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Toxicological, Enzymatic, and Molecular Assessment of the Insecticide Susceptibility Profile of *Triatoma infestans* (Hemiptera: Reduviidae, Triatominae) Populations From Rural Communities of Santa Cruz, Bolivia

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Abstract

A wide range of insecticide resistance profiles has been reported across Bolivian domestic and sylvatic populations of *Triatoma infestans* (Klug, 1834) (Hemiptera, Reduviidae), including some with levels proven to be a threat for vector control. In this work, the insecticide profile of domestic *T. infestans* was studied with standardized toxicological bioassays, in an area that has not undergone consistent vector control. F1 first-instarnymphs hatched in laboratory from bugs captured in three communities from the Santa Cruz Department were evaluated with different insecticides. Moreover, the enzymatic activity of esterases and cytochrome P450 monooxygenases was measured in individual insects to evaluate the possible mechanism of metabolic resistance to pyrethroids. In addition, the DNA sequence of sodium channel gene (kdr) was screened for two point mutations associated with pyrethroid resistance previously reported in *T. infestans*.

All populations showed reduced susceptibility to deltamethrin and α-cypermethrin, albeit the RR_{50} values varied significantly among them. Increased P450 monooxygenases and permethrate

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esterases suggest the contribution, as detoxifying mechanisms, to the observed resistance to deltamethrin in all studied populations. No individuals presented either mutation associated to resistance in the kdr gene. The level of susceptibility to α-cypermethrin, the insecticide used by the local vector control program, falls within an acceptable range to continue its use in these populations. However, the observed RR_{50} values evidence the possibility of selection for resistance to pyrethroids, especially to deltamethrin. Consequently, the use of pyrethroid insecticides should be closely monitored in these communities, which should be kept under entomological surveillance and sustained interventions.

Keywords

Triatoma infestans; insecticide; resistance; pyrethroid; esterase

Chagas disease is an endemic parasitic infection that affects approximately 6 million people in the world, while 70 million are at risk of contracting the infection in 21 Latin American countries (WHO 2015). *Trypanosoma cruzi*, the etiological agent, is transmitted primarily by hematophagous triatomine vectors, of which *Triatoma infestans* (Klug 1834) (Hemiptera: Reduviidae, Triatominae) is the most important in the southern countries of Latin America (Schofield et al. 2006, Gürtler et al. 2008). The broad vectorial capacity of *T. infestans* results from the combination of its high *Tr. cruzi* transmission rate, its wide geographic distribution, and its ability to colonize human households and feed on humans and domestic animals (Gürtler et al. 1997, Gürtler et al. 2005, Noireau et al. 2009, Noireau 2009).

Bolivia has the highest *Tr. cruzi* infection prevalence and rate of on-going vector transmission in the Americas (WHO 2015). In a country where 55% of its territory (~600km^2) is considered endemic for Chagas, the impact of this disease on the population is remarkable. Over 600,000 people are estimated to be infected with *Tr. cruzi*, representing the 6% of its population, and is currently the leading country where transmission occurs, accounting for over 92% of new cases in the southern cone region (WHO 2015). Approximately 3.5 million people (30% of the population) are estimated to be at risk of infection (Medrano-Mercado et al. 2008), over 600,000 people are estimated to be infected, and 45,000 yearly deaths are attributed to the disease (WHO 2015). A study carried out in a Santa Cruz hospital showed that nearly 60% of symptomatic congestive heart failure cases were attributed to Chagas disease (Hidron et al. 2010). The *Tr. cruzi* infection prevalence in pregnant women was 19% in the city of Santa Cruz and 47% in Camiri, a provincial capital in the Bolivian Chaco (Kaplinski et al. 2015). In the Bolivian Chaco, people continue to live in infested houses, and in some villages, 80–90% of adults test positive for Chagas disease (Chippaux et al. 2008, Samuels et al. 2013).

Since the 1980s, triatomine control has been primarily based on the use of pyrethroid residual insecticides that commonly have deltamethrin, lambda-cyhalothrin, and α-cypermethrin as active ingredients (Morel 1999). Pyrethroids are currently the main tool for vector control because of their high efficacy and residual activity, together with low risk of environmental contamination and lower health risks associated to them (Pinchin et al. 1980, Zerba 1997, Gürtler et al. 2004, Cécere et al. 2006).

Triatoma infestans is the target of a regional vector control program, the Southern Cone Initiative (SCI), an intergovernmental agreement that aims to eliminate vector transmission by the massive spraying of households with residual pyrethroid insecticides. The intention of this program is to eliminate domestic populations of the vector (Dias 2007). By 2010, SCI achievements included the interruption of vectorial transmission of *Tr. cruzi* infection by *Triatoma infestans* in Uruguay in 1997, in Chile in 1999, in Brazil in 2006, in the Oriental region of Paraguay in 2008, and in the Peruvian provinces of Tacna and Moquegua in 2009 and 2010, respectively (Dias et al. 2002, Schofield et al. 2006, WHO 2015). In addition, 19 out of 21 involved Latin American countries achieved 100% screening of donated blood. In Uruguay and Chile, where transmission by the principal vector was interrupted in 1997 and 1999, respectively, and where there is no secondary vector of epidemiological significance, the risk of vector transmission is now considered negligible.

