UNIVERSIDAD DE CHILE -FACULTAD DE CIENCIAS -ESCUELA DE PREGRADO

"*Trypanosoma cruzi* could affect the approaching behaviour of *Mepraia spinolai* (Hemiptera: Reduviidae) to humans by altering vector nutritional status: a field test"

Seminario de Título entregado a la Universidad de Chile en cumplimiento parcial de los requisitos para optar al Título de Bióloga con mención en Medio Ambiente.

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INFORME DE APROBACIÓN SEMINARIO DE TÍTULO

Se informa a la Escuela de Pregrado de la Facultad de Ciencias, de la Universidad de Chile que el Seminario de Título, presentado por la **Srta. Daniela Estay Olea**

"*Trypanosoma cruzi* could affect the approaching behaviour of *Mepraia spinolai* to humans by altering vector nutritional status: a field test".

Ha sido aprobado por la Comisión Evaluadora, en cumplimiento parcial de los requisitos para optar al Título de Bióloga con mención en Medio Ambiente.

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RESUMEN

Los insectos hematófagos exhiben comportamientos complejos en la búsqueda de alimento respondiendo a diversos estímulos olfativos y visuales del hospedero. *Mepraia spinolai* es el vector silvestre del protozoo flagelado *Trypanosoma cruzi,* agente causante de la enfermedad de Chagas en humanos, en la zona semiárida-Mediterránea de Chile. En este estudio se evaluó si existe asociación entre el comportamiento de acercamiento hacia hospedero humano con el estatus de infección y la condición nutricional de *M. spinolai.* Para esto, se capturaron manualmente 498 individuos en la Reserva Nacional Las Chinchillas, Chile, en seis intervalos consecutivos de 10 min cada uno (tramos), utilizando un humano como atractor. Cada individuo fue pesado, fotografiado y medido, con lo que se estimó el estado nutricional usando un Índice de Masa Corporal Estandarizado que independiza los valores de Índice de Masa Corporal del estado de desarrollo. El estatus de infección fue analizado en el contenido intestinal mediante un PCR en tiempo real usando contenido intestinal. Se detectó que el estado nutricional de triatominos no infectados fue mayor al de los infectados ($p = 0.005$). Posteriormente se evaluó si el estado nutricional se asociaba al tramo de acercamiento a hospedero humano, registrado como intervalo de captura, mediante una regresión logística ordinal separando infectados de no infectados. Entre los infectados, aquellos con mayor estado nutricional se acercaron primero ($p < 0.002$); no detectándose esta diferencia en los insectos no infectados. *Trypanosoma cruzi* podría alterar el comportamiento de búsqueda de *M. spinolai* en condiciones naturales, probablemente deteriorando el estado nutricional y alterando las capacidades de detección del vector.

ABSTRACT

Hematophagous insects exhibit complex behaviours when searching for blood-meals, responding to visual and olfactory host stimuli. *Mepraia spinolai* is a wild vector of *Trypanosoma cruzi*, causative agent of Chagas disease in humans, in the semiarid-Mediterranean ecosystem of Chile. In this study, we evaluated the association between the approaching behaviour to human host with *T. cruzi* infection status and nutritional condition of *M. spinolai*. To this end, we captured 498 individuals in Las Chinchillas National Reserve, Chile, in six consecutive 10 minute-time-span, using human as bait. Captured vectors were weighed, photographed and measured to calculate their nutritional status by means of a Standardized Body Mass Index, which makes Body Mass Index values independent from the developmental stage. *Trypanosoma cruzi* infection was assessed by real-time PCR using intestinal content. Nutritional status of uninfected triatomines were higher than that from infected ones ($p = 0.005$). Ordinal logistic regressions were performed separately for infected and uninfected groups to evaluate if the nutritional status was associated to the approaching behaviour to human host, recorded as the time-span of capture. Among the infected, those with higher nutritional status approached first ($p < 0.002$); there was no effect of nutritional status in the uninfected group. *Trypanosoma cruzi* might affect the foraging behaviour of *M. spinolai* under natural conditions, probably deteriorating nutritional status and altering vector detection abilities.

