Infection, Genetics and Evolution 71 (2019) 197–204

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

Survivorship of wild caught *Mepraia spinolai* nymphs: The effect of seasonality and *Trypanosoma cruzi* infection after feeding and fasting in the laboratory

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A R T I C L E I N F O

Keywords:
Mepraia spinolai
Trypanosoma cruzi
Vector survival
Fasting

A B S T R A C T

Chagas disease is caused by *Trypanosoma cruzi*. Vector survival is an important variable affecting vectorial capacity to determine parasite transmission risk. The aims of this study are to evaluate vector survival under fasting/starvation conditions of wild-caught *Mepraia spinolai* after feeding and fasting, the pathogenicity of *T. cruzi* infection, the parasite burden and seasonal variation in parasite discrete typing units (DTU). The survivorship of *M. spinolai* nymphs after two continuous artificial feedings was evaluated, assessing their infection with microscopic observation of fecal samples and PCR. Later, insects were fasted/starved until death. We performed qPCR analyses of parasite load in the fecal samples and dead specimens. *T. cruzi* genotyping was performed using conventional PCR amplicons and hybridization tests. Infection rate was higher in *M. spinolai* nymphs in summer and spring than in fall. Parasite burden varied from 3 to 250,000 parasites/drop. Survival rate for starved nymph stage II was lower in insects collected in the spring compared to summer and fall. TcII was the most frequent DTU. Mainly metacyclic trypomastigotes were excreted. We conclude that *M. spinolai* infection rate in nymphs varies among seasons, suggesting higher transmission risk in warmer seasons. However, nymphs stage II collected in spring are more sensitive to starvation compared to other seasons. TcII in single or mixed infection does not seem relevant to determine vector pathogenicity. These results of vector survivorship after fasting/starvation are important to determine the competence of *M. spinolai* as a vector of *T. cruzi*, since they excrete metacyclic trypomastigotes and the parasitism with *T. cruzi* seems to be poorly pathogenic to the vector under a severe fasting/starvation condition.

1. Introduction

The protozoan parasite *Trypanosoma cruzi* (Kinetoplastea: Trypanosomatidae), the causative agent of Chagas disease, is transmitted by hematophagous triatomine insects (Triatominae, Reduviidae) and most of them are sylvatic species. There are three endemic and sylvatic vector species on the Pacific side of the Southern Cone of South America, all hemimetabolous insects with diurnal activity. *Mepraia gajardoi* inhabits southern Peru and northern Chile (Frias et al., 1998); *Mepraia parapatrica* (*Frías-Lasserre, 2010*) and *Mepraia spinolai* (Frias and Atria, 1998) in endemic, more southern areas. Although the colonization of human dwellings by *Mepraia* species is infrequent, there are situations where human economic activities are performed in close contact with *Mepraia* habitat with potential human-vector contact, especially in summer when these triatomines exhibit greater activity and larger home ranges (Botto-Mahan et al., 2005). Sylvatic triatomines inhabit less stable environments than domestic ones, irregular intakes of blood meals and seasonally variable. Six discrete typing units (DTUs) have been described (TcI-TcVI) in *T. cruzi*, which are groups of parasites with similar DNA (Zingales et al., 2012), and a new entity Tcbat, found within the TcI clade has recently been described (Lima et al., 2015).

Some studies have evaluated the potential virulence of *T. cruzi* on

Abbreviations: DNA, Deoxyribonucleic acid; DTUs, Discrete typing unit; kDNA, Kinetoplastidic DNA; PCR, Polymerase chain reaction; qPCR, Quantitative PCR

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https://doi.org/10.1016/j.meegid.2019.04.002
Received 12 December 2018; Received in revised form 8 March 2019; Accepted 2 April 2019
Available online 03 April 2019

triatomines (Botto-Mahan, 2009; Schaub, 1988; Elliot et al., 2015). On one hand, *T. cruzi* showed a low effect on the life history traits of experimentally infected *Triatoma infestans*, evaluated by vector mortality rate and development time under optimal feeding conditions (Schaub, 1988). On the other hand, results of experimental infections and reinfections in *M. spinolai* with *T. cruzi* under regular feeding did cause delays in development and some harm, measured as vector mortality, especially in nymph stages IV-V (Botto-Mahan, 2009). This issue has been controversial since *T. cruzi* is thought to be nonpathogenic to its vector hosts, but it can be harmful to vectors under adverse conditions such as food deprivation (Schaub, 1989). Fasting is an important factor in infection reduction in triatomines which could alter *T. cruzi* transmission competence (Dias, 1934; Chowdhury and Fistein, 1986). Fasting is frequent in nature and collected triatomines are generally starved and weak (Schaub, 1994). Triatomine mortality caused by *T. cruzi* is an important variable to understand vector competence and population dynamics; therefore, it is necessary to know its relevance to assess Chagas disease epidemiology and *T. cruzi* transmission. *Mepraia spinolai* can survive in human dwellings and there are reports of a domiciliation phase in this vector (Canals et al., 2000; Cattan et al., 2002; Botto-Mahan et al., 2015a).