However, the Gran Chaco region, a 1.3-million-km^2 ecological zone shared among Bolivia, Argentina, and Paraguay, is an exception to the success achieved in other regions (Dias et al. 2002, Silveira 2002, Gürtler et al. 2007). Several areas of the Gran Chaco have been targeted with intensive vector control efforts without success (Gürtler et al. 2007, Mougabure-Cueto and Picollo 2015, Pessoa et al. 2015). Abundant populations of *T. infestans* live in peridomestic structures of the Gran Chaco rural houses, habitats where the pyrethroid formulations showed lower efficacy probably due to no persistent residual effect outdoors (Gürtler et al. 2004, Cécere et al. 2006). The recommended strategy of repeating an insecticide application every six months is usually not met in these areas for a number of reasons, including infrastructure, material, and human resources availability, among the main operational problems. In this context, residual populations of triatomines are likely to expand and eventually re-establish vectorial transmission of *Tr. cruzi* (Gürtler 2009). Reports of rapid re-infestation after spray campaigns, emergence of insecticide resistance, and the presence of sylvatic *T. infestans* populations challenge the strategy of the SCI in this region (Schofield and Dias 1999, Noireau et al. 2000, Rojas de Arias et al. 2004, Lardeux et al. 2010, Waleckx et al. 2012). The emergence of resistant triatomine populations after chemical treatments has been demonstrated, including several reports of resistance to pyrethroid insecticides from populations in the Gran Chaco region (Vassena et al. 2000, Picollo et al. 2005, Santo Orihuela et al. 2008, Toloza et al. 2008, Germano et al. 2010, Lardeux et al. 2010, Santo-Orihuela and Picollo 2011, Depickère et al. 2012).

The present work was carried out as part of a comprehensive epidemiological study in an area of the Bolivian Chaco with extremely high Chagas prevalence, cardiac disease burden, and serologic evidence of recent transmission and persistent domestic infestation (Samuels et al. 2013, Clark et al. 2015, Kaplinski et al. 2015; Fernandez et al. 2015). Our main objective was to evaluate the susceptibility of *T. infestans* populations from the Eiti region (Santa Cruz, Bolivia) to pyrethroid and nonpyrethroid insecticides.

In addition, we sought to establish the mechanisms responsible for the reduced susceptibility of these vector populations by evaluating the enzymes involved in deltamethrin degradation. In this sense, we analyzed the contribution of P450 monooxygenases and pyrethroid esterases as well as the possible presence of two mutations in the voltage-gated sodium

channel (kdr) gene associated with pyrethroid knockdown resistance (kdr) in *T. infestans* (Fabro et al. 2012, Capriotti et al. 2014).

Materials and Methods

Study Area and Previous Control History

The Eiti health sector was selected for intervention based on data provided by the Bolivian Ministry of Health Chagas control program, which reported high likelihood of active *Tr. cruzi* transmission. The Eiti health sector (19° 43′52.4994″ S, 63° 23′9.4812′ W; 800 m a.s.l. [meters above mean sea level]) is a catchment area composed of 18 villages with a total estimated population of 8,320 persons located in Gutierrez Municipality, Cordillera Province, Santa Cruz Department in Bolivia (Samuels et al. 2013).

The area had been sprayed with DDT and dieldrin (organochloride insecticides) by the National Malaria Eradication Service in the late 1950s and early 1960s. In the 1980s, HCH (γ-hexachlorocyclohexane) was used in the region, and subsequently, during the 1990s, the insecticide of choice was switched to pyrethroids (λ-cyhalothrin WP 12.5% and deltamethrin SC 2.5 and 5%). Beginning in 2002, α-acypermethrin SC 20% was the insecticide utilized in the region by the national control program (Alarico et al. 2010). According to the local vector control program records, the first systematic spray campaign targeting domestic *T. infestans* in these communities began in early 2000. Blanket-spraying with α-cypermethrin 20% was conducted in 2000 and 2003. From 2005 to 2009, focal spraying of infested houses was conducted by the national entomological control program of the Department of Health Services (SEDES) Chagas program. Moreover, beginning in 1997, the company Inesfly (Dias and Jemmio 2008) participated in a series of projects involving house improvement and painting with the microencapsulated formulation (Inesfly 5A IGR) in areas near Camiri (Bolivia) and La Rioja and Santiago del Estero (Argentina) (Amelotti et al. 2009, Alarico et al. 2010). This initiative included sectors of two of the communities evaluated in our work (El Cruce and Itapicoe, Fig. 1), and thus, a small number of houses in our study area were improved or rebuilt, and treated with the insecticide paint sometime from 1998 to 2009. No systematic spraying against triatomines was performed in the area from 2003 to the time of this study. Since then, individual houses were treated by community members if triatomines were detected within the houses and insecticide was available, but no official spraying programs were carried out (Samuels et al. 2013).

