Introduction

The Gran Chaco eco-region and adjacent areas extending from Argentina, Bolivia, and Paraguay are highly endemic for Chagas disease. Despite the current campaigns for the elimination of the major vector *Triatoma infestans* (Hemiptera: Reduviidae), transmission and reinfection persist in wide areas of this region. This fact interrupts the success in eliminating vectorial transmission because control programs have been conducted against domestic populations of *T. infestans* using residual insecticide spraying of human dwellings, and did not involve the potential reinfection process from sylvatic areas. Therefore, this new epidemiological scenario is a priority issue for the Southern Cone Initiative.

In the last decades, the sylvatic populations of *T. infestans* have been reported in Bolivia, Argentina, Chile, and Paraguay. These records have challenged the hypothesis that this vector is strictly domestic and peridomestic. Recent studies have shown that remnant triatomine populations exist in intradomiciles after spraying campaigns in endemic communities are highly connected to sylvatic colonies and they could be involved in restoring the reinfection process. To support this new paradigm, recent genetic studies in the Andes showed that first-generation migrants within different populations have provided evidences of insect movement from the sylvatic to the intra- and peridomestic areas, enhancing the hypothesis of vector transmission risk from the invasion of human habitats by sylvatic populations of *T. infestans*. These data show the relevance of sylvatic *T. infestans* populations in their possible role in the recolonization of treated areas and potential exchange between sylvatic and intraperidomestic triatomine populations in a wide geographical area of Grand Chaco under epidemiological risk of transmission of *Trypanosoma cruzi*.

Moreover, other several sources of reinfection by *T. infestans* were identified after house-insecticide spraying in the Gran Chaco region. In Chuquisaca, Bolivian Chaco, a molecular genetic study suggests that triatomine populations are probably residues coming from peridomestic habitats and they are characterized with an active dispersal ability and migration. In the dry and humid Argentinean Chaco, potential sources of reinfection such as external foci (neighboring localities or unknown foci) and residual foci (persistent bug populations surviving insecticide application or insecticide resistant bugs) were also identified. Furthermore, in other areas where sylvatic *T. infestans* foci were potentially discarded, reinfecting populations of *T. infestans*, which were determined by quantitative morphology, corresponded to mixed survivor populations drift from treated areas and neighboring habitats, whose phenotype may have originated from genetic derivation or selection caused by the insecticide.

In the Paraguayan Chaco, rapid reinfection of triatomine populations was observed in indigenous dwellings. Previous genetic studies on sylvatic samples have shown similar haplotypes in sylvatic and domiciled individuals of *T. infestans* captured in the Paraguayan Central Chaco. However, it was unknown if these populations were survivors.
from residual spraying, control failure, pyrethroid resistant, or if they came from external reinfestation. As part of a longitudinal research in rural areas of the Paraguayan Chaco, we investigated the house infestation with *T. infestans* in a well-defined study area and analyze the potential origin of the *T. infestans* reinfestation in two rural localities after insecticide spraying using the geometric morphometry of triatomine wings. We tested if the *T. infestans* captured in postspraying periods exhibits close similarities with those captured during prespraying in the same locality, in the closest neighbor locality, in the sylvatic environment near to them, and in a locality with high inhabitants interchange from where they could have arrived by passive transportation. Sources of feeding and *T. cruzi* infection analysis of triatomines were considered for assessing the potential risks of vector-borne transmission after spraying.

**MATERIALS AND METHODS**

**Study sites.** The fieldwork was carried out in 12 de Junio and Casuarina, two indigenous communities of Central Chaco in Paraguay (Figure 1). The area corresponds to xeromorphic woods of the Gran Chaco eco-region with some characteristic species such as *Aspidosperma quebrachoblanco*, *Schinopsis balansae*, *Bulnesia sarmientoi*, *Prosopis nigra*, *Calycophyllum multiflorum*, and *Stetsonia coryne*. The climate in Chaco Central is characterized by extreme heat in the summer and mild temperatures in the winter. High temperatures have been registered to reach 45°C in spring and summer times and low temperatures reached −2°C in winter times. Wind blows at an average speed of approximately 11.9 km/h that increases up to 14.0 km/h in the winter.