Vector transmission depends on many direct factors such as the animal reservoir, infectious bites, vector density, bite rate and vector survival/mortality rates (Canals et al., 2017), thus it is important to understand the factors that determine vector mortality. Here, for the first time we evaluate the effect of *T. cruzi* on field collected *M. spinolai* nymphs after laboratory feeding and starvation. To accomplish this, we collected triatomines in three consecutive austral seasons (summer, fall and spring) to answer the following questions: 1) Does *T. cruzi* infection in *M. spinolai* vary seasonally? 2) Does *T. cruzi* infection affect nymph mortality under fasting/starvation conditions? 3) Does the parasite burden and *T. cruzi* DTU composition affect vector mortality?

2. Material and methods

2.1. Macrobiotic and climatic characteristics of the triatomine collection area

Triatomine nymphs were collected from the rural locality Los Pozos, near Las Chinchillas National Reserve (31°20′28.74″ S, 71°14′1.58″ W, Chile; Fig. 1 AB, see flowchart in Fig. 2) in three seasons: summer (December 21–March 20), fall (March 21–June 20) and spring (September 21–December 20). This site is a hyper-endemic area of Chagas disease with semiarid-Mediterranean climate, presenting scarce plant cover and a mixture of rocks, pebbles, stones and sand, characterized by the presence of a bromeliad (*Puya berteroniana*) and domestic (*Capra hircus*, *Canis familiaris*, *Felis silvestris*, *Ovis orientalis*), native (*Abrothrix olivaceous*, *Lycalopex culpaeus*, *Octodon degus*, *Phyllotis darwini* and *Thylamys elegans*), and introduced (*Oryctolagus cuniculus*) mammals. The triatomine summer group of 136 individuals was collected in mid-March 2016, (late austral summer) with a 12.5:11.5 light:dark photoperiod,and extreme minimum and maximum temperatures of 13.8–46.4 °C (average 25.4 °C). The triatomine fall group of 122 individuals was collected in May 2016, corresponding to austral mid-fall with a 11.5:12.5 light:dark photoperiod and extreme minimum and maximum temperatures of 9.9–29.3 °C (average 16.4 °C). The triatomine spring group of 123 individuals was collected in October 2016, corresponding to austral mid-spring with a 12.5:11.5 light:dark photoperiod and extreme minimum and maximum temperatures of 11.3–45.1 °C (average 23.5 °C). Triatomine collection was performed on private land with the owner's permission. Insects were collected from 11:00 to 16:00 h by one trained researcher. *M. spinolai* were manually collected during the period of maximal activity beneath rocky outcrops composed of hundreds of rocks. Triatomines were transported in polystyrene foam boxes with folded paper as refuge, arriving to the laboratory one day after capture, and then kept individually in a climate chamber at 26 °C, with relative humidity of 70% and a 14:10 h light: dark photoperiod.

2.2. Triatomine fecal sample collection

Within two weeks after collection, 352 triatomines were weighed before and after being fed with uninfected *Mus musculus* anesthetized with 2% sodium pentothal. After feeding, each insect was classified by nymph stage and kept separately in a plastic container with small compartments of 3.2 × 3.6 cm to allow individual follow-up. Fecal samples were collected 30 min after feeding to full engorgement, and mixed with 100 μl of destilled water. An additional fresh sample was diluted 1:10 in saline buffer (NaCl 0.9% *w*/w) to be examined microscopically to determine the presence of parasite forms. This procedure was repeated for 307 nymphs after a second and last feeding 40 days later. Triatomines were maintained in a growth chamber under the same conditions previously described. Dead insects and ecdyses were recorded every 3 days after each feeding. We obtained fecal samples from all triatomines fed twice, without pressing the abdomen to avoid internal damage, allowing follow-up over time. In the case of dead infected triatomines after the two feedings the entire hindgut was dissected hydrated and its DNA extracted (see Fig. 2).

2.3. DNA extraction and T. cruzi infection determination

Each fecal sample (1st feeding: $N = 352$; 2nd feeding: $N = 307$) was extracted in a final volume of 200 μl with conditions already described in the EZNA Blood DNA Mini kit (OMEGA BIO-TEK, Norcross, GE, USA) (Egaña et al., 2016). *T. cruzi* infection was determined three times by PCR directed to minicircle kDNA using standardized conditions and oligos 121 and 122 in a volume of 50 μl (Wincker et al., 1994). PCR assays were repeated when negative results were obtained. In those cases, the extracted DNA was concentrated by evaporation. Three different PCR assays were repeated using variable volumes of the concentrated DNA used as template. A negative sample resulted when all PCR attempts with extracted DNA failed to detect a 330 bp amplicon. In addition, a sample of the hindgut was obtained from 15 dead infected insects (8 dead between the 1st and 2nd feeding, 7 dead after the 2nd feeding) for DNA extraction, PCR, qPCR and *T. cruzi* genotyping (see Fig. 2).