Framed within a wider epidemiological study, we carried out a baseline entomological evaluation and a vector control intervention in seven neighboring communities (Fig. 1) chosen nonrandomly based on size, relative lack of recent interventions, and proximity to our laboratory, as previously described (Samuels et al. 2013). During the entomological evaluation that took place in November-December 2011, 508 houses from the seven communities were evaluated for triatomine insects. Local villagers were trained by our personnel and a SEDES technician to perform timed manual collections within the houses and main peridomestic structures. No dislodging agent was used.

Over 40% (n = 205) of the houses were infested and a total of 1,022 *T. infestans* were captured in either domiciles or peridomestic structures (i.e., storage rooms). About 45% of

the captured insects were screened by microscopy at 40× in search for mobile trypanosomatids, yielding a *Tr. cruzi* prevalence of 39.7% ($n = 182$). At the end of our entomological survey in December 2011, a massive blanket-spraying using α-cypermethrin 20% provided by SEDES was conducted by local habitants supervised by our team.

Insects

For this study, *T. infestans* captured during the entomologic surveillances in several houses of the communities of Guasuanti (19° 47′10.08″ S 63° 25′29.88″ W; 1,022 m a.s.l.), Itapicoe (19° 46′0.78″ S 63° 28′56.58″ W; 1,127 m a.s.l.), and El Cruce (19° 44′51.78″ S 63° 26′45.42″ W; 1,086 m a.s.l.) were used. A pool of live insects of different developmental stages from each community was sent to CIPEIN and bred in the laboratory. All colonies were maintained at 28 ± 1°C, 50% RH, and a photoperiod of 12:12 (L:D) h. Insects were fed on pigeons on a weekly basis. Rearing conditions are described in detail elsewhere (Picollo et al. 1976, Nuñez and Segura 1987).

The F1 generation obtained from the field specimens (F0) were used to conduct the bioassays. For all the experiments, laboratory-reared unfed first instars (5–7-d-old, mean weight 1.3 ± 0.2 mg) were tested following the World Health Organization protocol (WHO 1994).

The susceptible reference strain of *T. infestans* used to determine the baseline was the NFS strain, from Santiago del Estero (Argentina), which has been reared in laboratory without insecticide exposure since December 2004 (Roca-Acevedo et al. 2011). This strain is derived from a domestic population with no exposure to insecticides and collected in an area where insects have successfully been controlled with deltamethrin.

Bioassays

Bioassays were performed according to the WHO protocol (1994). Briefly, *T. infestans* nymphs received a topical application of 0.2 μl of acetone solution of insecticide on the dorsal abdomen, using a 10 μl Hamilton (Nevada, USA) syringe with automatic repeating dispenser. The control group received pure acetone. The first step is carried out as a screening assay to determine doses of insecticide, discarding doses that cause 0 and 100% of mortality. Initially, four doses of insecticide were assayed with a dilution factor of 1 to 10 between doses. Later, three replicates of at least four more insecticide doses in a range that produced between 10 and 90% mortality were conducted. Table 1 shows the number of bugs (N) used in each bioassay.

The concentrations evaluated ranged from 10^{-4} to 12^{-2} mg/ml for all insecticides. Dosages were expressed as nanograms (ng) of active ingredient per insect (Table 1).

Treated insects were kept inside a plastic glass with folded paper at 28–30 °C and 50–70% RH. Mortality was evaluated after 24 h by placing the insects at the center of a circular filter paper of diameter 11 cm; those nymphs able to walk to the border of the paper were considered alive (WHO 1994). Mortality data were corrected to adjust for variability and natural mortality of the controls (Abbott 1987).

The insecticides evaluated in the bioassays were technical-grade deltamethrin (99.0%), fenitrothion (99.0%), fipronil (97.5%), and α-cypermethrin (97.9%), all from Ehrestorfer (Augsburg, Germany), and the synergist piperonyl butoxide (PBO) 90.3% (ICN, USA). Serial dilutions were prepared with analytical-grade acetone from Sintorgan SACIF (Buenos Aires, Argentina).

Statistical Analysis

To estimate the lethal dose (in nanograms of insecticide per insect) that kills 50% of treated individuals (LD50), mortality data from each *T. infestans* population against each insecticide evaluated were pooled and analyzed based on probit analysis (Litchfield and Wilcoxon 1949) with POLO Plus software (LeOra Software 2002 Berkeley, California, USA). Resistant ratios (RR50s) and 95% confidence intervals (CI) of each population were calculated by comparison of the dose–response curves between studied populations and the susceptible reference strain NFS (Robertson et al. 2007).

Populations were considered statistically different from the reference strain if the LDR 95% CI did not include the number 1.0 (Robertson et al. 2007, Russell 2007).

