	Cruzi 1 (Forward)	ASTCGGCTGATCGTTTTCGA	300 nM
	Cruzi 2 (Reverse)	AATTCCTCCAAGCAGCGGATA	
Triatomine 18S ribosomal DNA subunit [b]	18S (Forward)	TCCTTCGTGCTAGGAATTGG	300 nM
	18S (Reverse)	GTACAAAGGGCAGGGACGTA	
Cytochrome b vertebrate gene [c]	Cyt b (Forward)	CCCCTCAGAATGATATTTGTCCTCA	500 nM
	Cyt b (Reverse)	CCATCCAACATCTCAGCATGATGAAA	
Spliced leader intergenic region [d]	TcI SL-IR (Forward)	GCTTTGTGTTCTCGCACTCCA	200 nM
	TcI (Reverse)	CGATCAGCGCCACAGAAAGT	
Cytochrome c oxidase subunit II [d]	TcII COII (Forward)	GGATTACATCTACGGCTGACACC	100 nM
	TcII COII (Reverse)	CGAGAGTGATTATTTGGTGGGAGATA	
NADH dehydrogenase subunit 1[d]	TcV ND1 (Forward)	AGTTTTATTAATCTTATCAGGATTTGGTG	400 nM
	TcV ND1 (Reverse)	CCATCTGTGATAGGTGTTAATATTCC	
18S ribosomal DNA [d]	TcVI 18S (Forward)	CGTAGGCGTGGTCGGGT	500 nM
	TcVI 18S (Reverse)	TATTCCCGTTAAAGGCCCTTGT	

[a] (Piron et al., 2007);
[b] (Paim et al., 2012);
[c] (Boakye et al., 1999);
[d] (Muñoz-San Martín et al., 2017).

melting (HRM, hereafter) curve of the amplicons was performed, increasing the temperature from 72 °C to 92 °C at ramping increments of 0.1 °C/s, recording the changes in fluorescence with changes in temperature (dF/dT), which were analyzed using the Rotor-Gene® Q Series Software v.2.3.4 (QIAGEN). To identify potential blood meal sources of *M. spinolai,* each of the unknown vertebrate Cyt b samples was paired with each of the Cyt b from the references, comparing both the melting temperatures (Tm, hereafter) and the HRM curve (Peña et al., 2012). Reference species were chosen according to their relevance as reservoirs of *T. cruzi* or due to their proximity to rural anthropic environments (Correa et al., 1984; Chacón et al., 2016; Ihle-Soto et al., 2019; Jiménez and Lorca, 1990).

The sampling of domestic animals used as references was approved by the Animal Bioethics Committee of the Facultad de Ciencias Veterinarias y Pecuarias of Universidad de Chile (Certificado 4/10/2013). Sylvatic vertebrate samples were obtained in previous studies, authorized by the Servicio Agrícola y Ganadero (SAG) Resolución 8080, 23/11/2011.

2.5. Determination of Trypanosoma cruzi discrete typing units

Four separate real-time PCR assays for DTU genotyping of four of the seven DTUs of *T. cruzi* were performed for each triatomine sample positive for *T. cruzi*, as described (Table 1). Genomic DNA extracted from four representative *T. cruzi* strains for each of the tested DTUs were included as positive controls in each of the assays: Dm28c (TcI), Y (TcII), MN cl2 (TcV) and CL Brener (TcVI), along with a no-template control, all in duplicate.

2.6. Inclusion criteria for this study

We required that the sample presented a positive result for the *T. cruzi* conserved satellite nuclear region, and a positive result for the vertebrate Cyt b gene, to be included in this study; subsequently, we proceeded with the DTU determination if the sample presented only one blood meal source (Fig. 2).

2.7. Statistical analyses

Statistical analyses were performed using the software R (v. 3.6.3, 2020) in RStudio (RStudio Team, v. 1.4.1103), using the packages dunn.test and lawstat, and GraphPad Prism v.6.01 for Windows (GraphPad Software, La Jolla CA, US).

We tested for normality of the parasitic load with the Shapiro-Wilk test. We compared the parasitic load distribution between developmental instars, provinces, blood meal sources and *T. cruzi* DTUs using the Kruskal-Wallis tests with post-hoc Dunn's pairwise tests adjusted using the Bonferroni correction, or the Mann-Whitney test, as appropriate. We applied the Fisher exact test to determine if there were differences in the *T. cruzi* DTUs frequencies among developmental instars and Provinces. For all tests, p-values ≤ 0.05 were considered statistically significant.

3. Results

3.1. Parasitic load

We included a total of 735 samples of *M. spinolai* positive to the *T. cruzi* conserved satellite nuclear region (Fig. 2). From these, 194 corresponded to first instar nymph (26.4%), 217 to second (29.5%), 217 to third (29.5%), 40 to fourth (5.4%), 24 to fifth instar (3.3%), and 43 were adults (5.9%). The distribution of *T. cruzi* parasitic load ranged from 3.02×10^{-5} to 1.29×10^{9} par-eq/g, with a median of 2.31×10^{2} par-eq/g.