2.4. Trypanosoma cruzi satellite DNA Real-Time PCR assays

Assays ($N = 60$) were performed using *T. cruzi* nuclear satellite DNA primers Cruzi 1 (5′ ASTCGGCTGATCGTTTTCGA) and Cruzi 2 (5′ AAT TCCTCCAAGCAGCGGATA 3′) (Moreira et al., 2013) in a final volume of 20 μl containing 2 μl DNA template, 5× HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne, Taru, Estonia), 0.3 μM of each primer and nuclease free water. Cycling conditions 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 GmbH, Hilden, Germany). After all the amplification cycles, a melting curve was run. Each sample was tested in duplicate (see Fig. 2).

2.4.1. Parasite standard calibration curve

T. cruzi DNA standards for absolute quantification were obtained from parasite-free fecal samples spiked with 10^6 copies of nuclear satellite DNA/ml (cnsDNA/ml, hereafter), considering that one parasite cell harbors approximately 200 fg of DNA (Piron et al., 2007; Duffy et al., 2009). After the extraction process, 10-fold serial dilution was performed with nuclease-free water to cover a range between 10^6 and 0.1 cnsDNA/ml. Due to variability in the number of copies of the nuclear satellite DNA previously described (Kooy et al., 1989; Moreira et al., 2013; Ienne et al., 2010), the standards were made with clonal reference strains Dm28c (TcId) and Y (TcII) to reduce the differences in the detection limits (Ramírez et al., 2015; Duffy et al., 2013).