Evaluation of Deltamethrin Susceptibility After Pre-Treatment With the Synergist PBO

PBO has been extensively used as a general monooxygenase inhibitor and as a synergist for pyrethroid and other insecticides (Georghiou and Mellon 1983). Synergists act by blocking metabolic pathways that would otherwise break down pesticides, thus restoring susceptibility to the insecticide. When the treatment with PBO causes reversal from resistance to susceptibility, an oxidase metabolic pathway is likely involved in the pyrethroid resistance of that given population.

To determine whether the presence of metabolic resistance to deltamethrin in these populations is mediated by P450 monooxygenases, an assay exposing insects to PBO before a bioassay was carried out (Vassena et al. 2000). Briefly, the exposition was performed in circular glass containers, whose floor had an area of 95 cm^2 and were 6-cm-high. The container bases were impregnated with 1.5 ml of solution of 3.17 mg/ml of PBO in acetone. After acetone evaporation (1 h), the final concentration of PBO in the base of the containers was 500 mg/m^2. The containers used as control were only impregnated with acetone. Three replicates of 10 first-instar nymphs per container were exposed to PBO for 60 min. Thirty minutes later, 0.2 µl of deltamethrin diagnostic dose (DD) was applied topically; control insects were treated with the 0.2 µl of acetone. Nymphs were kept in the same post-treatment conditions as described for the evaluation of insecticide activity and mortality evaluated after 24 h.

The DD assay determined whether the insect reference population (NFS) was fully susceptible to deltamethrin. The DD was defined as the 99% lethal dose (LD99) for deltamethrin of a reference susceptible insect population (2 ng/insect) and was chosen for the topical application of first nymphs from field samples after PBO treatment (WHO 1994, Picollo et al. 2005, Gurevitz et al. 2012).

Pyrethroid Esterase Activity

Enzymatic activity was evaluated by the hydrolysis of 7-coumaryl permethrate (7-CP), a fluorescent substrate synthesized at CIPEIN, used to determine pyrethroid hydrolysis activity on individual insects (Santo Orihuela et al. 2006, 2008, 2013). The enzymatic hydrolysis of 7-CP produces 7-OHC (7-hydroxicoumarin), and the concentration of 7-OHC is easily monitored by measuring fluorescence. Live first-instar nymphs with the same characteristics as those used in bioassays (Table 2) were homogenized in 220 µl of phosphate buffer (0.05 M), pH 7.2, using a plastic mortar and pestle. The whole procedure was carried out on ice to avoid enzymatic degradation. The reaction was initiated by adding 10 µl of 7-CP (3.5mM, 2-methoxy ethanol) to 190 µl of each homogenate and posterior incubation at 25 °C for 33 min, at pH 7.2. Assays were carried out in black, 96-well, polystyrene, flat-bottomed microtiter plates (PerkinElmer Life and Analytical Sciences) at 25 °C. Fluorescence was measured with an excitation wavelength of 390 nm and an emission wavelength of 440 nm using a Fluoroskan Ascent Microplate Fluorometer (Thermo Scientific, Helsinki, Finland). Activity was measured every 3 min for 30 min. The relative fluorescence units (RFU) were corrected for background hydrolysis and nonspecific fluorescence of substrate and transformed to picomoles per minute (activity units) by using a calibration curve per replicate with dilutions of 7-OHC (68.5, 342.69, 685.44, and 1370.8 total picomoles per well). Results were analyzed with Ascent (Thermo Scientific, Helsinki, Finland) and Microsoft Excel 2010 (Microsoft) software.

Cytochrome P450 Monooxygenase Activity

Monooxygenase activity was measured according to the direct fluorometric test developed for individual abdomens of *T. infestans*, using 7-ethoxycoumarin (7-EC, Sigma-Aldrich Co) as substrate (De Sousa et al. 1995, González Audino et al. 2004, Picollo et al. 2005). The abdomens of living first-instar nymphs (Table 2) were placed individually into wells of a 96-well microplate containing 100 µl of 0.05 M phosphate buffer, pH 7.2, and 3.5mM 7-EC. The reaction was stopped after 4-h incubation at 30 °C by adding 100 µl of glycine buffer (10^{-4} M), pH 10.4. To precipitate the abdomens in the wells, microplates were centrifuged at 2,000 *g* for 30 s in a refrigerated centrifuge for microplates (4237 R, ALC International SRL, Cologna Monzese, Italy) before and after the incubation of the enzymatic reaction at 30 °C. For each population, similar wells receiving glycine buffer before incubation were used as blanks. The hydrolysis of 7-EC produced 7-OHC, whose fluorescence was monitored with an excitation wavelength of 390 nm and an emission wavelength of 440 nm measured in a Fluoroskan Ascent Microplate Fluorometer (Thermo Scientific, Helsinki, Finland). Assays were conducted in black, 96-well, polystyrene, flat-bottomed microtiter plates (Perkin Elmer Life and Analytical Sciences) at 25 °C. Results were analyzed with Ascent software (Thermo Scientific, Helsinki, Finland) and Microsoft Excel 2010 (Microsoft).

