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Commercial coconut palm as an ecotope of Chagas disease vectors in north-eastern Venezuela

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Abstract

Background & objective: There are few reports of *Coccus nucifera* (Palmae) infestation by triatomines (Hemiptera, Reduviidae, Triatominae), vectors of *Trypanosoma cruzi* (Kinetoplastida: Trypanosomatidae), the causal agent of American Trypanosomiasis. The aim of this study was to determine if this palm is an appropriate ecotope for *Rhodnius prolixus* and *Triatoma maculata*, the main vectors in Venezuela.

Methods: Dry and green leaves, humid debris, interfoliaceus meshes and bracts from *C. nucifera* from north-eastern Venezuela were examined for the presence of triatomines. Samples of the intestinal content of vectors, macerated in isotonic saline solution and haemolymph were examined microscopically for the presence of *Trypanosoma* spp. The parasites were isolated and characterized using biological parameters and PCR. Triatomine blood meal sources were determined using ELISA.

Results: A total of 14 palms were examined in which viable eggs of both species of vectors were found in 13 palms (92.85%). A total of 242 *R. prolixus* and 144 *T. maculata* adults were collected, of which 98% of *R. prolixus* and 70% of *T. maculata* individuals were infected by *T. cruzi* (TcI genotype) and 13% of *R. prolixus* individuals showed a mixed infection with *T. rangeli,* the other American trypanosome. ELISA testing for possible triatomine blood-meal sources revealed that these vectors are essentially eurytrophic and zoophilic, although *R. prolixus* also eventually used human blood as a nourishment source.

Interpretation & conclusion: The results obtained suggest that *C. nucifera* is an appropriate vegetal niche for these triatomine species in Venezuela. The presence of this commercial palm may represent a useful environmental bioindicator of risk for Chagas disease.

Key words *Coccus nucifera*; ecotope; *Rhodnius prolixus*; *Triatoma maculata*; Venezuela

Introduction

Trypanosoma cruzi (Kinetoplastida: Trypanosomatidae), the causal agent of Chagas disease, develops in an ecopathogenic complex in which the parasites circulate between numerous selvatic mammalian reservoirs and insect vectors (Hemiptera, Reduviidae, Triatominae). Both bioecological and social factors promote the dispersion of this wild enzootia, facilitating the development of the so called peri- and domestic transmission cycles in which humans and several domestic mammals act as reservoirs of the disease. In addition, a few triatomine species have become adapted to colonize houses, including those in highly urbanized areas[1–2]. Despite the importance of *T. cruzi* for public health, basic aspects of its sylvatic and peridomiciliar transmission cycle have not yet been fully elucidated[3].

Among the numerous triatomine ecotopes, palms (Palmae) are possibly the most extensively studied. Nevertheless, although the infestation of many palm species has been widely reported for Venezuela, there are few reports of *Coccus nucifera* (coconut palm) as an appropriate vegetal niche for triatomines[4–10]. *Rhodnius pallescens* has, however, been found colonizing coconut palms in Colombia[3,11]. These reports led us to investigate the role of commercial coconut palms as niches for triatomines in peridomiciliar ecotopes in endemic rural areas of north-eastern Venezuela.

Material & Methods

Study areas: The study was carried out in the following villages: Eneal I, Eneal II, Valle del Neverí, Pica del Neverí, Guaipalomo and Angostura (Bolívar Municipality; north Anzoátegui State, Venezuela). All the villages surveyed (10° 08' N – 64° 37' W) lie just six meters above sea level, in a dry littoral tropical forest biome with humidity coming from the mountains. The temperature ranges from 25–29°C, mean annual rainfall 500–600 mm[3] and 75–85% relative humidity[12]. There were a total of 112 houses and 320 people in the study area. Coconut palms were randomly selected in a radius of 1–5 m from inhabited houses with improved construction. Sampling was done from January 2008 to July 2009.

Dissection of palms and isolation of Trypanosoma spp from collected triatomines: A total of 14 *C. nucifera* palms ($\overline{x}$ =19 yr old) were studied[4]. Dry and green leaves, humid debris, interfoliaceus meshes and bracts were examined for the presence of triatomines. Collected adult triatomines and first and fifth instars were identified according to Lent and Wygodzinsky[13]. Other stages were identified in the laboratory by the ontogenical sequencing of collected specimens.