1. Introduction

One of the most relevant activity in the life of a hematophagous insect is to find its next meal, especially after a prolonged fasting period. Search for a blood-meal responds to an integrated behaviour, which is divided into three phases: appetite search, activation and orientation, and attraction (Lehane, 2005). Search for appetite occurs when the insect is directed by hunger without an orientation behaviour, where it could find a host by contact stimuli. Activation and orientation are generated after receiving host stimuli, changing their behavioural patterns to one oriented. Attraction corresponds to the establishment of contact with the potential host (Sutcliffe, 1987). In the case of blood-sucking hemipterans (Reduviidae: Triatominae) the search activity is a complex behaviour, because triatomines present a high number of antennal receptors associated with this activity, given their status of temporary ectoparasites (Lehane, 2005). Triatomines are strictly hematophagous organisms, therefore, to increase the probability of completing their life cycle, they shall know the regular location of their hosts (Lehane, 2005). Host location is achieved by receiving stimuli emitted by potential prey, such as CO_2 emanations, heat and shape, although depending on the time elapsed since the last blood intake, CO_2 emanation can attract or repel the insect (Bodin et al., 2009). Even though, some of the mechanisms behind host location have been described (Guerenstein and Lazzari, 2009; Moreno et al., 2006), little is known on the extrinsic factors altering host seeking behaviour in triatomines (Ramírez-González et al., 2019).

Triatomines are important biological vectors, given the role they play in the transmission of the protozoan parasite *Trypanosoma cruzi* to several mammalian species (Panzera et al., 2004), including humans in which it causes Chagas disease. It is estimated that 7 million people are infected with *T. cruzi*, leading to approximately 22,000 deaths per year throughout the Americas (de Fuentes et al., 2018). In Chile, *Mepraia spinolai* is the main wild vector of *T. cruzi*, distributed between 26º and 33°S (Frías-Lasserre, 2010; Garrido et al., 2019), and its habitat includes bird nests, rock crevices, caves and bromeliads, as well as abandoned houses (Lent and Wygodzinsky, 1979; Schofield et al., 1982; Canals et al., 1997; Ihle-Soto et al., 2019). This triatomine presents accentuated sexual polymorphism once reaching maturity, with invariable micropterous females, and micropterous, macropterous or brachypterous males (Lent and Jurberg, 1967), however, only macropterous males have been reported as capable to fly (Berazay-Puente et al., 2017). Several mammal species serve as bloodmeal of *M. spinolai* and are hosts of *T. cruzi*, including rodents, carnivores, marsupials and introduced lagomorphs (Botto-Mahan et al. 2009, 2010; Oda et al., 2014; Ihle-Soto et al., 2019). In some areas, *M. spinolai* populations can reach over 70% of *T. cruzi* infection with spatial variation in their abundances and infection prevalence (Bacigalupo et al., 2006; Coronado et al., 2009; Correa et al., 2015; Ihle-Soto et al., 2019; San Juan et al., 2020).

Mepraia spinolai exhibits a diurnal behaviour, using its antennal receptors to detect and approach hosts (Moreno et al., 2006). A concentration between 300-400 ppm above the environmental CO_2 level induces vector orientation and approach to the CO_2 source (Bacigalupo et al., 2006). Triatomines carry on their antennae sensilla, structures with sensory cells, mainly olfactory ones to detect host odour (Guerenstein and Lazzari, 2009), however, scarce information has been reported on the effect of *T. cruzi* on host detection ability by infected triatomines. One laboratory study detected that the triatomines *Triatoma pallidipennis* and *T. longipennis* infected with *T. cruzi* are more active and orientate more times towards human odour than control odour, distilled water (Ramírez-González et al., 2019). In the same line, a study showed that *M. spinolai* individuals experimentally infected with *T. cruzi* detected and orientated towards their hosts faster and exhibited a 45% higher biting rate compared to uninfected *M. spinolai* individuals (Botto-Mahan et al., 2006). Another important variable to be considered in the study of detection and approaching behaviour by vectors is their nutritional status, which has been estimated considering their weight and length (Schofield, 1980). Variation in nutritional status may affect population density, capacity or probability of flight initiation in *T. infestans* (Schofield et al., 1992), and in other cases, sensory response alterations in host searching by *Rhodnius prolixus* (Castillo-Neyra et al., 2015).