Activity values of pyrethroid esterase and P450 monooxygenase of individual nymphs from different populations are expressed as picomoles of 7-OHC per minute and per insect (pmol/min/i). Analysis of variance, nonparametric Kruskal–Wallis, and Dunn were used to compare the values of 7-CP or P450 enzymatic activity per minute and per insect among populations using Instat V. 3.01 (GraphPad Software, San Diego, CA, USA).

Screening for Mutations in the kdr Gene

A fragment of the DNA sequence of the sodium channel gene was amplified and screened for two point mutations associated with pyrethroid knockdown resistance (*kdr*), previously reported in *T. infestans* (Fabro et al. 2012, Capriotti et al. 2014).

Initially, we attempted to use the screening methodology previously published (Fabro et al. 2012), but we failed to successfully amplify the target region or amplification was not reproducible across samples from different populations. We therefore optimized PCR conditions and redesigned the primers based on the complete sequences obtained in two specimens of our data set that amplified the target region with the original amplification protocol. We found a combination of a new primer (Tifw2plm 5′-GAT ATC AAT TAT GGG TCG AAC TG-3′) with a reverse primer (Ti rev3= 5′-TTA ACC CGA ACA AGA ATA TA-3′) previously published (Fabro et al. 2012, Capriotti et al. 2014) that successfully amplified the target region in all populations. Briefly, DNA was purified from each individual using the QIAamp DNA purification kit (QIAGEN), following the manufacturer's recommendation for DNA extraction from tissues. PCR amplification reaction was carried out with Accustart II PCR superMix (Quanta Bio) in a final volume of 25 µl, using 1 µl of each primer at 10 pmol/µl and 3.5 µl of DNA template. Amplification conditions were optimized as follows: 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 52 °C for 50 s, and 72 °C for 4 min; and a final elongation step at 72 °C for 15 min. The reaction produced a fragment of approximately 560 bp containing the two mutational sites of interest. Amplification products were checked by electrophoresis on 2% agarose gels stained with Gelred (Biotium), and positive samples were purified with MultiscreenPCR plates (Millipore) following manufacturer's standard protocol. Cycle sequence reactions for both forward and reverse strains were carried out using the BigDye terminator Cycle sequencing kit v3.1 (Applied Biosystems), as recommended by the manufacturer, and later purified with the BigDye XTerminator purification kit (Applied Biosystems). Direct sequencing of the PCR product was obtained with a 3500 ABI automated DNA sequencer (Applied Biosystems). Sequences were analyzed with SeqMan Pro 12.2.0 (DNASTAR, Lasergene12) and aligned for comparison with Bioedit 7.2.0 (Hall 1999). The genotype of susceptibility/resistance of each individual was determined by direct observation of the nucleotide substitutions in the target sites (L1014 and L9225).

Results

Toxicity to Insecticides With Different Modes of Action in Bolivian *T. infestans* First Instar

The LD50 values of the NFS strain for deltamethrin and α-cypermethrin were 0.13 and 0.075 ng per insect, respectively, and all three populations from Santa Cruz showed significantly higher RR50 values. For deltamethrin, significant differences were also observed among populations; Guasuanti had the lowest RR50 (2.71), whereas El Cruce and Itapicoe exhibited RR50s fivefold higher than NFS (13.90 and 10.62, respectively). Moreover, the populations from El Cruce and Itapicoe showed significant albeit minimal, reduction of susceptibility to fenitrothion compared with NFS (RR50s 2.36 and 1.65, respectively). The RR50 value for insects from Guasuanti was no different from NFS.

None of the studied populations showed significant differences from the reference strain in the susceptibility against fipronil.

Table 1 shows the toxicity of deltamethrin, α-cypermethrin, fenitrothion, and fipronil against *T. infestans* from El Cruce, Itapicoe, and Guasuanti communities and the reference susceptible strain NFS.

P450 Activity Evaluated by a Synergist

After PBO exposure, the insects received a topical application of acetone solutions of deltamethrin, which resulted in 100% mortality for all three populations (data not shown). Field populations of *T. infestans* pre-treated with PBO showed deltamethrin susceptibility similar to that of the susceptible NFS reference strain.

Pyrethroid Esterase and Cytochrome P450 Monooxygenase Activity

All populations exhibited increased 7-CP esterase activity (Table 2) in comparison with the reference strain NFS (18.63, 18.51, and 17.91 vs. 11.35 pmol/min/I). In contrast, the values of P450 monooxygenase activity were highly variable among populations (Table 2).

Screening for Mutations in the kdr Gene

The sequence analysis of 15, 17, and 7 insects from El Cruce, Itapicoe, and Guasuanti, respectively, showed that no individuals presented either of the two mutations in the target sites L1014 and L9251 of the sodium channel gene.