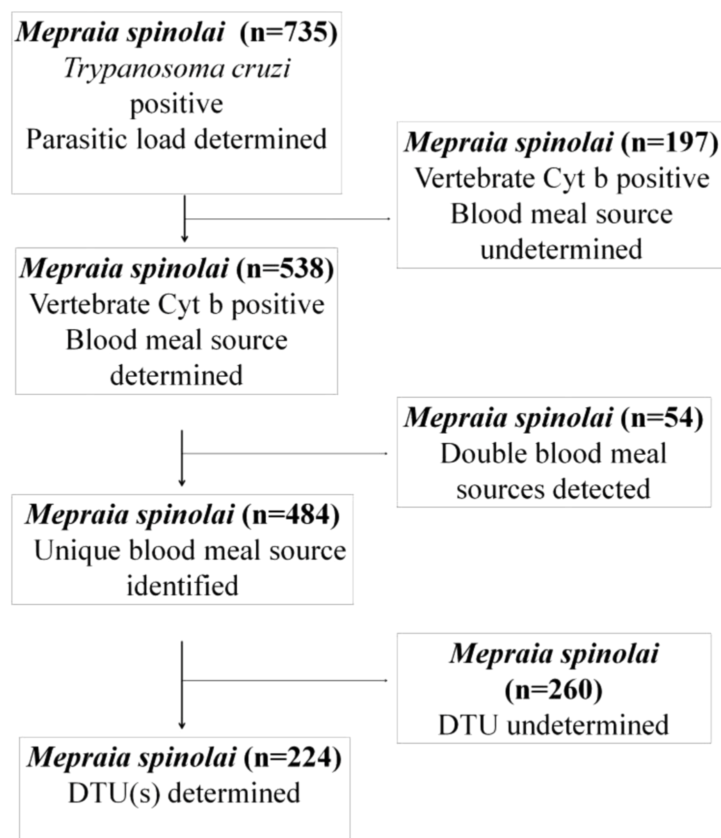

Fig. 2. Triatomine sample analysis scheme.

3.2. Blood meal sources

A specific HRM profile was obtained for each reference species analyzed, and the software was able to discriminate and recognize the analyzed samples as different genotypes. When comparing samples against the references, confidence percentages equal or over 70% were considered a match. Of the 735 samples positive for *T. cruzi*, it was possible to identify the blood meal sources using the HRM technique in 538 (73.2%) of them (Fig. 2, Supplementary Table 1); in 484 only one blood meal source was identified (Fig. 2, Supplementary Table 1), while in 54 two blood meal sources were identified (Fig. 2, Supplementary Table 2; Supplementary Table 3); these double blood meals were discarded from further analyses, as the possible relation of each blood meal source was not going to be discriminated. Nevertheless, vectors with one blood meal source compared with the double ones did not show significant differences in their parasitic loads (Mann Whitney test U = 11,940, p = 0.298). Human was detected in 65 *M. spinolai* specimens. In 197 samples (26.8%), in spite of having amplified the Cyt b fragment, it was not possible to identify the vertebrate species involved (Fig. 2, Supplementary Table 1). Detailed information for each specimen analyzed is available in Supplementary database 1.

3.3. Trypanosoma cruzi discrete typing units (DTUs)

From the 735 *T. cruzi* positive samples, 484 presented a unique blood meal source, so they were included in our DTU determination (Fig. 2), obtaining at least one positive result in 224 of them (46.3%). Single infections, that is, with only one DTU detected, corresponded to 138 samples (61.6%): 88 triatomines presented TcI (39.3%), followed by 29 with TcVI (12.9%), 17 with TcII (7.6%) and four with TcV (1.8%). Mixed infections, that is, with two or more DTUs, were detected in 86 triatomines (38.4%): 40 of them presented the mixture TcI+TcII+TcVI (17.8%), followed by 25 triatomines with TcII+TcVI (11.1%), 10 with TcI+TcII (4.5%), nine with TcI+TcVI (4%), one with TcI+TcV (0.5%), and one with TcI+TcII+TcV+TcVI (0.5%).

3.4. Trypanosoma cruzi load according to blood meal source and DTUs

The number of vectors fed uniquely on each blood meal source with DTUs determined is available in Table 2. Parasitic load in naturally infected *M. spinolai* with DTU(s) determined and a unique blood meal source ranged from 5.9×10^{-5} to 1.29×10^{9} par-eq/g, with a median of 2.3×10^{3} par-eq/g. The parasitic load was not normally distributed (Shapiro-Wilk normality test, $p < 0.0001$).

When comparing their parasitic loads according to the unique blood meal sources (Fig. 3), no significant differences were found (Kruskal-Wallis test, $\chi^2 = 12.10$, df = 15, p = 0.671). The most frequent blood meal source in the subsample with DTUs determined was *Abrothrix olivaceus* (Rodentia: Cricetidae) with 17.8%, followed by *Octodon degus* (Rodentia: Octodontidae) (17%), *Oryctolagus cuniculus* (Lagomorpha: Leporidae) (12.9%), *A. longipilis* (9.4%), and human (8.5%). In the four *M. spinolai* fed only on *Equus asinus* (Perissodactyla: Equidae) we could not identify their infecting DTU; the median of their parasitic load was 167.9 par-eq/g (Table 2).