Notwithstanding, to our best knowledge, it is unknown if the same kind of alterations occur under field conditions, especially considering the complexity of natural environments and the *T. cruzi* load of free-ranging triatomines. In this study, we examined if the approaching behaviour of the triatomine *M. spinolai* to humans is associated to its infection status and nutritional status, assessing *T. cruzi* infection by real time PCR and nutritional status as body mass index in an individual base.

2. Materials and methods

2.1. Study site

This study was carried out in a protected area, Las Chinchillas National Reserve (31° 30' S, 71° 06' W; Chile, Fig.1), a hyper-endemic zone of Chagas disease (Botto-Mahan et al., 2010). The climate is semi-arid Mediterranean-type with most rainfall concentrated between June and August. The mean annual precipitation is 185 mm, alternating long droughts and unusual years of high rainfall seemingly associated with El Niño events (Di Castri and Hajek, 1976). Vegetation is thorny and mainly represented by shrub and cactus species (Lubert and Pliscoff, 2006).

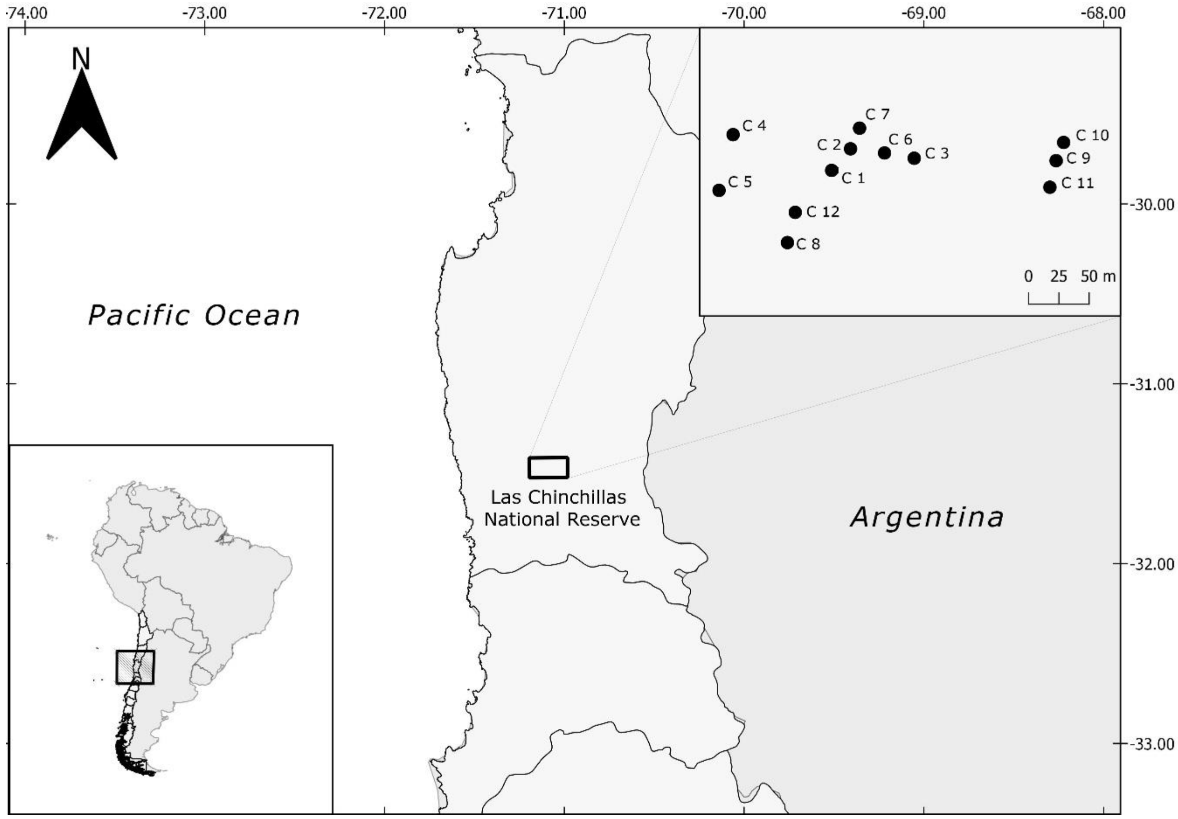

Figure 1. Map of the study site, Las Chinchillas National Reserve, Chile. C: Colony.