Insectivorous bats (*Eumops glaucinus,* Molossidae; *Glossophaga longirostris*, Phyllostomidae-Glossophaginae), and opossums (*Didelphis marsupialis*) were found in several palms.

Samples of the intestinal content of triatomines macerated in isotonic saline solution and triatomine haemolymph were examined microscopically as wet smears (400x) and Giemsa-stained smears (1000x) for the presence of *Trypanosoma*[7,14]. Fecal material from positive triatomines was inoculated subcutaneously (200 metacyclic trypomastigotes/g body weight) into groups of five NMRI mice raised at our laboratory facilities ($\overline{x}$ = 12 g wt). Tail blood samples were examined three days after inoculation and thereafter three times weekly until the onset of chronicity or death[15,16].

The identification of *Trypanosoma* species was performed by xenodiagnosis of positive mice with 12 reared III instar nymphs of *R. prolixus* in order to observe the typical *T. cruzi* and/or *T. rangeli* triatomine-mammal-triatomine cycle[7,14]. Blood parasites were cultured first in blood agar medium (Difco, Thomas Scientific, New Jersey, USA) in a 5-fluorocytocin and gentamycin solution and then in Roswell Park Memorial Institute Medium (RPMI) at 27°C. Haemolymph flagellates from naturally infected triatomines were maintained using the same protocol.

Molecular characterization of Trypanosoma isolates: DNA extraction and polymerase chain reaction (PCR) for amplification of the D7 divergent domain of the 24Sα rRNA gene (D71, 5'-AAGGTGCGTCGACAGTGTGG-3' and D72 5'-TTTTCAGAATGGCCGAACAGT-3' primers); the non-transcribed spacer of the mini-exon genes (TC 5'-CCCCCCTCCCAGGCCACACTG-3', TC1 5'-GTGTCCGCCACCTCCTTCGGGCC-3' and TC2 5'CCTGCAGGCACACGTGTGTGTG-3' primers) and the size-variable domain of the 18S rRNA gene (V1, 5'-CAAGCGGCTGGGTGGTTATTCCA-3' and V2, 5'-TTGAGGGAAGGCATGACACATGT-3' primers), were performed following the Brisse *et* *al* protocols[17] for the following codified triatomine isolates[18]: TPRX/VE/2008/ANG; TPRX/VE/2009/ANG78; TPRX/VE/2009/El1; TPRX/VE/2009/El3; TPRX/VE/2009/El12; TMAC/VE/2008/Piq1; TMAC/VE/2008/Pique62; and TMAC/VE/2008/Pique63.

Identification of the blood meal sources of collected triatomines: Blood meal sources were determined for 105 *T. maculata* and 101 *R. prolixus* randomly selected from those collected in the coconut palms, using an enzyme-linked immunosorbent assay (ELISA). Briefly, the intestinal contents of the triatomines were incubated for 1 h at 18°C and then left to dry on paper. Then, 5 µl of 5% bovine serum albumin (BSA) were added and the mixture was incubated for a further 1 h at 18°C. The paper disks were inserted in an ELISA plate with 150 µl of commercial anti-rabbit IgG, anti-mouse IgG, anti-rat IgG, anti-hen IgG, anti-horse IgG, anti-human IgG and anti-dog IgG conjugated to alkaline phosphatase (Santa Cruz Biotechnology, Inc. Antibodies), or with artisanal antisera from opossum blood sera produced in New Zealand albino rabbits and conjugated to alkaline phosphatase; all paper disks were washed after incubation.

The conjugated antisera were diluted (1/1000) in PBS pH 7.2-Tween20, 0. 05–5% low fat milk and incubated for 30 min at 37°C, 150 µl of p-nitrophenyl phosphate in diethanolamine buffer (DEA) were then added and the mixture incubated for a further 20 min. The reaction was stopped with 1M NaOH (100 µl/well) and read at 405 nm in an ELISA Spectra Classic (TECAN, Austria). The cut-off value for a positive reaction with commercial conjugates was OD (optical density) = 0.2 and with opossum conjugates, OD = 0.4. The intestinal content of reared *R. prolixus* fed on blood from different animals, was used as a control.

Ethical guidelines: The experiments involving animals in this study comply with the current laws of Bioethics as set down by the Venezuelan Ministry of Science and Technology, and were approved by the Ethics Review Committee and Animal Management Committee, FONACIT (Fondo Nacional para la Ciencia y Tecnología).