When comparing parasitic loads between single (n = 138) and mixed (n = 86) infections, mixed infections showed higher parasitic loads than single infections (Mann Whitney test U = 1369, $p < 0.0001$) (Fig. 4, Graphical Abstract). The parasitic load of single infections ranged from 5.9×10^{-5} to 6.07×10^{7} par-eq/g, with a median of 238.6 par-eq/g. In the mixed DTUs, it ranged from 5.6×10^{0} to 1.29×10^{9} par-eq/g, with a median of 1.27×10^{7} par-eq/g.

The parasitic load of the 260 samples without DTU determined was significantly lower than those with DTU(s) determined (Mann Whitney test U = 16,384, $p < 0.0001$), presenting parasitic loads very close to the limit of detection of our *T. cruzi* assay.

Notably, TcI was the most frequent DTU in single and mixed infections, present in 149 of the 224 individuals analyzed (66.7%), and it was detected at least in one specimen fed on each of the blood meal sources detected in our study (Fig. 5), except in *E. asinus* (Supplementary database 1).

Thirteen *M. spinolai* had DTU(s) determined in the Province of Elqui (5.8%), 86 in Limarí (38.4%) and 125 in Choapa (55.8%). We found

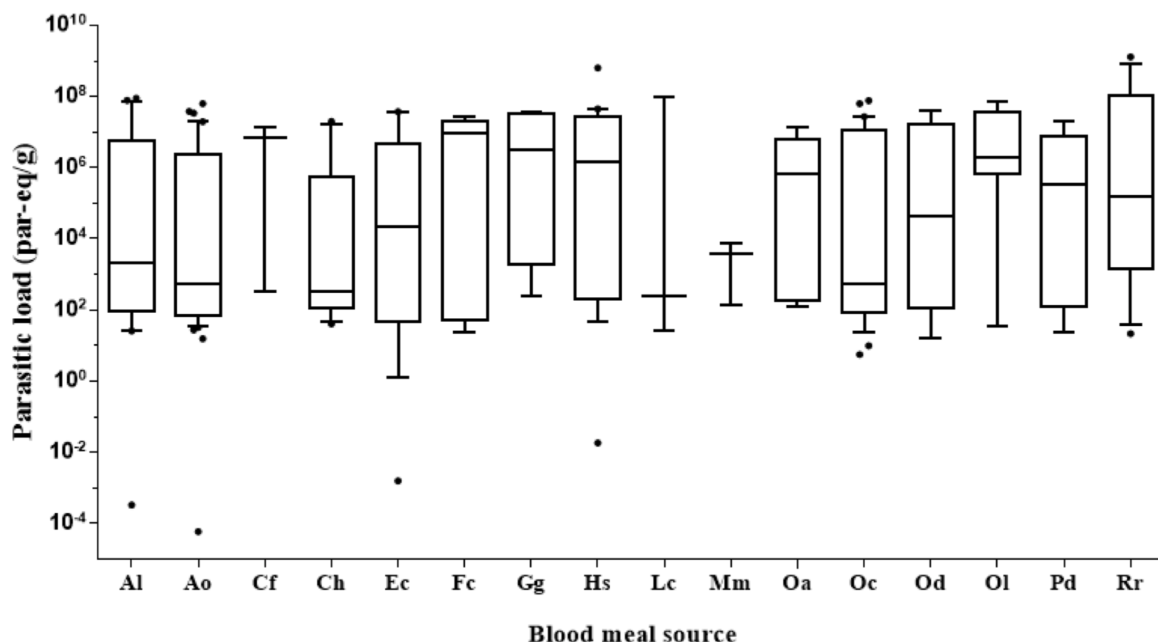

Fig. 3. Box plots showing the comparison of *T. cruzi* parasitic load, presented in logarithmic scale, according to the unique blood meal source of 224 *M. spinolai*. The line inside the box represents the median, and the box extends from the lower to the upper quartiles. Whiskers indicate the 90th and 10th percentiles and dots represent the outliers. Al: *Abrothrix longipilis*, Ao: *Abrothrix olivaceus*, Cf: *Canis lupus familiaris*, Ch: *Capra hircus*, Ec: *Equus caballus*, Fc: *Felis catus*, Gg: *Gallus gallus*, Hs: *Homo sapiens*, Lc: *Lycalopex culpaeus*, Mm: *Mus musculus*, Oa: *Ovis aries*, Oc: *Oryctolagus cuniculus*, Od: *Octodon degus*, Ol: *Oligoryzomys longicaudatus*, Pd: *Phyllotis darwini*, and Rr: *Rattus rattus*.