2.2. Kissing bug collection

During January 2018, we collected individuals of *Mepraia spinolai* from 12 different colonies. In each colony, one trained researcher manually collected kissing bugs in six consecutive 10 minute-time-span (total time: 1-hour), in sunny days between 11:00 and 13:00 h, the time of day with maximum insect activity (Canals et al., 1997). Captured insects were classified by stage of development and weighed (precision: ±0.1 mg). In addition, a ordinal categorization of the abdomen shape to describe from extended fasting to recently engorged was performed as follows: extremely concave abdomen (1), moderately concave abdomen (2), flat abdomen (3), moderately convex abdomen (4), and extremely convex abdomen (5). These measurements were performed the same day of capture, and the engorgement was assessed by the same experienced person. These five categories were defined in order to include the most extreme as well as intermediate forms of abdomen, which were easily observed by a trained eye. Finally, triatomines were individually stored.

2.3. Processing of M. spinolai individuals and nutritional status

At the laboratory, after euthanized with a 48-h cold shock at -20 ºC, insects were photographed on graph paper. Total body length and maximum abdomen width were measured on the photographs using the program Image J (version 1.x Schneider et al., 2012). With these values and the weight, we estimated a nutritional status for each insect as a Body Mass Index (BMI), calculated using the following equation (Peig and Green, 2010):

$$BMI = \frac{M}{(L_{tb} * W_{ma}) * 10000}$$

where M is the mass of the individuals, L_{tb} is total body length, and W_{ma} is maximum abdomen width. To obtain a *BMI* value independent from the stage of development, this value was standardized with the equation (Yu et al., 2009):

$$SBMI = \frac{BMI - V_{min}}{V_{max} - V_{min}}$$

where *SBMI* is the standardized body mass index, V_{min} is the minimum value of *BMI* in that specific age (nymph and adults) and V_{max} is the maximum value of *BMI* in that specific age.

2.4. DNA extraction from faeces and intestine of triatomines

Triatomines were subjected to abdominal extrusion to obtain both intestinal content and intestine samples. Each sample of approximately 25 mg was mixed with 20 μL of freenucleases water. Whole DNA was isolated from faecal and intestine samples using the DNeasy® Blood & Tissue Kit (QIAGEN, CA, USA). The manufacturer's recommendations were followed but the samples were centrifuged for 4 min at 17000 ×g to dry the DNeasy membrane, and the final elution volume was 100 μl. Samples were stored at −20 °C until molecular analysis.

2.5. Real-Time PCR assays of Trypanosoma cruzi Satellite DNA

Assays were performed using *T. cruzi* nuclear satellite DNA primers Cruzi 1 (5'-ASTCGGCTGATCGTTTTCGA-3') and Cruzi 2 (5'-AATTCCTCCAAGCAGCGGATA-3') (Piron et al, 2007) with concentration of 0.4 μM, 5× HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne, Taru, Estonia) and 5 μl DNA template, with a final volume of 20 μl. Cycling conditions were 15 min at 95 °C followed by 50 cycles at 95 °C for 15 s, 65 °C for 20 s, and 72 °C for 20 s, finishing with a default melting curve. All the assays were run in a QuantStudio 3 Real-Time PCR System (Thermofisher, USA) with each sample in duplicate. To consider a triatomine as infected, two conditions had to be met: amplification before a Ct of 42 (after that it was corroborated by electrophoresis), and a melting temperature between 83.0 to 87.0 ºC. The melting temperature varies depending on the strains used (Duffy et al., 2009).