The villages of 12 de Junio and Casuarina are located in Presidente Hayes Department and are distributed over approximately 500 km²; they have 570 multiethnic inhabitants and the distance between villages is around 15 km. A total of 111 dwellings are distributed between in 12 de Junio (N = 60) and Casuarina (N = 51) villages. The average number of people per household in the village of 12 de Junio is 6.7, whereas the average year of schooling is 0.6. The origin of this ethnic group is unclear but Fabre (2005) divides them into two groups. The first lived in the Chaco bank of the Paraguay River in Puerto Casado (now Puerto Victoria) and the second group moved into the area of the Mennonite farms. Inhabitants of 12 de Junio correspond to a group of “Angaité” who originally lived in the area of Puerto Casado, Alto Paraguay Department (bordering the eastern region). The average number of people per household in the Casuarina village is 5.2, whereas the average year of schooling is 2.9. Most people live in dwellings with trunk wall and mud-brick walls without plaster.
manual compression sprayers (X Pert Hudson®, Chicago, IL) domiciliary T. infestans localities were included. A sample of intra-

at least 10 intradomiciliary number of males. Pre- and postspraying collected sites (with spraying periods (3, 9, and 12 months) due to the low num-

ber of triatomines captured in the domestic or peridomestic areas during 30 minutes per house. The triatomine bugs collected were placed in plastic glasses identified with the name of the household head, house number, and specific site of collection. dwellings were sprayed with lambda cyhalothrin (10% wettable powder) at 30 mg/m^2 using manual compression sprayers (X Pert Hudson®, Chicago, IL) by experimented field workers from the Chagas Disease National Control Program. Immediately after insecticide spraying, knocked down triatomines were collected, as well.

A self-sealing plastic bag was given to each family for placing any triatomine bug captured in the domestic or peridomestic areas. The intradomiciliary captures were carried out in all months following the same procedure previously explained. House infestation pertains to the finding of at least one T. infestans in domestic or peridomestic sites.

The collection of sylvatic T. infestans was carried out five times by 2 days each over the months of May to August 2010 in areas around the road between 12 Junio and 10 Leguas, which are separated 8 km from each other as described in Rolón and others (2011). Sylvatic triatomines were captured on dry branches and nests of birds, using manual revision of demarcated areas throughout the day with the help of Nero, a 9-month-old gray German shepherd dog. The 10 Leguas village was simultaneously intervened with a blanked spraying campaign as well as 12 de Junio; however, reinfestations or adult captures were not detected in their human dwellings in any of the months of postspraying and few adults were captured during the baseline, then was not included in the sample.

Morphometric geometric of wings. We used females T. infestans collected during the pre- (baseline) and post-

spraying periods (3, 9, and 12 months) due to the low num-

ber of males. Pre- and postspraying collected sites (with at least 10 intradomiciliary T. infestans) from 12 de Junio and Casuarina localities were included. A sample of intradomiciliary T. infestans from Puerto Casado collected in 2005 was incorporated to analysis due to the potential original settlement of 12 Junio inhabitants. Sylvatic triatomines, collected in 2010 from the neighborhood of 12 de Junio, were incorporated to the analysis.

A total of 101 wings from females of T. infestans were removed with forceps and mounted between microscope slides and coverslips using a commercial adhesive. Images were obtained using a digital camera (Moticam 1000 1.3 MP Live Resolution), using software Motic Image Plus 2.0 (Kowloon, Hong Kong, China) connected to a stereo-

coscopic microscope (Quimis 08021363, model Q714Z-2; Diadema, Sao Paulo, Brazil). A total of seven “type I” landmarks (venation intersections, according to Bookstein, 1991) were selected (Figure 2). We did not use all possible landmarks due to the limitation of having few specimens mainly in the smallest group. Only right wings were included in the analyses to avoid pseudoreplication.

To compare the overall wing size between populations of T. infestans, we used the isometric estimator known as “centroid size” derived from coordinate data. Centroid size is defined as the square root of the sum of the squared dis-
tances between the center of the configuration of landmarks and each individual landmark. Shape variables as described by Bookstein (1990) were obtained using the Generalized Procrustes analysis superimposition algo-
rithm. The resulting variables are called partial warps (PWs). The method is based on the superimposition of each individual using least-square criterion, eliminating effects of scale, orientation, and position of the objects. The shape variables define the positional changes at each landmark in relation to a consensus shape.

Statistical analysis. Landmark repeatability was tested in 100% of wings, digitized twice by the same person. The measurement error was estimated by the “repeatability” index (R) as described by Arnaqvist and Martensson (1998), which can be regarded as the Pearson correlation coefficient between two measurements. Kruskal–Wallis tests corrected by Bonferroni’s method were used to analyze the isometric size. The shape proximity was analyzed using Mahalanobis distances. These were derived from shape variables and their statistical significance was computed by permutation tests (1,000 runs each) after Bonferroni correction. The distances were used in an unweighted pair-group method with arithmetic average (UPGMA) cluster analysis to produce a dendrogram. To detect allometry (i.e., to establish whether variation in shape was affected by variation in size), the shape variables were regressed on the centroid size by multivariate regression analysis.