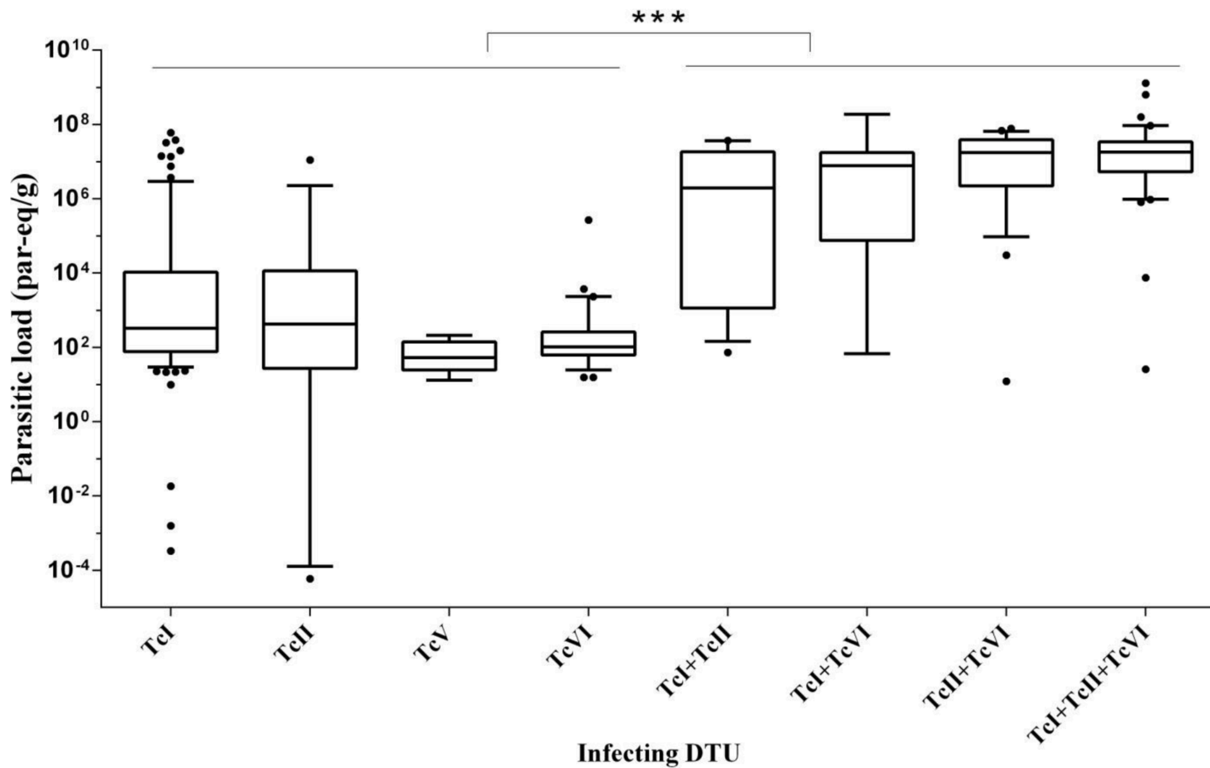

Fig. 4. Box plots showing the comparison of *T. cruzi* parasitic load, presented in logarithmic scale, according to the infecting DTU in 224 *M. spinolai*. The line inside the box represents the median, and the box extends from the lower to the upper quartiles. Whiskers indicate the 90th and 10th percentiles and dots represent the outliers. Mann Whitney test: *** $p < 0.0001$.