2.6. Statistical analyses

To characterize and evaluate the variations of the infection of the captured individuals, we compare difference in the age structure in the uninfected and infected triatomines, we used a Kolmogorov-Smirnov test. To assess whether the categorization of the shape of the abdomen is associated with the Standardized Body Mass Index, we constructed a Generalized Linear Model (GLM) with a quasibinomial distribution. A Kruskal-Wallis analysis was performed to detect the effect of infection on the SBMI. We used two ordinal logistic regressions, testing infected and uninfected group separately, to evaluate if *SBMI* (explanatory variable) was associated to the approaching behaviour (response variable), recorded as the interval of capture (time-span: 1 to 6). All the analyses were performed with the R software (version 3.6.0, R Development Core Team 2019) and JMP-Pro (version 14).

3. Results

3.1. Trypanosoma cruzi infection

A total of 498 kissing bugs were collected (mean ± SE: 41.58 ± 0.90 insects/hour*researcher), including 457 nymphs and 42 adults (See detailed information in Electronic Supplementary Material, ESM1-Table S1). A total of 273 insects were infected with *T. cruzi* (54.8%). When assessing age structure in both infected and uninfected groups, first instar nymphs were overall the most represented age stage captured (N = 122, and N = 162, respectively; Fig. 2; Supplementary data - Table 1). The frequencies of the different age structure in the infected and uninfected groups differed ($P < 0.001$) with a higher relative frequency of first instar nymphs in the uninfected group than in the infected group (72.0% versus 44.6%, respectively).

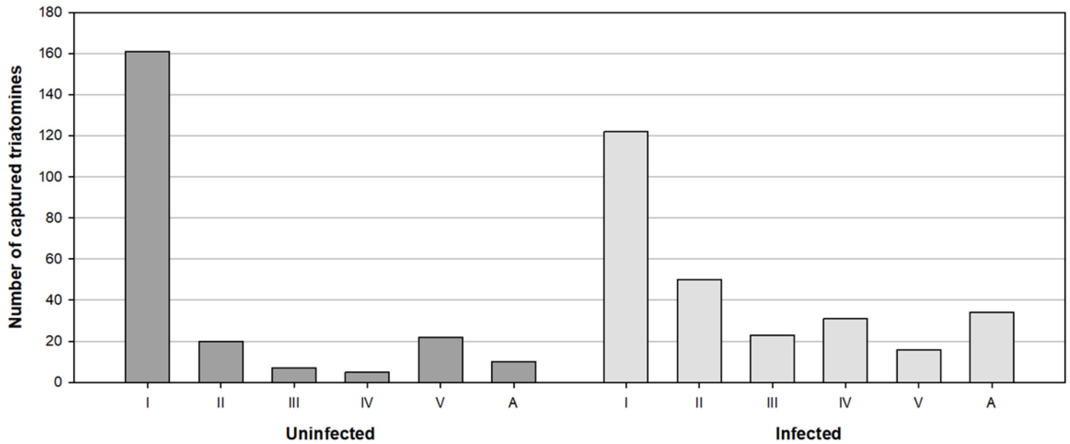

Figure 2. Bar plot of total number of captured triatomines by age, in uninfected and infected groups. I, II, III, IV and V correspond to first, second, third, fourth and fifth instar nymphs, respectively. A corresponds to adults (males and females combined).

3.2. Nutritional status, infection status and interval of capture

The SBMI was significantly related to the abdomen shape ($P < 0.001$), where extremely concave abdomen (1) obtained the lowest SBMI and extremely convex abdomen (5) the higher SBMI (Fig. 3). The SBMI was significantly higher in uninfected triatomines compared to those infected ($P = 0.005$; Fig. 4). When assessing the relationship between nutritional status on the time-span of the triatomine arrival, mostly infected individuals with higher SBMI arrived in the first-time interval of capture ($P = 0.002$; Fig. 5A). We did not detect an effect of the SBMI on the arriving time of uninfected triatomines ($P = 0.10$; Fig. 5B).