Postspraying specimens from 12 de Junio were entered one by one to the discriminant analysis of prespraying and sylvatic samples (reference group) and assigned to these reference groups with which they had the shortest Mahalanobis distance. Each individual classification was performed on shape variables computed from the total of
reference specimens plus the individual to be classified; thus, for each classification, shape variables of the reference specimens were recomputed after adding only one post-intervention individual as proposed by Dujardin and others (2010) and Gaspe and others (2013).26,36 The percentage of postintervention specimens assigned to each reference group was computed.

The geometric coordinates of each landmark were digitized using tpsDig2, version 2.09 (free software developed by Rohlf, available at www.life.bio.sunysb/morpho). VAR was used for the precision test in the digitization of landmarks and for the nonparametric comparisons of centroid size, MOG for Procrustes superimposition, generation of PW, assignment of “unknown specimens,” and validated reclassification tests; PAD for computing the Mahalanobis distances; COV for examination of residual allometry within shape variables. The UPGMA dendogram was obtained using Phylip, version 3.6 (Seattle, WA).37 through PAD. The modules VAR, MOG, PAD, and COV developed by J. P. Dujardin are included in the CLIC package (free software package available at www.mome-clic.com).

**Infection with **T. cruzi** and blood meal sources.** Infection with T. cruzi was determined by microscopic observation of feces at 400× magnification for all T. infestans captured alive.

Blood meal contents were examined on 49 sylvatic T. infestans collected in the neighborhood of 12 Junio and 10 Leguas, and 12 intradomical T. infestans from 12 de Junio and 10 Leguas (N = 9). All triatomines were dissected, coded, frozen, and stored at Centro para el Desarrollo de la Investigación Científica, Paraguay. A random sample of 30 (61%) sylvatic and a total 87 (100%) intradomiciliary bugs were tested against human, dog, chicken, cat, and goat antiserum. Antisera from wild animals were not included in these assays because they were not available commercially. A direct enzyme-linked immunosorbent assay (ELISA) was used to identify blood meal sources of triatomines.38

**RESULTS**

**House reinfestation by **T. infestans**.** Baseline dwelling infestation by T. infestans in 12 de Junio of 70% (42 houses) and Casuarina 43.1% (22 houses) dropped sharply in both localities up to the first-month postspraying (0%) and remained with 1.6% and 11.8% until the 6-month follow-up, respectively. The locality of 12 de Junio did not present peri-domestic habitats and Casuarina showed 5.9% (3/51) of peri-domestic infestation. All infested dwellings after the insecticide spraying were previously infested at baseline with T. infestans (Figure 1). Infection with T. cruzi was not detected in any of the evaluated triatomine bugs.

**Morphometric analyses.** Comparison of two digitization sets for the same specimens showed good repeatability for centroid size (R = 0.99) and the relative warps (R = 0.93). Kruskal–Wallis tests give no significant values (P > 0.01) for comparisons of wings size between pre- and post-spraying pairs from each population of T. infestans. Similarly, there were no significant differences in the size of the wings of sylvatic individuals when compared with pre-populations and/or postspraying ones (Kruskal–Wallis test, P > 0.01) (Figure 3).

**Wing shapes of females **T. infestans** collected at pre-(B) and postspraying (A) in the villages studied as well as those captured in sylvatic environments (Syl) in the Paraguayan Chaco, showed some significant differences as indicated by the discriminant analysis (Figure 4). The canonical factor 1 explained 40% of the variance, whereas the canonical factor 2 explained the 20% (60% of the total variation). Permutation test shows that the populations exhibited significant differences in the Mahalanobis distances (P < 0.003; Table 1).

The cross-checked classification of pre- and postspraying specimens from 12 de Junio showed that 42–46% were correctly reclassified; similar values were registered for pre- and postspraying specimens from Casuarina

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**Figure 3.** Wing centroid size distribution for *Triatoma infestans* females collected before (B) and after (A) pyrethroid spraying at “12 de Junio” (12J), “Casuarina” (Cas), and “Puerto Casado” (PC) localities in Paraguayan Chaco. Syltically (Syl) bugs were captured next to 12J. Vertical lines (blue) under the quantiles represent specimens. Each box denotes the median as a line across the middle and the quartiles (25th and 75th percentiles) at its ends. This figure appears in color at www.ajtmh.org.