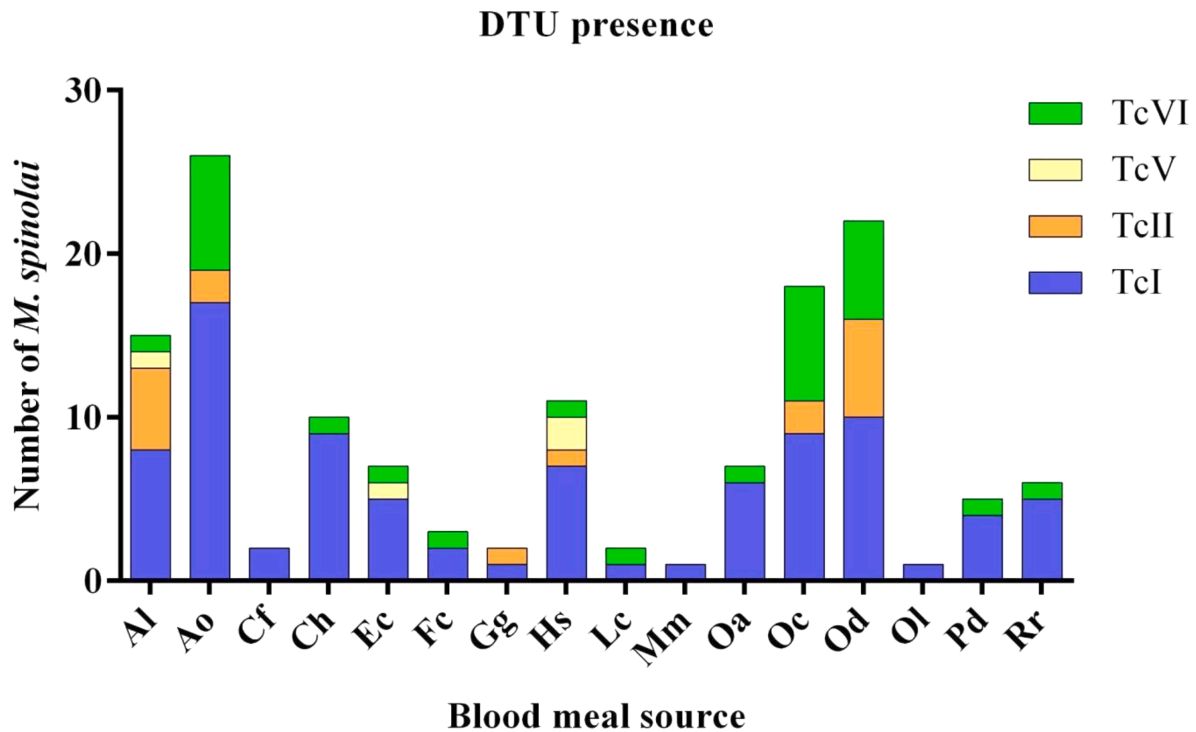

Fig. 5. Stacked bar chart presenting single DTU infections detected in *M. spinolai* fed on each of the unique vertebrate blood meal sources tested. Al: *Abrothrix longipilis*, Ao: *Abrothrix olivaceus*, Cf: *Canis lupus familiaris*, Ch: *Capra hircus*, Ec: *Equus caballus*, Fc: *Felis catus*, Gg: *Gallus gallus*, Hs: *Homo sapiens*, Lc: *Lycalopex culpaeus*, Mm: *Mus musculus*, Oa: *Ovis aries*, Oc: *Oryctolagus cuniculus*, Od: *Octodon degus*, Ol: *Oligoryzomys longicaudatus*, Pd: *Phyllotis darwini*, and Rr: *Rattus rattus*.

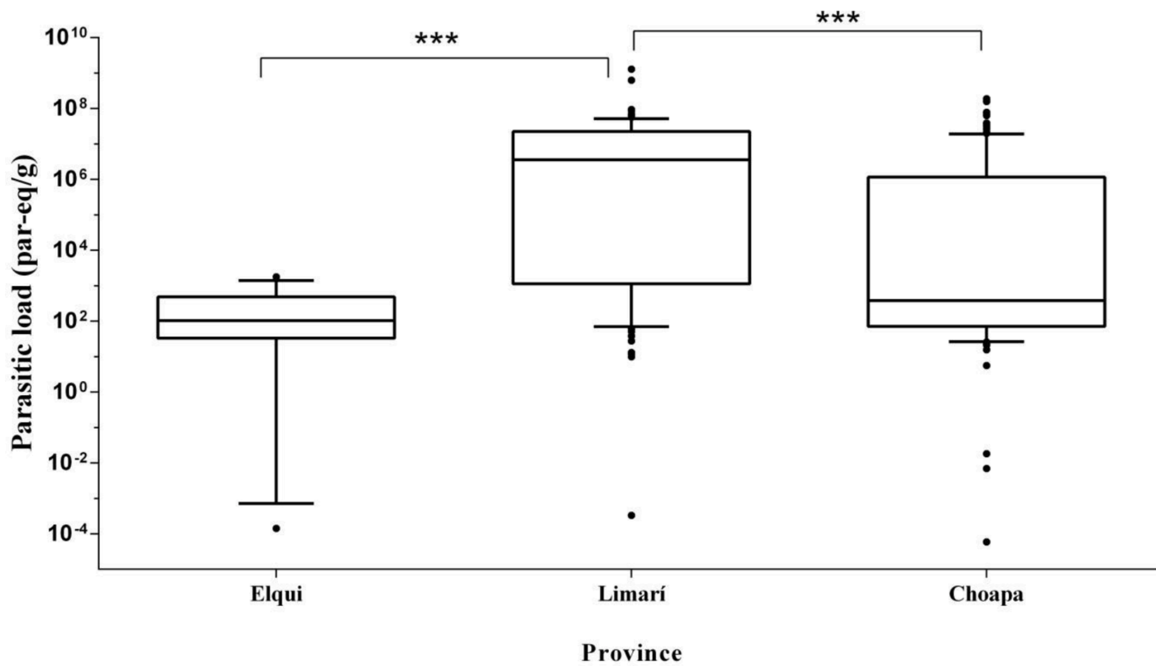

Fig. 6. Box plots showing the comparison of *T. cruzi* parasitic load, presented in logarithmic scale, according to Province in 224 *M. spinolai*. The line inside the box represents the median, and the box extends from the lower to the upper quartiles. Whiskers indicate the 90th and 10th percentiles and dots represent the outliers. *** Dunn's post-hoc test $p < 0.0001$.

differences in the parasitic loads by Province (Kruskal-Wallis test, $\chi^2 = 37.01$, df = 2, $p < 0.0001$); post-hoc Dunn's pairwise tests revealed significant differences between triatomines from Limarí over those from Choapa and Elqui ($p < 0.0001$ for both comparisons; Fig. 6). The single and mixed DTU infections by Province had significant differences (Fisher's exact test, $p < 0.0001$), with Limarí having a higher number of mixed infections.