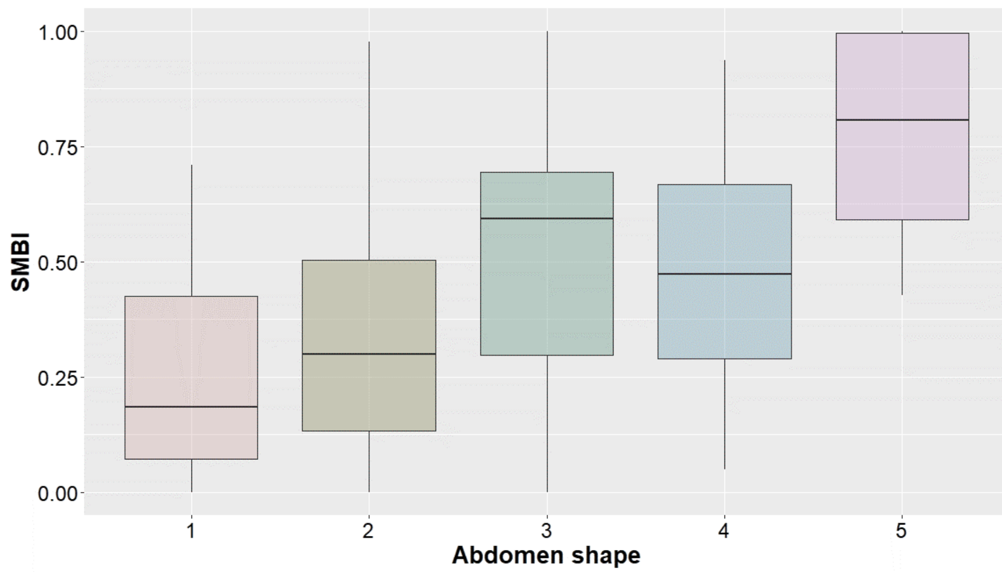

Figure 3. Boxplot of nutritional condition (SBMI) by abdomen shape. (1) extremely concave abdomen; (2) moderately concave abdomen; (3) flat abdomen; (4) moderately convex abdomen; (5) extremely convex abdomen.

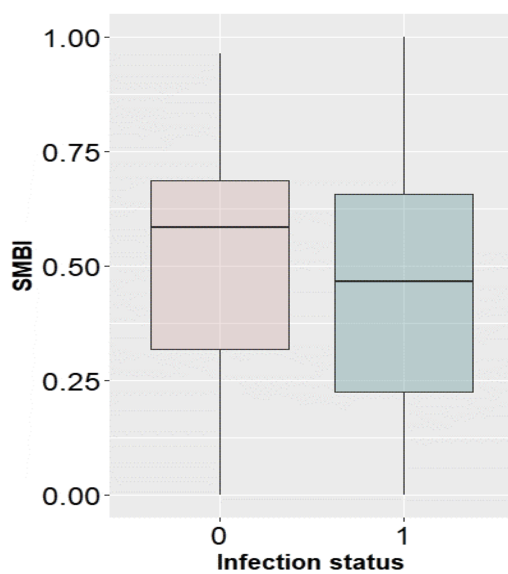

Figure 4. Boxplot of nutritional condition of triatomines (SBMI) by infection status. (0) Uninfected triatomines; (1) Infected triatomines.