Statistical analysis: The data were processed using the Microsoft Excel program and analyzed with either ANOVA, the Kolmogorov-Smirnov test or simple regression, according to each particular case. All tests were done using the Statgraphics 5 Plus software.

Results

Palms from six villages in north-eastern Venezuela (n=14, mean height = 18.2 m) were identified as *Coccus nucifera* Linneo (Palmae) or 'coconut palm', native to tropical ecosystems in both the Old and New Worlds[19]. The palms had a mean of 35 dry leaves, 30 green leaves and 12 bracts.

Hatched and fresh triatomine eggs (0–245; mean = 53 eggs/palm) were observed in all the plant parts examined, except for the green leaves, of 13/14 palms. Of the physiognomic characters tested, the only correlation found was a strong positive correlation between palm length and the number of triatomine eggs (simple regression, r = 0.704465, Table 1).

Overall, 242 *R. prolixus* and 144 *T. maculata* adults were found in 11 out of the 14 palms examined. There were no significant differences in the distribution of instars (relative abundance of individuals in each instar) among palms for either of the triatomine species studied ($F = 0.72861$, $p > 0.05$, df = 5, for *R. prolixus*; and $F = 0.409013$, $p > 0.05$, df = 5 for *T. maculata*) (Table 2). A strong positive correlation (r = 0.704465) between palm height (11.8–32 m) and the number of individuals successfully raised from viable eggs to adult (in the laboratory) was found.

Almost all of the *R. prolixus* individuals collected were infected by *T. cruzi* (98%), while the proportion of infected *T. maculata* individuals was lower

Table 1. Physiognomical characteristics and triatomine egg infestation in coconut palms (*Coccus nucifera*) from villages in north-eastern Venezuela

Villages	Dissected palm No.	Length (m)	Approx. age (yr)	No. of dry leaves	No. of green leaves	No. of bracts	No. of hatched and fresh eggs
Eneal I	1	19.5	25	46	0	12	60
	2	11.8	13	12	52	15	103
	3	22	23	33	38	18	30
	4	15	16	18	42	10	0
Eneal II	5	17.8	18	28	32	12	25
	6	12.6	12	40	26	7	10
Valle del Neverí	7	15.7	14	43	36	11	14
	8	18	23	30	43	16	80
Guaipalomo	9	20	20	47	21	17	10
	10	14	13	30	46	11	15
	11	21.5	22	25	32	18	72
Pica del Neverí	12	16.3	14	36	28	10	41
	13	19	24	45	0	5	35
Angostura	14	32	26	56	23	12	245
Av.		18.2	18.8	35	30	12	53

Table 2. Distribution of *R. prolixus* and *T. maculata* instars and the relative proportion of individuals infected with *T. cruzi*/palm in coconut palms from villages in north-eastern Venezuela

Palm No.	*Rhodnius prolixus* instars							Infected by *T. cruzi*	*Triatoma maculata* instars							Infected by *T. cruzi*
	I	II	III	IV	V	A	Total	No. (%)	I	II	III	IV	V	A	Total	No. (%)
1	2	1	4	4	0	2	13	11 (84.6)	1	1	1	2	1	2	8	5 (62.5)
2	0	1	3	4	2	3	13	13 (100)	0	0	1	3	3	3	10	7 (70)
3	2	2	5	5	1	5	20	20 (100)	1	0	1	1	0	1	4	2 (50)
4	0	0	0	0	0	0	0	0 (0)	0	0	0	0	0	0	0	0 (0)
5	1	2	3	4	1	3	14	13 (92.9)	1	0	0	4	1	2	8	5 (62.5)
6	0	0	3	2	0	0	5	5 (100)	2	1	0	3	3	3	12	3 (25)
7	0	1	2	3	1	4	11	10 (90.9)	1	1	1	3	4	2	12	5 (41.7)
8	0	1	6	3	0	3	13	13 (100)	0	1	2	3	2	1	9	4 (44.4)
9	1	0	3	3	0	4	11	10 (90.9)	0	0	1	2	3	1	7	4 (57.1)
10	0	0	0	0	0	0	0	0 (0)	0	0	0	0	0	0	0	0 (0)
11	6	4	7	8	6	5	36	36 (100)	0	0	0	0	0	0	0	0 (0)
12	7	9	7	5	5	10	43	43 (100)	4	6	4	4	5	8	31	28 (90.3)
13	0	0	0	0	0	0	0	0 (0)	5	7	4	3	4	6	29	24 (82.8)
14	8	10	6	5	3	31	63	63 (100)	1	3	2	1	0	7	14	14 (100)
Total	27	31	49	46	19	70	242	237 (98)	16	20	17	29	26	36	144	101 (70)

I–V —Larval instars; A—Adults.