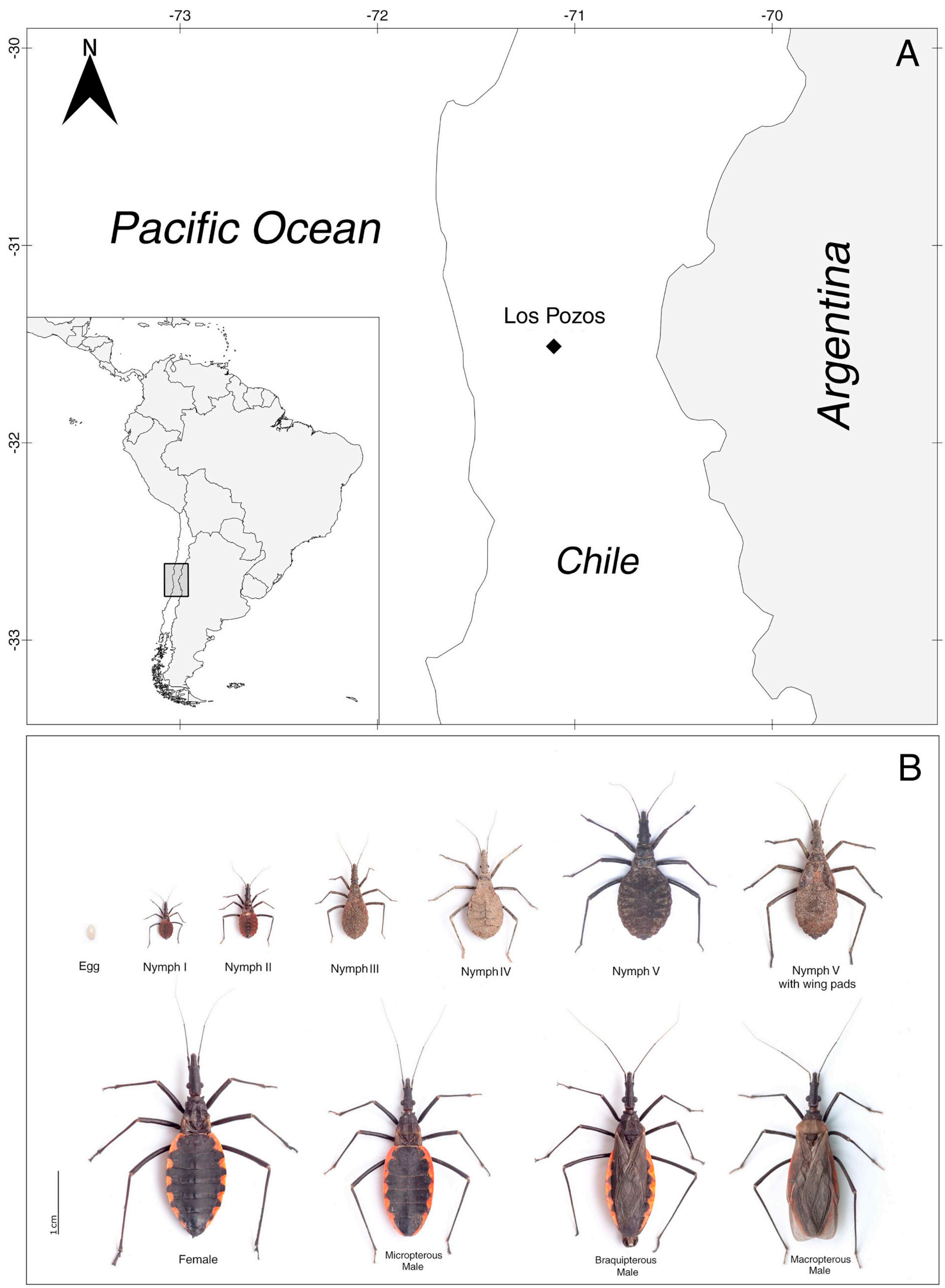

Fig. 1. A: Map showing the collecting site: Los Pozos, Chile. B: Complete development of *Mepraia spinolai*, including the egg, nymphal stages, and adults. Fifth-nymphal stages with and without wing pads are shown. The micropterous female and the polymorphic male (micropterous, brachypterous and macropterous morphs) are shown. Photographs by V. Valdés.

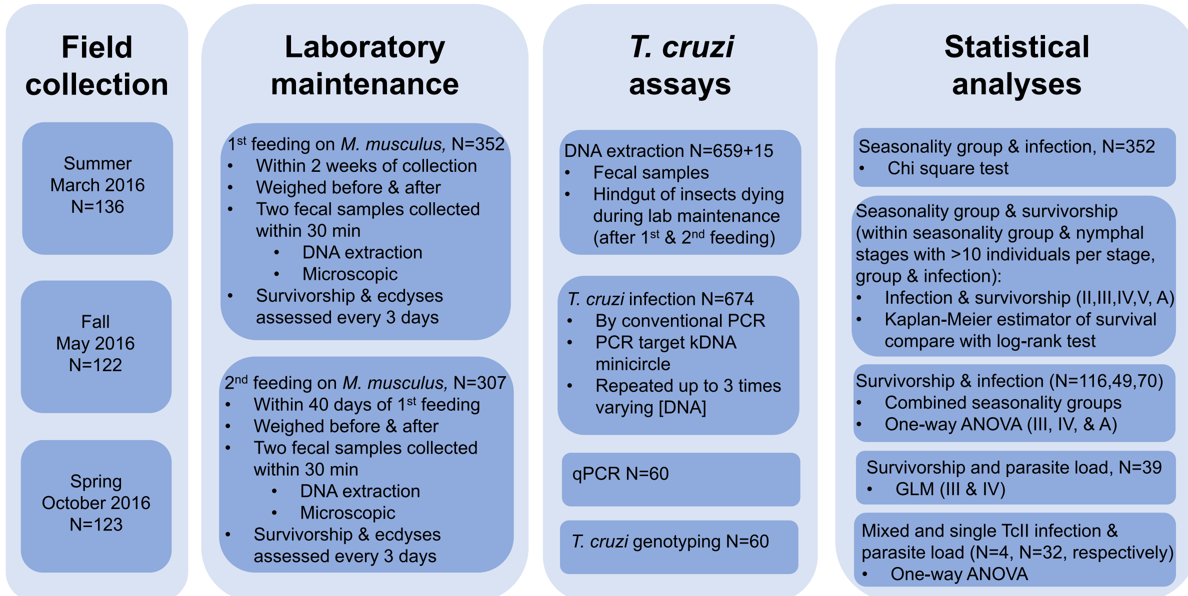

Fig. 2. Flowchart of the experimental design of this study, including the protocols for field collection, laboratory maintenance, *Trypanosoma cruzi* assays, and statistical analyses. Sample sizes are shown in parentheses.

2.4.2. Heterologous internal amplification control Real-Time PCR Assays

All samples were co-amplified with 1 pg/µl of a sequence of 183 bp from tonoplast intrinsic protein 5;1 (TIP 5;1) of *Arabidopsis thaliana* (GenBank accession number NM_114612) generated by gBlocks® Gene Fragments (Integrated DNA Technologies, San Diego, CA, USA) used as a heterologous internal amplification control (IAC) to discount carry-over of PCR inhibitors. Assays were performed using primers IAC Fw (5′ ACCGTCATGGAACAGCACGTA 3′) and IAC Rv (5′ CTCCCGCAACAAA CCCTATAAAT 3′) (Ramírez et al., 2015) at a final concentration of 0.2 µM and at a melting temperature of 58 °C. The other qPCR assays conditions were the same as described above.

2.4.3. Control standard calibration curve

The standard curve for the IAC was performed with 2, 0.2, 0.02 and 0.002 pg/µl of the gBlocks® Gene Fragments.

2.4.4. Normalization and quantification of parasite loads

The parasite equivalents of DNA samples were calculated considering the amplification curve of standard *T. cruzi* DNA and the results were normalized according the heterologous IAC results.