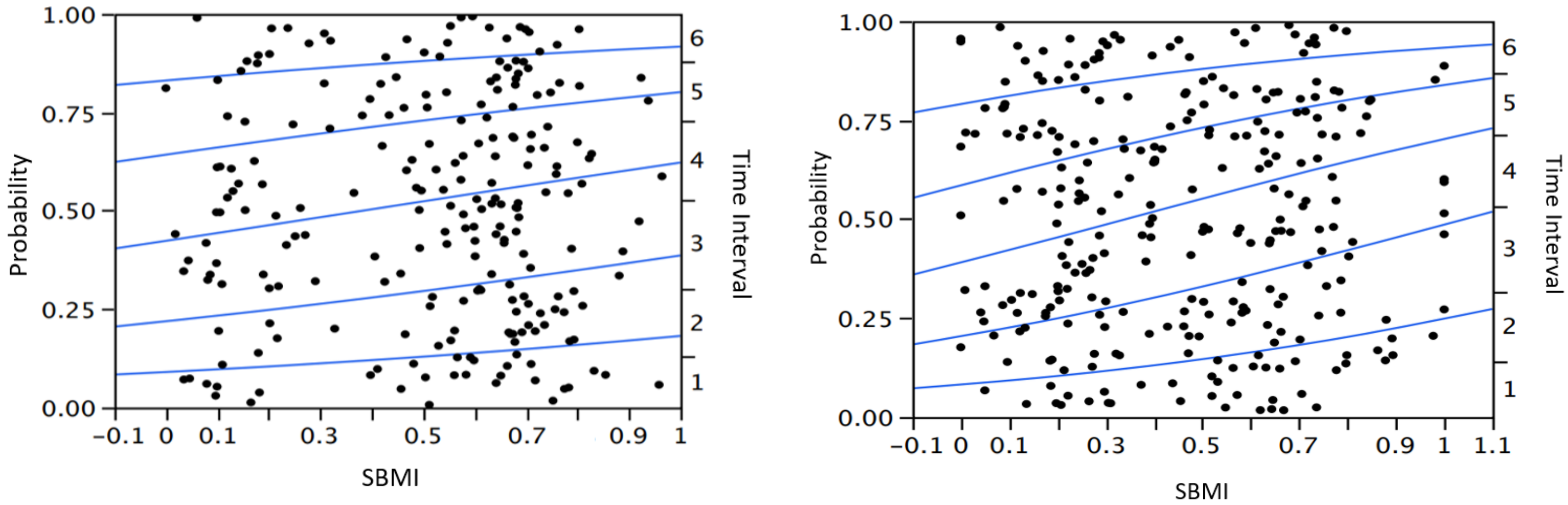

Figure 5. Scatter plot of the ordinal logistic regression for capture time interval (10 min-timespan: 1 to 6) in relation to the standardized body mass index (*SBMI*). The area under the curve (blue lines) represents the probability of an individual of a determined *SBMI* value (black dot) to be captured in each capture time interval. Left panel: Uninfected triatomines; Right panel: Infected triatomines.

4. Discussion

In this study, we found that the approaching behaviour to human host by *M. spinolai* is related to *T. cruzi* infection status and nutritional status, in which infected kissing bugs with higher nutritional status approach first to a human host compared with infected bugs with lower nutritional status. This pattern is not detected in uninfected bugs.

More than half of the total triatomines captured were infected with *T. cruzi* (54.8%), a prevalence within the range of 39.7% - 76.1% described for the area (Coronado et al., 2009; Botto-Mahan et al., 2010; Ihle-Soto et al., 2019). Infection in kissing bugs present temporal and spatial variation, and detection depends of the fasted/starved levels in insect vectors (Egaña et al., 2014). The capture frequency of developmental stages differed between the groups, where the uninfected group was mainly represented by first instar nymphs, and very low representation for other nymphal stages. In the case of the infected group, this difference was less marked. Because triatomines mainly acquire *T. cruzi* from blood-meals on infected mammalian host (Garcia et al., 2010) or by coprophagy on infected bugs' faeces (Lehane, 2005), infection probability increases with the progress of developmental stages. Additional studies should be carried out in other seasons, for example the cold months of winter, to evaluate how the capture frequency of different developmental stages and *T. cruzi* infection of triatomines change, as described by other studies on *M. spinolai* in which triatomine infection rate is higher in winter than summer (Ihle-Soto et al., 2019).

In general, sylvatic triatomines are 50% lighter than bugs collected in domestic and peridomestic habitats (Schofield, 1980; Lopez et al. 1999), and probably sylvatic *M. spinolai* follows this pattern. Our results showed that the SBMI may be considered an accurate indicator of the nutritional status of a triatomine, due to the strong relationship detected with the *M. spinolai* abdomen shape. Although the abdomen shape is the first approximation of the nutritional status in triatomines, it should be noted that this categorization is an observational measure that require trained observers. Therefore, assessing nutritional status by using measures of length, width and weight of triatomines to calculate BMI is a more accurate data. A blood meal may reach 5–10 times the insect's body weight, changing the abdomen shape, in which the signals of end-feeding is provided by abdominal stretch mechanoreceptors (Anwyl, 1972; Chiang and Davey, 1988). As expected, the uninfected groups presented a better nutritional status than the infected group. Previous studies have suggested that *T. cruzi*-infected triatomines have a lower weight than those uninfected, need more time to reach maturity and present lower survival, specially before the last stage (Botto-Mahan, 2009; Schaub, 1992). Kollien and Schaub (2000) suggested that trypanosomatids may compete for trace nutrients with triatomines. In addition, the acquisition of *T. cruzi* generate an immune response in the triatomine (Garcia et al., 2010), which also may generate a decrease in its nutritional status or body condition in general.