Table 3. Distribution of *R. prolixus* and the relative proportion of individuals coinfected with *T. rangeli*/ palm from villages in north-eastern Venezuela

Palm No.	I Instar	II Instar	III Instar	IV Instar	V Instar	Adult	Coinfected by *T. rangeli*	
							No.	%
1	0	0	1	2	0	0	3/13	23.1
2	0	0	1	1	0	1	3/13	23.1
3	0	0	1	0	0	1	2/20	10
4	0	0	0	0	0	0	0/0	0
5	0	0	1	2	0	0	3/14	21.4
6	0	0	0	0	0	1	1/5	20
7	0	0	1	1	0	1	3/11	27
8	0	0	1	1	0	1	3/13	23.1
9	0	0	1	1	0	0	2/11	18.2
10	0	0	0	0	0	0	0/0	0
11	0	0	0	0	0	0	0/36	0
12	0	0	0	0	0	0	0/43	0
13	0	0	0	0	0	0	0/0	0
14	1	2	1	0	1	7	12/63	19
Total	1	2	8	8	1	12	32/242	13.0

(70%) (Table 2). In addition, 13% of *R. prolixus* were infected by both *T. rangeli* and *T. cruzi* according to parasitological and/or molecular criteria[7,14,17] (Table 3). *Trypanosoma rangeli* was not, however, observed in *T. maculata*. Our attempts to maintain *T. rangeli* in RPMI medium were fruitless.

The number of individuals of each triatomine species in relation to their alimentary sources is shown in Fig. 1. According to the ELISA analyses of the blood sources of *T. maculata* and *R. prolixus*, these species were eclectic in their alimentary habits; feeding on birds, rodents, opossums, rabbits, dogs and horses. Only *R. prolixus* rarely used humans as a blood source. There was no statistically significant difference between the frequency distribution of blood sources between the triatomine species (Kolmogorov-Smirnov test, $p > 0.05$).

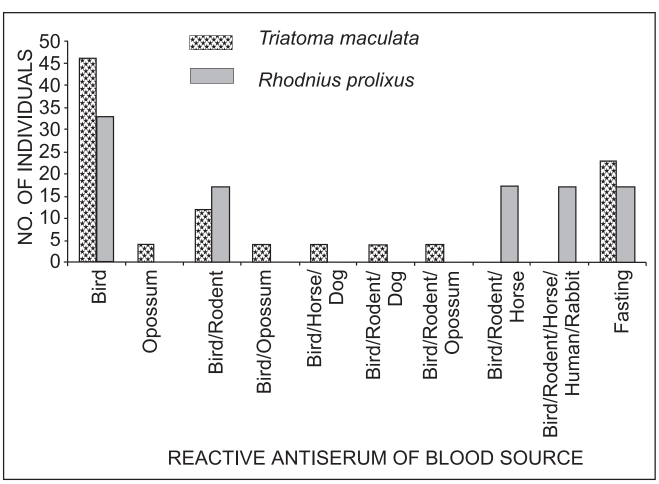

Fig. 1: Blood meal sources of *Rhodnius prolixus* and *Triatoma maculata* collected from *Coccus nucifera* in north-eastern villages in Venezuela

The results of the molecular analysis by PCR using the three molecular markers analyzed in *T. cruzi* isolates: the non-transcribed spacer of the mini-exon genes, the D7 divergent domain of the 24Sα rRNA gene, and the size-variable domain of the 18S rRNA gene, showed an TcI lineage pattern as revealed by the amplification of the products at of 350, 110 and 175 bp, respectively, in the eight parasite strains isolated from collected triatomines (Figs. 2A, B, C).