**Figure 4.** Scatterplots of discriminant analysis showing the variation in wing shape of females of *T. infestans* collected during pre-spraying (B) and postspraying (A) interventions in housing locations of 12 de Junio (12J), Casuarina (Cas), Puerto Casado (PC), and those captured in sylvatic environments (Syl) in the Paraguayan Chaco. The polygons enclose the individuals of each group. The centroids of each population are represented by squares and the ball inside represents each individual. This figure appears in color at www.ajtmh.org.
Wings of bugs captured in sylvatic environment (Syl) were included. Brackets show the sample size for each population. Puerto Casado (PC) localities from Paraguayan Chaco. Wings of bugs spraying (A) with pyrethroid at 12 de Junio (12J), Casuarina (Cas), and specimens of *Sylvatic na* (*N* = 33) from Puerto Casado prespraying (B) were assigned to other reference groups, Casuarina and postspraying population. A low percentage (11%) of bugs were reactive to the antisera tested (13.3%), 22 samples were not reactive (73.3%), and four samples (13.3%) were "border-line reactive" (low reaction in the ELISA test, and were collected in nest of *Tabara major* and dry branches of "quebracho blanco" and "palo santo." Sylvatic triatomines had human blood as meal source although they were captured in sylvatic areas with a distance of more than 1.5 km from the nearest house of both nearby villages. The sylvatic females showed blood meals from several hosts but chicken blood was not detected in any of these specimens. In relation to intradomicile triatomines blood meals in both villages, in a total of 12 and nine individuals with intestinal content from 12 de Junio and 10 Leguas, respectively, the human meals were predominant in both villages (41.7% and 33.3%), chicken meals were more frequent in 12 de Junio (33.3% and 11.1%), whereas dog meals were predominant in 10 Leguas (16.7% and 22.2%), and goat the blood meals were 8% and 11.1%, respectively. One specimen was reactive to antiserum to cat from 10 Leguas (11.1%).

**DISCUSSION**

Our study seems to be the first to evaluate wings similarity of *T. infestans* from the neighboring wild environment using geometric morphometric tools and shows a remarkable morphometric wings similarity between sylvatic and domestic populations and has suggested potential evidences on active dispersal ability and migration of these sylvatic individuals. Our results revealed that when the intradomicile postspraying individuals from 12 de Junio were entered one by one to the discriminant analysis, most were assigned to the sylvatic group. An entomological survey was conducted in all dwellings of 12 de Junio and Casuarina, resulting in a significant *T. infestans* infestation. However, in the entomological monitoring of postspraying at 3, 6, 9, and 12 months, very low triatomin abundance per house was detected, mostly adults inside the dwellings, because in these villages peri-domestic structures did not exist. With the support of a trained dog, a search of sylvatic areas around 12 de Junio was performed 2 years after the spraying, which allowed us to capture sylvatic individuals of *T. infestans* for the first time in the Paraguayan Chaco. These sylvatic triatomines were genetically similar to those found in domiciles, as well as those captured in the monitoring postspraying.12,13 Unlike what was observed by us,
previous studies in Argentina and the eastern region of Paraguay have attributed the reinestation by *T. infestans* after spraying to residual foci in peridomestic structures. However, in the absence of peridomestic structures in the localities of our study area, with the exception of three dwellings in Casuarina, triatomines collected during post-spraying periods may have been residual, sylvatic, or brought by passive transportation.

Our morphometric studies show that wing shapes of triatomines from Puerto Casado, an original community of this ethnic group, situated about 400 km from 12 de Junio, did not differ statistically from those captured in 12 de Junio (pre- or postspraying). This finding could be attributed to the fact that this village had its origin in a surrounding population of Puerto Casado, Alto Paraguay as described by Fabre (2005). Passive transport could have happened 27 years ago when they moved from Puerto Casado to 12 Junio or by successive visits of relatives from their former locality, very common practices in this ethnic group. In our study, morphometric results also show that triatomines from 12 de Junio did not have statistically significant differences between individuals captured in the baseline survey from those captured during postspraying periods as well as from sylvatic ones, and cytb genetic analysis previously obtained from these sylvatic individuals showed some haplotypes that were not found in the baseline insects, but they were present in some postspraying individuals. Although we cannot confirm whether the sylvatic foci were preexisting or not to the wide-community spraying of the study area, *T. infestans* populations would have a potential displacement between domestic and sylvatic areas, caused by spraying, as suggested by Ceballos and others (2011).