The impact of this protozoan parasite on the approaching behaviour of *M. spinolai* was related with the nutritional status. This result, in which infected insects with the best nutritional status arrived in the first interval of capture, could be due to low physiological impact of *T. cruzi* on triatomines with higher nutritional condition. A previous study reported that infected *Triatoma infestans* under optimal nutritional condition presented a mortality rate of 9%, a figure slightly higher than the 4% reported for uninfected *T. infestans* (Schaub, 1988). However, Schaub and Lösch (1989) found that *T. cruzi* reduces the starvation resistance by about 15%, this could be related to the fact that uninfected triatomines arrive at any capture interval, regardless of their nutritional status.

There is experimental evidence that individuals of *M. spinolai* infected with *T. cruzi* find hosts almost twice as fast as uninfected bugs (Botto-Mahan et al., 2006), probably indicating that *T. cruzi* alters the feeding behaviour of this triatomine depending on the nutritional status. In addition, infected *Rhodnius prolixus* decrease their locomotory activity in the absence of host cues, this could be considered an energy saving mechanism to avoid loss of nutritional resources (Marliére et al., 2015).

In summary, our findings suggest that under natural conditions *T. cruzi* may induce changes in the nutritional status and, in turn, in the approaching behaviour of *M. spinolai*. Even though, it is desirable to count on field studies to gain reality, some variables cannot be established such as the time of *T. cruzi* infection or starvation. To improve the understanding of the alterations *T. cruzi* may produce in *M. spinolai*, future studies should evaluate how variation in parasitic loads could be explaining part of the large variability detected in the approaching behaviour of infected triatomines, especially considering the starvation and the time dependent effects might cause in the outcome of this protozoan-triatomine interaction.

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Appendix

Table S1. Total number of each developmental stages per colony. The values in parentheses correspond to the number of infected triatomines. I, II, III, IV and V correspond to first, second, third, fourth and fifth instar nymphs, respectively. A corresponds to adults (males and females combined).

	Developmental stages						
Colony	I	II	III	IV	V	A	**Total**
1	18 (8)	0 (0)	6 (5)	6 (6)	2 (2)	4 (2)	36 (23)
2	7 (6)	7 (4)	11 (7)	3 (2)	2 (2)	9 (6)	39 (27)
3	77 (41)	4 (4)	1 (1)	2 (1)	0 (0)	2 (2)	86 (49)
4	0 (0)	3 (3)	0 (0)	1 (1)	1 (1)	1 (1)	6 (6)
5	26 (10)	10 (8)	0 (0)	0 (0)	1 (0)	0 (0)	37 (18)
6	8 (3)	1 (0)	2 (2)	5 (5)	9 (1)	3 (2)	28 (13)
7	17 (5)	6 (6)	3 (3)	4 (4)	1 (0)	6 (6)	37 (24)
8	26 (15)	14 (11)	4 (2)	5 (5)	5 (3)	3 (2)	57 (38)
9	30 (15)	3 (3)	0 (0)	0 (0)	5 (4)	5 (4)	43 (26)
10	13 (6)	4 (4)	0 (0)	0 (0)	1 (0)	4 (4)	22 (14)
11	32 (9)	13 (7)	1 (1)	5 (4)	2 (1)	2 (1)	55 (23)
12	29 (4)	5 (0)	2 (2)	4 (2)	8 (1)	4 (3)	52 (12)
Total	283 (122)	70 (50)	30 (23)	35 (30)	37 (23)	43(33)	**498 (273)**