We found significant differences when comparing parasitic loads according to developmental instar (n 1st = 39; 2nd = 62; 3rd = 77; 4th = 15; 5th = 5; adults = 26) (Kruskal-Wallis test, $\chi^2 = 15.56$, df = 5, p = 0.0082), but they were significant only when comparing the 2nd nymphal instar with the 3rd (Dunn's post-hoc test p = 0.0031), being lower in the latter (Fig. 7). The single and mixed DTU infections by developmental instar had significant differences (Fisher's exact test, p = 0.037), with the 3rd nymphal instar having a higher number of single infections.

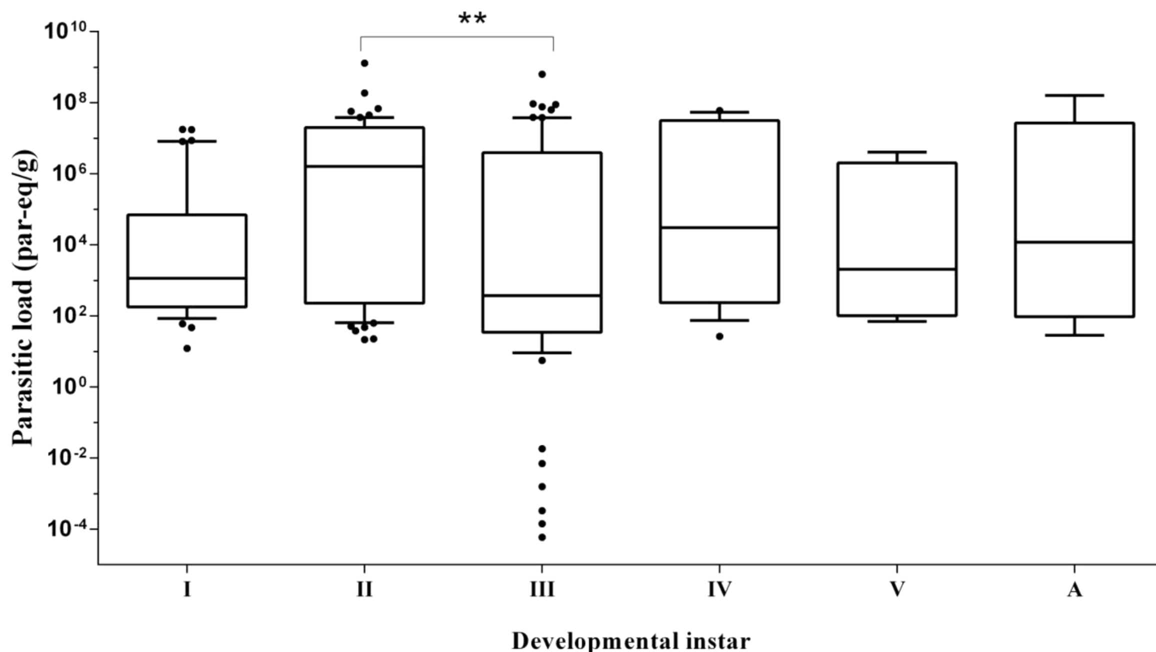

Fig. 7. Box plots showing the comparison of *T. cruzi* parasitic load, presented in logarithmic scale, according to developmental instar in 224 *M. spinolai*. The line inside the box represents the median, and the box extends from the lower to the upper quartiles. Whiskers indicate the 90th and 10th percentiles and dots represent the outliers. ** Dunn's post-hoc test p = 0.0031.

4. Discussion

This is the first report on the parasitic load of naturally infected *Mepraia spinolai* that relates this variable to the blood meal source of the triatomine and the DTU of *T. cruzi*. There were no significant differences in parasitic loads according to blood meal source, but we found higher *T. cruzi* loads in triatomines infected with more than one DTU, i.e., mixed infections.

As observed before in field captured triatomines (Moreira et al., 2017; Valença-Barbosa et al., 2021), we detected a wide range of parasitic load values: 3.02×10^{-5} to 1.29×10^{9} par-eq/g. There is one report on parasite burden in field captured *M. spinolai* that assessed the *T. cruzi* load by qPCR in its feces, after laboratory feeding, showing low to high amounts per microliter, in which they stated that this value would not be directly related with the lifespan of the vector (Mc Cabe et al., 2019). Unfortunately, different measurement units prevent direct comparison with our results. Furthermore, in one of these studies, starved insects and younger instars were discarded (Valença-Barbosa et al., 2021). In *M. spinolai* populations, mainly composed of young starved instars (Bacigalupo et al., 2006; Estay-Olea et al., 2020), more than 85% in our case, this would probably have underrepresented the source populations. A field study of *M. spinolai* found that infected ones presented lower standardized body mass index (Estay-Olea et al., 2020), so preselection by nutritional status could also bias the results regarding *T. cruzi* frequency of infection. Moreira et al. (2017) used a conventional PCR screening before pursuing parasitic load quantification, with the downside that conventional PCR has reduced sensitivity compared to some qPCR techniques (Hagström et al., 2019; Saavedra et al., 2016). Therefore, we consider our approach for quantifying the parasitic load to be representative of natural *M. spinolai* populations living in the Coquimbo Region.