2.5. Trypanosoma cruzi genotyping

For genotyping, PCR-DNA for blot analysis was performed using 10 µl of each PCR product ($N = 60$). Four *T. cruzi* clones (sp104cl1, CBBcl3, NRcl3 and v195cl1), corresponding to TcI, TcII, TcV and TcVI, respectively, were used to generate DTU-specific probes (Arenas et al., 2012). Construction of minicircle probes and radiolabeling was performed as described (Arenas et al., 2012). The PCR products were subjected to agarose electrophoresis, transferred onto Hybond N^+ nylon membranes (Amersham, Piscataway, NJ, USA), and cross-linked by ultraviolet light for DNA fixation. After transferring PCR products, four equivalent membranes were pre-hybridized for at least 4 h at 55 °C. Each membrane was then hybridized overnight with a *T. cruzi* DTU-specific DNA probe labeled with ^{32}P with a Klenow DNA polymerase 1×10^6 cpm membrane. After hybridization, membranes were washed under high stringency conditions and then exposed using the Molecular Imager FX (Bio-Rad Laboratories, Hercules, CA, USA). DNA amplicons (30–300 mg DNA) were electrophoresed onto 2% agarose gel, after which the DNA was denatured and then transferred to nylon membranes. Four copies of identical membranes containing DNA blots were hybridized against the panel of the specific DNA probes to recognize specific lineages of TcI, TcII, TcV and TcVI, which are the representative *T. cruzi* DTUs circulating in Chile, as described (Barnabé et al., 2001; Rozas et al., 2007; Coronado et al., 2009; Muñoz-San Martín et al., 2017) (see Fig. 2).

2.6. Statistical analyses

The association between seasonality and *T. cruzi* infection status was tested by χ^2 tests. To test for differences in survivorship among the different groups (summer, fall and spring) and between infected and uninfected nymphs from each group, the Kaplan-Meier estimator of the survival function was obtained and then the survival functions for each group or infection status were compared using a Log-rank test with Bonferroni correction (CRAN Task View: Survival Analysis; https://cran.r-project.org/web/views/Survival.html). For the survival analyses, only nymphs from the same stages were compared. Analyses were performed only with sample sizes ≥10 individuals per nymph stage, group and infection status (nymphs II, III, IV, V and adults, depending on the comparison). In addition, we tested for differences in survivorship between infected and uninfected nymphs (summer, fall and spring groups combined) with one-way ANOVAs. Log transformations were performed to obtain normality.

We used a logistic regression analysis to test whether parasite load was related to microscopic *T. cruzi* detection probability. In addition, we assessed if *M. spinolai* survivorship depended on parasitic load using a General Linear Model (GLM) with normal distribution and identity link. Finally, we evaluated whether the presence of TcII in single or in mixed infection affected the parasite load and survivorship of *M. spinolai* using one-way ANOVA. Analyses were performed with the R software (Team R Core 2014, version 3.0.2) (see Fig. 2).

3. Results

3.1. Blood ingestion and association between infection-seasonality

Overall, the average amount of blood ingested by nymph stage I, II, III, IV, V and adults was 55, 83, 105, 140, 240 and 450 mg, respectively. The results of *M. spinolai* infection assayed for the three groups of

Table 1
Number of individuals of the different stages of *Mepraia spinolai* (I to V, and adult) collected during the three seasons. *Trypanosoma cruzi* infected insects that died after first feeding are shown in parentheses.

Season of collection	Stage at collection	Deaths during transportation	Deaths between 1st and 2nd feeding	Infection status after 2nd feeding		
				Infected	Non-infected	Total
Summer	I	4	1	0	0	5
	II	1	0	1	12	14
	III	5	13(4)	11	24	53
	IV	1	7	3	13	24
	V	1	1(1)	4	2	8
	Adult	2	0	16	14	32
	Total	14	22(5)	35	65	136
Fall	I	0	5	0	0	5
	II	2	6	2	12	22
	III	1	1	0	26	28
	IV	0	2	3	13	18
	V	0	0	1	12	13
	Adult	3	0	1	32	36
	Total	6	14	7	95	122
Spring	I	2	2	0	0	4
	II	2	3(2)	4	7	16
	III	3	3(1)	20	35	61
	IV	1	1	15	12	29
	V	1	0	0	6	7
	Adult	0	0	4	2	6
	Total	9	9(3)	43	62	123

insects (summer, fall and spring) are shown in Table 1. This table also indicates the number of insects that died during transportation and between the first and the second feeding (mostly first stage nymphs). They probably died from the interaction between starvation and the stress related to transportation. The infectious status of each nymph was used to obtain the infection rate of each group. Insects collected in summer and spring displayed the higher infection rates, with 32.8% (40 out of 122) and 40.4% (46 out of 114), respectively. Insects collected in fall presented the lowest infection rate (6.0%; 7 out of 116). Significant differences in infection rates were obtained for insects collected in the fall compared with those collected in summer ($\chi^2 = 26.9$, d.f. = 1, $p < .001$) and spring ($\chi^2 = 38.2$, d.f. = 1, $p < .001$), respectively. No difference was detected in infection rate for those insects collected in summer and spring ($\chi^2 = 1.46$, d.f. = 1, $p = .228$).