Discussion

The natural foci of American trypanosomiasis are extremely diverse and notably ubiquitous due to the enormous variation in habitats and reservoirs of *T.*

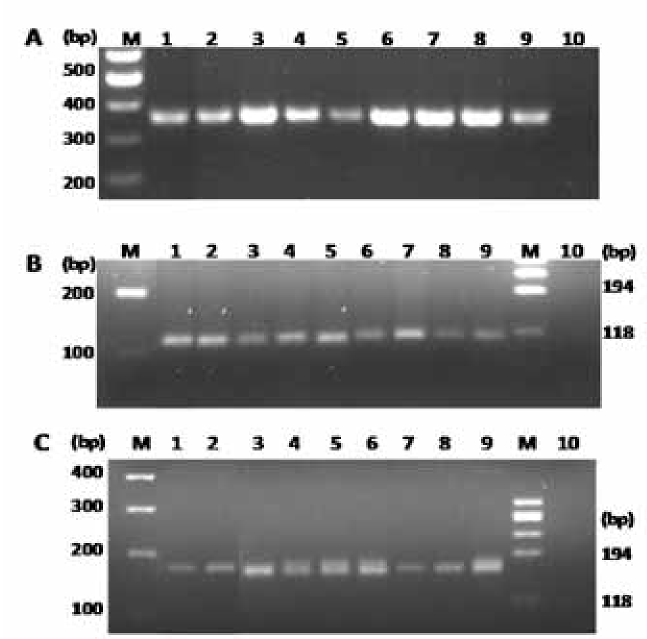

Fig. 2: Molecular characterization of *Trypanosoma cruzi* isolates from triatomines collected from coconut palms, by PCR analysis (Brisse *et al*[17]): (A) D7 divergent domain of the 24Sα rRNA gene revealed using primers D71 and D72. (110 bp corresponds to the TcI lineage); (B) non-transcribed spacer of the mini-exon genes (350 bp– TcI lineage); (C) 18S rRNA gene size-variable region revealed using primers V1 and V2 (175 bp–TcI lineage). (1) Reference TcI strain MDID/VE/1976/Dm28; (2)TMAC/VE/2008/Piq1; (3)TPRX/VE/2008/ANG; (4) TMAC/VE/2008/Pique63; (5)TMAC/VE/2008/Pique62; (6)TPRX/VE/2009/El12; (7) TPRX/VE/2009/ANG78; (8)TPRX/VE/2009/El1; (9)TPRX/VE/2009/El3; (10) negative control. M left and M right indicate 100 bp ladder (Biotools B&M Labs, S.A. Madrid, Spain) and IX 9φ X174/*Hae* III indicate digested (Roche, Mannheim, Germany) DNA molecular markers, respectively

cruzi and the marked eurytopy and eurytrophy of the vectors[7].

The wide neotropical distribution of the palms, their varied antropic uses and morphological variability permits the formation of trophic chains; from invertebrates to poikilothermic and homeothermic vertebrates, which provide the triatomines with readily available reservoirs as blood sources. This explains why the palm-triatomine adaptation is the most suitable for the tribe *Rhodniini*, and thus the importance of the palms as natural ecotopes for this zoonosis[4,8,20,21]. All these factors combined confer great ecoepidemiological importance to palms as environmental bioindicators of wild and peridomestic triatomine ecotopes and, consequently, as risk components for Chagas disease[3].

Reports of the presence of triatomines in commercial coconut palms, and more specifically the sympatric presence of *R. prolixus* and *T. maculata* in these palms in Venezuela, are scarce[4]. In this study, in spite of the low number of plants examined and insects collected, the infection percentages for *T. cruzi* in *R. prolixus* and *T. maculata* (98 and 70%, respectively) can be considered high when compared with other results. In addition, the presence of *T. rangeli* in 13% of the collected *R. prolixus* demonstrates the importance of *C. nucifera* as an environmental bioindicator of areas with a high risk of transmission of both trypanosomes. The positive correlation between coconut palm height and viable triatomine eggs found in this study, suggests that both palm microclimate (temperature, ventilation and humidity) and vertebrate colonization are important for vector biogeocenosis.

Despite their high genetic variability, *T. cruzi* isolates have been classified into two major phylogenetic lineages, *T. cruzi* I and *T. cruzi* II based on zymodemes and different genetic markers[18,22–24]. A preferential association of *T. cruzi* genotypes with sylvatic or domestic transmission cycles has been described. Parasites belonging to the *T. cruzi* II genotype are preferentially associated with human infection and domestic cycles in regions from the American Southern Cone where Chagas' disease is endemic[24–27], while *T. cruzi* I parasites are associated with the sylvatic cycle and a low prevalence of symptomatic patients[24,25,28]. However, a recently published paper showed that 74% of Venezuelan isolates from acute chagasic patients were typed as *T. cruzi* I[29]. Other studies also suggest that *T. cruzi* I predominates in both human and sylvatic cycles, at least in Mexico and Guatemala[30].