We were unable to identify the blood meal source in 26.8% of our samples, possibly due to the time lapsed from the triatomines last meal, or more likely because of the absence of a matching vertebrate species as reference (Canals et al., 2001; Chacón et al., 2016), as all these samples had positive vertebrate Cyt b amplification; the HRM technique does not permit the identification of unexpected blood meal sources, which constitutes one of the limitations of our study. However, we identified the blood meal sources in most of our samples. As there is a chance that an identified sample could actually correspond to a species closely related to a reference, these results should be considered potential blood meal sources. Future studies using this technique should consider sequencing for undetermined blood meal sources, and for confirmation of the identified species (Omondi et al., 2015; Owino et al., 2019; Peña et al., 2012). Notably, this is the first study finding 17 different blood meal sources in triatomines using a qPCR technique coupled with HRM; previous studies only compared against 14 references or less (Cantillo-Barraza et al., 2015; Hernández et al., 2016; Minuzzi-Souza et al., 2018; Peña et al., 2012; Peña-García et al., 2014); nevertheless, sequencing studies usually provide more resolution (Arias-Giraldo et al., 2020; Dumonteil et al., 2018, 2020; Gorchakov et al., 2016; Murillo-Solano et al., 2021), so it is likely that in the future the latter technique will prevail if the required funding is available and/or the costs are reduced.

Over 42.6% of the species identified as a blood meal source corresponded to wildlife, which confirms the diet of *M. spinolai* (Canals et al., 2001; Chacón et al., 2016; Molina et al., 2004). The most frequently detected blood meal sources in our study were rodents: *A. olivaceus*, which has up to 42% of *T. cruzi* infection (Rozas et al., 2005), and *O. degus*, with 37% of infection (Botto-Mahan et al., 2015); followed by rabbits, reported with 37.9% of infection in this area (Botto-Mahan et al., 2009), constituting natural reservoirs for the wild transmission cycle of *T. cruzi.* In addition, the high number of triatomines fed on humans stands out (65), demonstrating the risk posed by *M. spinolai*, as these were *T. cruzi* positive triatomines with a detectable parasitic load. The Coquimbo Region is a Chagas disease endemic zone with human seroprevalence of 2.8%, higher than the Chilean national average of 1.2% (MINSAL, 2016); this may be explained in part by accidental vectorial transmission from these opportunistic vectors.

In this study, we did not find a relation between the blood meal sources with the level of *T. cruzi* parasitic load. Something similar was previously reported, without a clear pattern relating *T. cruzi* infection with blood meal sources (Zárate et al., 1980). Possibly, the blood meal source results reflect only the most recent hosts, while infection with *T. cruzi* lasts for the entire life of the insect (Dumonteil et al., 2018), being able to find trypomastigotes in insects maintained without feeding for long periods of time (Garcia et al., 1995). These were triatomines captured from the field, for which we did not know their last date of feeding, nor of previous blood meals. In this sense, *M. spinolai* can survive starvation for up to 7 months (Mc Cabe et al., 2019).

Regarding the DTUs, we detected a higher percentage of single infections over mixed ones, as previously reported (Coronado et al., 2009; Ihle-Soto et al., 2019). Our results confirm that TcI is the most frequent DTU in *M. spinolai*, as other studies have stated (Coronado et al., 2009; Ihle-Soto et al., 2019), so TcI may have a higher probability of being transmitted to mammals by this vector. TcI was also the most frequent DTU reported in South America, both in domestic and sylvatic cycles (Brenière et al., 2016). We could not identify the infecting DTU in a large number (260) of *T. cruzi* positive samples. It is possible that some corresponded to DTUs not tested here, or could correspond to one of the DTUs tested, but with slight genetic differences that prevented annealing, as reported for hybridization tests (Ihle-Soto et al., 2019). However, most of these samples presented lower parasitic loads, which were probably insufficient for detection by the DTU genotyping real-time PCR assays (Muñoz-San Martín et al., 2017).