3.2. Infection, parasitic burden and genotyping

Microscopic fecal observations revealed mainly metacyclic trypomastigotes and sometimes spheromastigotes (parasite form was not recorded by specimen). All infected insect samples were *T. cruzi* genotyped; even some samples without qPCR analysis (see Table A1, Supplement material). The parasitic burden results ranged from 1257.3 to 0.015 cnsDNA/µl (see summary by stage in Table 2). These results indicate that a single fecal drop might contain between 25,140 and 3 parasites, respectively; the results of qPCR have a correction factor of 200×, corresponding to the fecal sample volume from which DNA was obtained. Some *T. cruzi* genotyping was performed twice, using fecal samples from the first and the second feedings. The results of qPCR were obtained from fecal samples after the second feeding. In most cases, *T. cruzi* genotyping was identical in the first and second fecal samples, except when indicated in Table A1. These corresponded to cases of mixed infections with more than one *T. cruzi* DTU, which changed in DTU composition from the first to the second fecal sample. Fig. 3 shows results of *T. cruzi* genotyping in some fecal samples with single and mixed infections. Fig. 3A and B show results of representative insect samples from the first and the second feeding, respectively. We also studied *T. cruzi* infection, parasite burden and *T. cruzi* genotyping in some of the infected insects after death. Even though these samples were extracted from the whole insect hindgut, they presented much lower parasite burden (0.15–15.0% parasites) than the corresponding fresh drop samples. Those corresponded to 11 insects, of which nine maintained positive results by qPCR, only seven were positive by PCR and the other four changed their status from infected to uninfected after they died from starvation. Only three out of the seven samples were genotyped–all had *T. cruzi* TcII (not shown). Tables 2 and A1 also include microscopic detection data of fresh fecal samples obtained at the second feeding. *T. cruzi* detection probability by visual observation of insect fecal samples (including nymph stages III and IV, combining the three seasons, $N = 39$) did not depend on the parasite load ($p = .133$). In addition, we did not detect an effect of parasitic burden on survivorship of nymphs III and IV combined ($\chi^2 = 1.29$; $p = .257$; N = 39).

3.3. Survivorship and survival curves of Mepraia spinolai

We did not detect statistically significant differences in the

Table 2
Infected *Mepraia spinolai* individuals grouped by stage (after the second feeding) showing range of post 2nd feeding survival (days), proportion of *Trypanosoma cruzi* detected in feces post feeding by optical microscope (OM), range of copies of nuclear satellite DNA/µl (cnsDNA/µl) by qPCR, *T. cruzi* DTUs present in single and/or mixed infections. N: sample size; ND: not detected. See detailed information in Supplement (Table A1).

Stage	N	Range of survival (days)	OM detection	cnsDNA/µl	DTUs
II	4	70–119	0.75	35.2–129.5	TcII
III	26	20–322	0.65	ND – 1257.3	TcI, TcII, TcV, TcVI
IV	15	78–353	0.53	0.12–540.1	TcI, TcII, TcV, TcVI
V	4	139–305	0.50	ND – 50.4	TcI, TcII, TcV
A	11	193–330	0.18	ND – 1079.2	TcI, TcII, TcV, TcVI

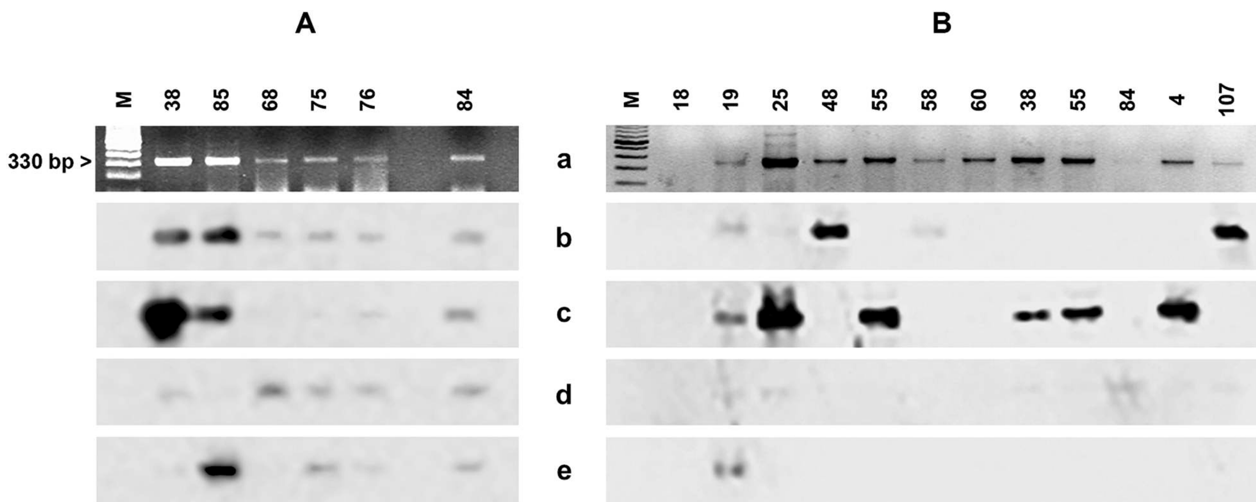

Fig. 3. A: Samples after the first feeding. B: Samples after the second feeding. Representative results of *T. cruzi* DTUs by means of hybridization tests on *Mepraia spinolai* samples. a) Minicircle PCR amplicons stained with ethidium bromide; b) Hybridization with TcI probe (sp 104 cl 1); c) Hybridization with TcII probe (CBB cl 3); d) Hybridization with TcV probe (NR cl 1); e) Hybridization with TcVI probe (V195 cl 1). Lane M, 100-base pair DNA ladder.