Discussion

Pyrethroid resistance occurrence in *T. infestans* populations may jeopardize the future effectiveness of costly vector control actions. In Bolivia, increasing number of reports denote that the frequency and geographical spread of resistance are much higher than ever thought in the early 1990s when control actions were designed (Lardeux et al. 2010, Bustamante Gomez et al. 2014, Bustamante Gomez et al. 2016), which calls for the increase in surveillance of the populations targeted for control. In this context, the insecticide resistance profile in all three *T. infestans* populations evaluated from the Santa Cruz Department showed lower RR values than populations observed in other areas of Bolivia, particularly when compared with domestic populations (Germano et al. 2010, Lardeux et al. 2010, Depickère et al. 2012, Bustamante Gomez et al. 2015, Gorla et al. 2015, Roca-Acevedo et al. 2015). However, the toxicological analyses showed significant levels of resistance (RR50s) toward deltamethrin and α-cypermethrin in all three communities. Moreover, the resistance level of the populations varied among them, showing fivefold higher RR_{50} values for deltamethrin in El Cruce and Itapicoe, compared with Guasuanti.

The RR50s obtained in these populations was not as high as in other areas that had presented difficulties for vector control (i.e., RR50: 541.6; Tierras Nuevas, Tarija, Bolivia; Germano et al. 2010). However, previous reports on the evaluation of susceptibility of *T. infestans* populations with the same methodology applied here demonstrated that RR50 values as low as 7.17 can jeopardize the vector control (Gurevitz et al. 2012).

Current PAHO guidelines determined that populations with RR50 > 5 (fivefold higher than reference populations) should be considered resistant (PAHO 2005, Pessoa et al. 2015). Above that RR50 level, PAHO guidelines recommend: 1) to investigate the operational failures in the vector control strategies performed by the Chagas Disease Control Program (CDCP); 2) to change the insecticide used for CDCP to another with a different mechanism of action; and 3) to continue monitoring the susceptibility profile of the altered populations through time. On the other hand, PAHO guidelines consider that if a field population presents RR50 < 5, the change in susceptibility observed would probably be due to individual variability, and thus, control activities could be continued with the same insecticide, although the addition of susceptibility monitoring activities is highly recommended.

Interestingly, the two populations with increased RR50 (El Cruce and Itapicoe) are located in the most easily accessible area of the Eiti health sector (Fig. 1). These two communities also presented the most developed conditions with regard to housing and infrastructure. Several projects supported by nongovernmental organizations and government initiatives were carried out in that accessible area of Eiti, which included building good-quality housing that replaced the traditional mud-stick/thatched structures. In addition, in both communities, trials were carried out with an insecticide paint or micro-encapsulated formulation, and initiatives that included re-building, plastering, and fully painting a few local houses (Dias and Jemmio 2008, Alarico et al. 2010). The easily accessible location of these two communities could have facilitated various interventions and increased the frequency of vector control measures in contrast to more isolated communities like Guasuanti. Therefore, it is likely that insect populations from El Cruce and Itapicoe had been under higher pyrethroid pressure and, consequently, present increased resistance values toward pyrethroids. However, because no systematic vector control actions have been on-going in this area and no insecticide sprayings had occurred since 2009, RR50 values were substantially lower than in other areas of Bolivia, such as Yacuiba (RR = 154.4; Santo Orihuela et al. 2008), located approximately 200km away from Eiti. This suggests that the selective pressure was insufficient to fix the resistance phenotype in these populations.

The susceptibility profiles against fenitrothion (organophosphate) and fipronil (phenylpyrazole) were evaluated to assess the possible action mechanisms against different kinds of insecticide compounds. Results showed no resistance to fipronil in any population. However, in El Cruce and Itapicoe, significant reduced susceptibility to fenitrothion was observed. Biochemical analysis of pyrethroid esterases (7-CP) showed increased activities for all Eiti populations, indicating a possible contribution of this enzymatic group to the altered susceptibility to pyrethroids and the observed alteration for fenitrothion (B. Brogdon Personal communication; Flores et al. 2006).

The contribution of this possible metabolic pathway mediated by esterases has been reported previously in *T. infestans* and other insect species (Hemingway and Ranson 2000; Picollo et al. 2005; Santo Orihuela et al. 2006, 2008; Barrios et al. 2010; Santo-Orihuela and Picollo 2011; Roca-Acevedo et al. 2015). Pyrethroids are mainly cleaved by esterase-mediated hydrolysis, yielding less-toxic compounds (Abernathy and Casida 1973).

Various works have been published related to insect P450 monooxygenases and their active metabolic role in insecticide resistance (Berge et al. 1998, Scott 1999, Feyereisen 2005, Bass and Field 2011, David et al. 2013). The contribution of this enzyme family to insecticide degradation has been demonstrated in *T. infestans* from different regions in Argentina, Brazil, and Bolivia (Vassena et al. 2000, Picollo et al. 2005, Santo Orihuela et al. 2008, Roca-Acevedo et al. 2011, Forlani et al. 2013). When all three populations from Eiti were treated with PBO before the bioassays, the resistance to deltamethrin reverted to total susceptibility. The synergism effect of PBO reverted the resistance to deltamethrin, suggesting that an oxidase metabolic pathway is involved in the pyrethroid resistance mechanism of these populations.