Notwithstanding these limitations, due to the high number of samples analyzed we were able to unveil that mixed infections - i.e., those with more than one DTU detected - showed significantly higher parasitic loads than single infections. This could be an artifact of the methodology, given that when there are more copies of a target it is easier to detect it and, conversely, when the parasitic load is low, both quantification and DTU determination are less achievable (Ramírez et al., 2015). However, the parasitic loads registered in the individuals presenting mixed infections are higher than just the sum of the parasitic load detected in specimens with single infections of those DTUs, so we postulate that these DTUs coexist in a mutually beneficial relation inside this vector. In the laboratory, the development of *T. cruzi* cultures as mixtures of strains differs from pure clonal genotypes, suspecting potentiation or inhibition reactions between them (Pinto et al., 1998). A short-term experimental infection of *R. prolixus* comparing single and mixed infections detected that mixed ones presented generally lower numbers than those infected with a TcI strain alone, but some parts of the digestive tract contained significantly more parasites in mixed infections, particularly metacyclic trypomastigotes after three weeks post infection (Araújo et al., 2014); it is possible that during long term infections in natural *M. spinolai* populations, the parasites from different DTUs are able to colonize its intestinal tract and multiply, even those with slower rates of development. In fact, a previous study comparing the capacity of *M. spinolai* and *T. infestans* to acquire *T. cruzi* infection from a common rodent reservoir two months post feeding showed that *M. spinolai* presented more mixed DTU infections (Campos et al., 2007). In this regard, the *T. cruzi* DTU composition - with TcII as single or mixed infection - did not appear to have an effect on the life expectancy of *M. spinolai* (Mc Cabe et al., 2019). It is possible that these higher parasitic loads in mixed infections do not have a detrimental effect on *M. spinolai* but could turn them into super-spreaders, with both high numbers and different types of *T. cruzi* that they can transmit to susceptible hosts. Here, these super-spreaders would encompass approximately 38% of the triatomine population. The existence of mammal super-spreaders was mentioned as negligible regarding the fruitful spread of this parasite, when studied focusing on the transmission from mammals to triatomines (Jansen et al., 2020), so it is possible that this

strategy is occurring at the vector level.

Higher parasitic loads were found in the Province of Limarí. In the case that this is caused by factors related to the sampling site, altitude could be a relevant variable, as those sampling sites presented a lower average altitude. In *Triatoma dimidiata* (Latreille, 1811), altitude was reported to be related to the immune response of the triatomine and to the virulence of *T. cruzi* (De Fuentes-Vicente et al., 2017). Another site factor that could be involved is the number of DTUs present in reservoirs, which would imply that in areas where more DTUs are available, the parasitic loads of the vectors would be higher, if our hypothesis of a positive relation between the number of infecting DTUs and the parasitic load is accurate. Unfortunately, in this study we did not monitor the infection in mammals from the sampled localities. However, we did find a higher proportion of mixed infections in *M. spinolai* from Limarí, which would support this theory. Other factors involved could be related to the genotype of the triatomine (Pech-May et al., 2019), or the microbiota present (Dumonteil et al., 2020), which were not determined in this study, or the effect of undetermined blood meal sources; in this sense, the proportion of specimens without blood meal source identification was distributed similarly by Province.

On the other hand, triatomine population composition could be related with these findings, if the infection level was related to the developmental instar of the vectors. When we compared the population structure from the three Provinces, we confirmed that they presented large variations in the number and proportion of developmental instars captured, but in all of them the most abundant were the 1st-3rd nymphal instars. We detected an overall difference among instars regarding parasitic load, with 2nd instar nymphs showing higher levels than 3rd instar nymphs, and when comparing single and mixed infections by developmental instar, we also detected differences, probably attributable to these instars, as the 2nd and 3rd nymphal instars comprised 62% of our DTU determined sample. So far, we have not found any records in the literature explaining differences in *T. cruzi* infection between nymphal instars. This requires further investigation. A previous study in *T. infestans* comparing sylvatic nymphs with adults that invaded nearby houses noticed that the nymphal instars harbored more mixed infections than the adults, and - assuming they originated from the same population - there could have been some DTU filtering process in the adults (Bacigalupo et al., 2012), and this may be occurring in different developmental instars of *M. spinolai*. Future studies should attempt to include more specimens per developmental instar and to perform laboratory infection of *M. spinolai* with blood spiked with single or mixed DTUs in different proportions, to validate our theory regarding the parasitic load and the infecting *T. cruzi* DTUs in this vector species.

Conclusions

There is a wide range of variation in the parasitic loads of *Mepraia spinolai*, with a large number of vertebrate species as blood meal sources, including wildlife, domestic animals and humans. The most ubiquitous DTU in this study was TcI. Mixed *T. cruzi* infections with different DTUs in *M. spinolai* are related to a higher parasitic load, so some individuals of the triatomine population will pose higher risk than others; also, they could be concentrated in some areas, which will require targeted preventive interventions and monitoring of the human population exposed.

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CRediT authorship contribution statement

Miguel Saavedra: Validation, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Antonella Bacigalupo:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing, Project administration. **María Victoria Barrera:** Investigation, Writing – original draft, Writing – review & editing. **María J. Vergara:** Investigation, Writing – original draft, Writing – review & editing. **Bárbara Álvarez-Duhart:** Formal analysis, Visualization, Writing – review & editing. **Catalina Muñoz-San Martín:** Conceptualization, Methodology, Validation, Investigation, Supervision, Writing – review & editing, Funding acquisition. **Rigoberto Solís:** Writing – review & editing. **Pedro E. Cattan:** Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors have no competing interests to declare.

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Supplementary materials

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