Trypanosoma cruzi II, was later subdivided into five groups: Tc IIa–IIe[18,30–32]. Brisse *et al*[17] reported simple methods for the characterization of *T. cruzi* isolates by agarose gel electrophoreses of the PCR products with only three genes: mini-exon, 24Sα rRNA, and 18S rRNA. This approach was used in this investigation and we obtained only the TcI pattern. A Second Satellite Meeting in 2009[33], recognized that the nomenclature for *T. cruzi* strains should be classified into six DTUs: *T. cruzi* I-VI. *Trypanosoma cruzi* I corresponds to the TcI originally defined in the First Satellite Meeting[18]. The fact that only the TcI lineage was identified in this study (the most abundant lineage among chagasic patients in Venezuela), reinforces the hypothesis that both domestic and peridomestic transmission cycles may be sustained by triatomines from surrounding palms[29,34].

Nevertheless, the scarce participation of humans as food sources for either species (as revealed by the ELISA test) indicates that this vegetal niche, although near to human dwellings, remains a closed ecotope for domestic parasite circulation in the study area. Birds were very important blood sources for both triatomine species, which is to be expected since poultry breeding in domestic and peridomestic environments and the colonization of birds inside palms (natural ecotopes of Passeriformes) is a common feature of rural communities in this country.

Rodents, dogs and horses also provided significant blood meal sources. These mammals are locally predominant due to the anthropization of the environment and the domestication and maintenance of livestock near coconut palms. Some mammals, such as bats and rodents were found inside coconut palms, and thus may be considered as potentially important *T. cruzi* reservoirs[7]. Nevertheless, the absence of some mammals as blood meal sources may be justified in function of their biomass, defensive behavior against triatomines, or that some antisera were not used. These results suggest that assessments of potential blood sources should be done in function of local epidemiological situations rather than *a priori* assumptions.

Rhodnius prolixus, T. maculata and *P. geniculatus* are the main vectors of *T. cruzi* in Venezuela. Although all these species are distributed throughout the country, they show successively lower grades of domiciliation. *Rhodnius prolixus* is the most proliferative and aggressive in its feeding habits and quick defecation and is responsible for the high incidence and dispersion of Chagas disease in Venezuela[5]. The adult stages of both *R. prolixus and T. maculata* habitually migrate to human dwellings: 29% of the *R. prolixus* and 25% of the *T. maculata* individuals collected were taken near human houses. It is suggested that this result, together with the high rates of triatomine infection, could be a risk factor to be considered in addition to the properties of *C. nucifera* as an appropriate natural ecotope.

The coconut palm is distributed in Venezuelan forests and coastal areas. There are scarce reports of *T. maculata* in this palm species in these areas[4,6,34]. Thus it is imperative that more research be undertaken into coastal *C. nucifera* as an ecotope for different triatomine species. The oral transmission of *T. cruzi* has produced Chagas disease outbreaks in Brazil and recently in Venezuela due to the consumption of sugarcane juice, fruit wine from the ‘bacaba’ palm (*Oenocarpus bacaba*), or other foods where triatomines are crushed in artisan factories together with the vegetable matter, contaminating it with metatrypo-mastigotes[35–37]. To obtain ‘coconut water’ from *C. nucifera,* consumers habitually ingest the liquid by drinking directly from the perforated fruit, thus touching their mouths to it. Fruit clusters can remain stored during long periods before their consumption, thus increasing the possibility of triatomine fecal contamination. Furthermore, Marques[38] developed a culture medium using ‘coconut water’ in which *T. cruzi* proliferates with metatrypomastigote production; thus the possibility of oral *T. cruzi* transmission by drinking from the coconut in this manner should not be overlooked.

Conclusion

This study represents an important contribution to the scarce knowledge on commercial coconut palms (*C. nucifera*) as natural and appropriate vegetal niches for the breeding and multiplication of *R. prolixus* and *T. maculata* in Venezuela. Both of these species are important Chagas disease vectors, with high rates of *T. cruzi* (TcI) infection. We hope that the results obtained lead to improved surveillance and control, especially of the re-infestation of human dwellings from surrounding palms. The area studied has its own epidemiological and micro-environmental peculiarities resulting from the particular human activities and social patterns found in this region, factors which should be taken into account when control programs are being planned.

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