survivorship of infected and uninfected nymph stage III (infected = 232.2 ± 14.2 days, N = 31; uninfected = 240.1 ± 8.1 days, N = 85; F = 0.237, p = .628), IV (infected = 197.7 ± 17.5 days, N = 21; uninfected = 218.2 ± 11.7 days, N = 38; F = 1.01, p = .318) or adults (infected = 208.2 ± 14.3 days, N = 21; uninfected = 236.5 ± 8.9 days, N = 49; F = 2.95, p = .091), combining the groups of all seasons.

Comparing the survival rate of *M. spinolai* nymphs collected in summer, fall and spring (combining infected and uninfected), we found statistically significant differences only in nymphs II (χ^2 = 28.2, p < .001, N = 38; Fig. 4). Specifically, nymphs II collected in summer and fall exhibited higher survival rate than those collected in spring (χ^2 = 19.9, p < .001; χ^2 = 14.9, p < .001, respectively). We did not detect differences in nymphs III (χ^2 = 1.7, p = .437, N = 116), IV (χ^2 = 3.0, p = .227, N = 59) or V (χ^2 = 1.9, p = .169, N = 25).

3.4. *Trypanosoma cruzi genotyping, survivorship and parasite burden*

TcII was the most prevalent *T. cruzi* DTU present in the insects under study; however, our results were not conclusive to suggest a difference in survivorship with a preferential parasite DTU composition. Nymphs stages III-IV with TcII in single (N = 32) or mixed (N = 4) infection did not show differences in their parasite burden (F = 0.63, p = .432) or in their survival (F = 1.80, p = .188).

4. Discussion

We report seasonal variation of *T. cruzi* infection in *M. spinolai.* The highest infection rates were in summer and spring, and the lowest in fall. A similar observation was detected before with *M. spinolai* of an endemic area in central Chile (Ordenes et al., 1996). Therefore, seasonal changes in the infection of the sylvatic *M. spinolai* can be translated into higher vector competence and transmission risk in warmer seasons. The parasite populations colonize the triatomine hindgut for

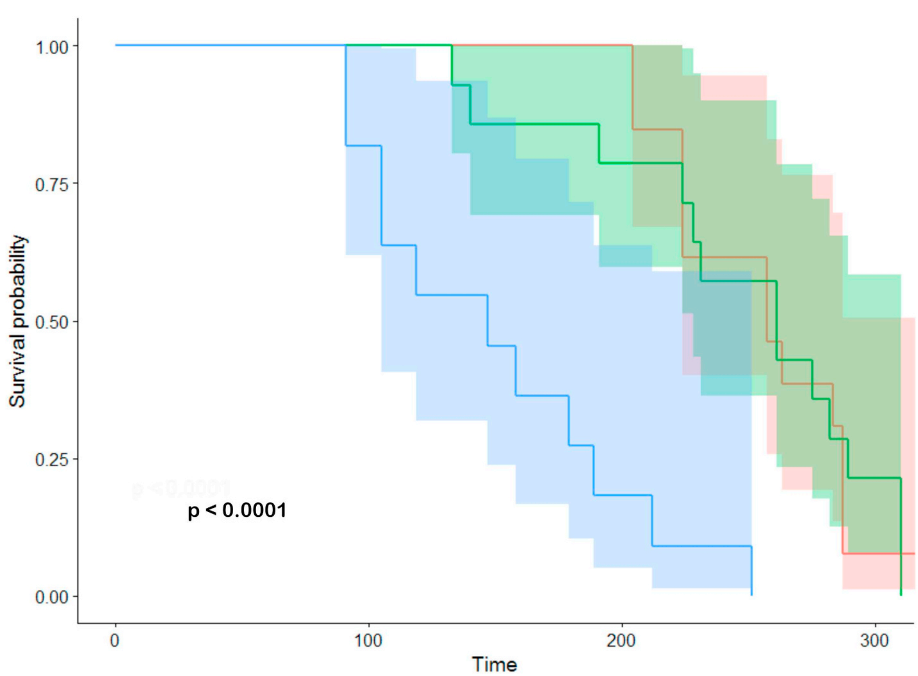

Fig. 4. Kaplan-Meier estimates of survivor function for *Mepraia spinolai* nymph stage II collected in summer (green), fall (red), and spring (blue) from Los Pozos, Chile. The x-axe is expressed in number of days. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