The biochemical analysis results on the activity of the cytochrome P450 monooxygenase (evaluated by 7-ethoxycoumarin-O-deethylation) showed a highly variable pattern among populations not consistent with the resistant profile observed with the bioassays. This could reflect the specificity of each isoenzyme of this large family involved in the resistance mechanism to each particular insecticide type (Schama et al. 2016). Therefore, the specific enzyme family we evaluated by this test was not likely responsible for the resistance observed. In this regard, Sawicki et al. (1986) reported the possibility that even slight structural changes resulting in steric effects in pyrethroid molecules can greatly influence the final susceptibility. Many authors have demonstrated that the P450 monooxygenases are involved in the metabolism of virtually all insecticides, leading to activation of the molecule in the case of organophosphorus insecticides, or more generally to detoxification (Hemingway and Ranson 2000). As new research is being developed in this topic, the complete sequencing of this enzymatic group is being characterized, which would allow for the development of new markers to detect individual enzymes involved in the resistance mechanism in each population (Grosso et al. 2016; Ibrahim et al. 2015).

Recently, Fabro et al. (2012) and Capriotti et al. (2014) demonstrated the presence of two point mutations in the sodium channel associated with pyrethroid knockdown resistance (kdr) in *T. infestans* populations in Chaco and Salta provinces of Argentina. However, the populations evaluated in this study showed no evidence of altered target site (kdr) and thus that is not likely to be the mechanism related to the observed pyrethroid resistance profile. This result is not surprising, as the presence of the named kdr mutations are associated to much higher levels of resistance to deltamethrin than the ones detected in these populations. (Fabro et al. 2012, Capriotti et al. 2014).

Final Remarks

The toxicological analysis by bioassays demonstrated that the triatomine populations studied in three separate communities in the Santa Cruz Department showed decreased susceptibility to pyrethroid insecticide.

The resistance pattern detected in this sample indicates the applications of α-cypermethrin in this area have not yet caused significant selective pressure of P450 monooxygenases to this particular insecticide. Therefore, in these populations, the observed level of susceptibility to α-cypermethrin (the insecticide used by the Bolivian vector control program) falls within the acceptable range to continue the use of this insecticide for vector

control. However, because all populations showed critical RR50s values, indicating reduced susceptibility to both deltamethrin and α-cypermethrin, it is expected that continuous and massive spraying with the same type of insecticide might select specific mechanisms toward α-cypermethrin resistance. Therefore, these populations have the potential to develop resistance to pyrethroids, which would lead to higher risk of vector control failure. The current recommendations include close entomological monitoring in the area, including periodical assessment of resistance level to pyrethroid and sustained well-conducted intervention activities.

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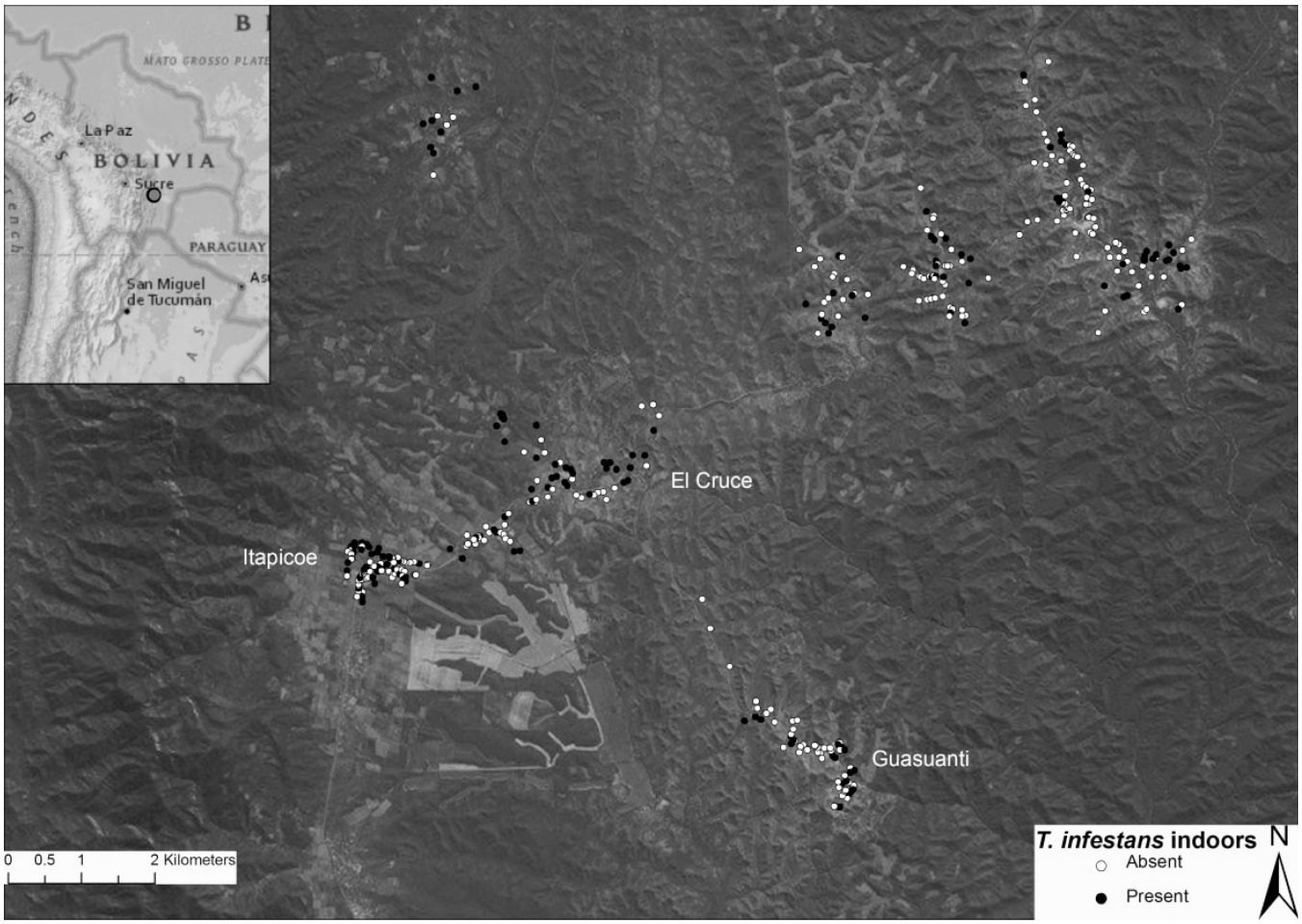