Morphometric studies of triatomine populations from La Rioja, Argentina, have shown the presence of mixed reinestation in dwellings attributed to survivors and to peridomestic populations. Our results indicate that mixed triatomine population occurs as well, when the shape of wings of postspraying individuals from 12 de Junio are sufficiently similar to those from sylvatic ones and these postspraying individuals are also similar to those captured during the prespraying. When the sylvatic individuals were analyzed by discriminant analysis, similar results were observed due to the sylvatic individuals were assigned to the postspraying group from 12 de Junio (results not shown).

Morphometric similarity in wings and gene flow observed previously between triatomines from pre- and postspraying captures, as well as from sylvatic ones, leads us to think in a dynamic mixed exchange between populations as has been highlighted in studies of La Rioja, or with potential involvement of other phenomena such as basal repopulation by residual foci due to faulty spraying, or by a potential reemergence of resistance to insecticides in use. On the other hand, pre- and postspraying triatomines from Casuarina were morphologically similar between them but different from 12 de Junio. In regard to this result, we consider as a possible explanation that these localities belong to different ethnic groups with different customs, and Casuarina dwellings present better quality and lower triatomine abundance. This differentiation could also be attributed to the diversity of construction materials, variety of hosts, instestation before insecticide spraying, as explained elsewhere and were mentioned as explicative factors in independent studies. Furthermore, other reasons could be related to the geographical distance between these two villages of 15 km that could be showing a certain structuring caused by geographical distances or by subsequent domiciliary sprays, which allow the differentiation of their different backgrounds.

In our study area, the origin of the postspraying triatomines remain uncertain; these postspraying individuals could come from intradomiciliary populations settled in the sylvatic area after a previous massive spraying, and maintain sufficient mobility, flying, or walking, as mentioned elsewhere allowing them to be captured inside the dwellings. Evidence supporting this finding in our study is the presence of intestinal content positive to human blood in adult triatomines captured between 1.5 and 3.4 km from the nearest dwelling and a fifth instar nymph fed on goat captured at 274 m of the nearest dwelling as well. The flight dispersal capacity of *T. infestans* range between 200 and 2,000 m would explain the result mentioned earlier. Furthermore, independently of the origin of sylvatic triatomines, different researchers have given great importance to the findings of sylvatic individuals in different countries and the impact that this evidence may have on control programs. In fact, this species generates a new challenge to the vector control programs because they can establish large colonies in nearby vegetation where chemical control is infeasible.

The low *T. infestans* densities observed could explain the absence of *T. cruzi* infection in these triatomines. Some studies have showed that low infection rates in domestic and peridomestic sites are associated with low *T. infestans* densities. On the other hand, control programs before and during surveillance phase have showed highly significant decrease of rate infection in domestic and peridomestic areas and recovery after blanket spraying could occur between 2 and 3 years. Our small sample of sylvatic triatomines

### Table 2

| Nearest village | Triatomine stage | Distance to the nearest house (in meters) | Blood meal source | Capture site |
|-----------------|------------------|-----------------------------|-------------------|--------------|
| 10 L            | 1♂               | 1,952                       | Human             | Nest of Tabara mayor in fallen Palo Santo tree |
| 10 L            | 1♀               | 274                         | Goat              | Dry branches of fallen quebracho blanco |
| 10 L            | 1♂               | 3,438                       | Dog               | Dry branches of fallen quebracho blanco |
| 12 J            | 1♀               | 3,345                       | Human, Cat        | Dry branch of quebracho blanco |
| 12 J            | 1♂               | 1,593                       | Human             | Fallen and dry Palo Santo tree |

10 L = 10 Leguas village; 12 J = 12 de Junio village; NV = nymph V.
was not infected with T. cruzi as well, and was captured with poor intestinal contents and mainly associated with goats or bird nets.9

Future studies should attempt to improve the understanding of the reinfection process incorporating sylvatic populations, independently of the species, in the analysis of field studies. Genetic studies could shed light on the potential origin of preexisting sylvatic triatomines in the Chaco; supported by the ancestral knowledge of old indigenous people who permanently indicate that the triatomines have always come from the sylvatic area when the north wind blows. Therefore, additional studies should look for strategies that modify the ones currently used by the control program to eliminate triatomine populations surviving sprayings, and seek new surveillance tools to prevent extra domiciliary reinfection pressure existing in these indigenous villages.

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