further quick excretion by diuresis after feeding (de Dias et al., 2015). In this optimal nutritional condition, competition between parasites and vector for nutrient resources is absent, with mortality rate of 9% among the infected *T. infestans* nymphs compared to 4% among the uninfected (Schaub, 1988). Another study of experimental infections of *M. spinolai* with one single feeding opportunity by stage and reinfections showed a significant effect of *T. cruzi* on survival rates among the later nymph stages (Botto-Mahan, 2009). A different situation occurs when insects are fasted/starved as in this study, when the insects were no longer fed after two consecutive feedings. Overall, insects collected in fall showed the lower infection rate (5%) compared with those from summer (31.3%) and spring (43.5%). Survival rates of *M. spinolai* nymph stage II after fasting revealed that insects collected in the fall presented the longest survival, compared to equivalent nymphs collected in summer and spring. It is worth to mention that at least the youngest nymphs collected in the spring would correspond to a different generation from those of the summer and fall. Interestingly, nymphs III-IV and adults withstand up to 7 months without feeding, a period which exceeds a long winter when the biotic and abiotic conditions are less favorable for vector feeding and development. Similar results of mortality rate during fasting were obtained in uninfected reared *Rhodnius prolixus* (Feliciangeli et al., 1980), natural populations of *Triatoma dimidiata* (Vargas and Zeledón, 1985), and in a few *M. spinolai* nymphs with undefined status of *T. cruzi* infection that survived up to 335 days (Gajardo-Tobar, 1952). Under the fasting/starved condition used here, insect survivorship was similar in the infected and non-infected insects (nymph stages III, IV, and adults). These results contrast with those reported in experimental infections with *T. cruzi* on starved *T. infestans*. The parasite in experimental infections reduces survivorship of starved *T. infestans* between 14 and 17% but only among nymph stages III-IV (Schaub and Lösch, 1989). In conclusion, our results of mortality in fasting/starved natural populations on infected *M. spinolai* are different to the equivalents in experimental infections of *T. infestans*, since we found no effect of the parasite on survivorship among nymph stages III-IV. We found that the survivorship of *T. cruzi* infected nymphs III-IV was not affected by the parasite burden (range: 250,000–3 parasites/drop). Finally, our results in infected dead *M. spinolai* suggest that fasting/starvation such as that tested in this study reduces infection. This observation was performed before using a low sensitive parasitological tool to quantify *T. cruzi*, microscopic examination of the feces of naturally and reared infected triatomines starved for 140 days (Vargas and Zeledón, 1985; Phillips and Bertram, 1967; Schaub et al., 1986). We found the same result of infection decrease in regularly fed infected *M. gajardoi* after three consecutive feedings (Egaña et al., 2016). All these results on infected triatomines under regular feeding or starvation suggest that insect vectors need to be reinfected to transmit *T. cruzi* and to behave as a competent vector. *T. cruzi* DTU composition in the infected insect does not seem relevant to determine mortality. The most prevalent *T. cruzi* DTU detected was TcII, but this result varied over time in this endemic area, probably due to changes in the transmission cycle due to alternative reservoir hosts where *M. spinolai* feeds (Botto-Mahan et al., 2015b; Campos-Soto et al., 2016). In a previous study in the same endemic area, TcII was only detected in naturally infected *M. spinolai* at the third feeding, suggesting that some *T. cruzi* DTU are residual and flourish only after feeding (Egaña et al., 2016). We detected many cases of mixed infections with more than one *T. cruzi* DTU, and we observed fluctuations in the *T. cruzi* DTU composition over time between the two fecal samples collected. We have previously found this result in *Mepraia* species naturally infected with *T. cruzi* followed with regular feeding (Egaña et al., 2016). TcII in mixed infections does not seem to cause more harm to *M. spinolai* than single infections.

To conclude, we answered the addressed questions. First, *M. spinolai* infectivity varied seasonally. Second, survivorship of *M. spinolai* nymphs II vary depending on the season of collection. Third, the parasite burden does not seem to be relevant to determine vector lifespan, nor the *T. cruzi* DTU composition of TcII in single or mixed infections.

Funding

This study was financially supported by CONICYT-FONDECYT-Chile N°1160080 to A. Solari, and N°1170367 to C. Botto-Mahan.

Authors' contributions

Conceived and designed the experiments: AS. Performed the experiments: AM, FY, RP, AL, SO, CM, and AS. Analyzed the data: AS, SO, CM, CBM. Contributed reagents/materials/analysis tools: AL, SO and AS. Wrote the paper: AS, CM, SO, and CBM. All authors read and approved the final manuscript.

Ethics approval

Protocol CBA#0987 FMUCH 2018. Comité de Bioética sobre Investigación en Animales. Facultad de Medicina. Universidad de Chile.

Acknowledgments

We especially thank Consuelo Sáez for Kaplan-Meier analyses, and Nicol Quiroga and Esteban San Juan for artwork. Photo of *T. cruzi* for the Graphical Abstract was downloaded from https://blogs.biomedcentral.com/bugbitten/2016/09/23/waste-disposal-system-parasites-new-target-drugs-neglected-tropical-diseases/.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.04.002.

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