Fig. 1.

Collection sites (houses) for bug samples in rural communities of Eiti Health Sector, Municipality of Gutierrez, Santa Cruz Department, Bolivia. Inset: Geographical location of the study area. Imagery credits: Esri, i-cubed, USDA, USGS, AEX, GeoEye, Getmapping, Aerogrid, IGN, IGP, and the GIS User Community. Copyright: © 2012 Esri, DeLorme, NAVTEQ.

Table 1

Toxicity of insecticides against *T. infestans* first instars from Eiti Department, Santa Cruz, Bolivia

Insecticide	Population	*n*	Slope ± SE	LD_{50} ng/insect (95% CI)	RR_{50} (95% CI)
Deltamethrin	El Cruce	116	1.08 ± 0.32	1.80 (0.85–5.85)	13.90 (6.24–30.98)
	Guasuanti	104	0.90 ± 0.21	0.35 (0.07–0.69)	2.71 (1.04–7.06)
	Itapicoe	90	1.58 ± 0.47	1.37 (0.37–241)	10.62 (5.15–21.88)
	NFS[a]	120	3.10 ± 0.26	0.13 (0.11–0.15)	—
α-Cypermethrin	El Cruce	140	1.2 ± 0.23	0.47 (0.23–0.77)	6.22 (3.07–12.61)
	Guasuanti	134	1.21 ± 0.21	0.42 (0.2–0.73)	5.61 (2.67–11.73)
	Itapicoe	130	1.03 ± 0.24	0.41 (0.16–0.73)	5.47 (2.47–12.09)
	NFS	90	1.99 ± 0.40	0.075 (0.051–0.106)	—
Fenitrothion	El Cruce	120	3.46 ± 0.61	25.6 (20.2–32.6)	2.36 (1.77–3.14)
	Guasuanti	88	1.47 ± 0.42	10.60 (4.4–19.0)	0.98 (0.52–1.86)
	Itapicoe	90	2.03 ± 0.50	17.80 (12.4–25.2)	1.65 (1.14–2.38)
	NFS[b]	90	2.07 ± 0.18	10.8 (4.0–26.6)	—
Fipronil	El Cruce	81	0.9 ± 0.27	0.052 (0.004–0.132)	0.22 (0.03–0.8)
	Guasuanti	81	0.96 ± 0.29	0.042 (0.004–0.11)	0.18 (0.02–0.69)
	Itapicoe	81	0.86 ± 0.25	0.072 (0.008–0.18)	0.33 (0.05–1.17)
	NFS	121	0.83 ± 0.17	0.178 (0.054–0.916)	—

n = number of bugs used per assay (total replicates). RR_{50} is considered significant if the CI does not contain the number 1 (Robertson et al. 2007).

[a]data from Roca-Acevedo et al. (2011).

[b]Data from Carvajal et al. (2012).

Table 2

Enzymatic activities of 7-coumaryl permethrate (7-CP) esterases and P450 monooxygenases

Population	7-CP esterases	*N*	P450 monooxygenases	*N*
El Cruce	18.63 (± 1.11)*b*	31	138.17 (± 17.29)*a*	32
Guasuanti	18.51 (± 0.84)*b*	32	352.20 (± 56.53)*c*	16
Itapicoe	17.91 (± 1.17)*b*	30	226.22 (± 40.45)*b*	23
NFS	11.35 (± 0.81)*a*	43	194.09 (± 15.83)*b*	40

The activity values are presented as the mean activity (pmol/min) per insect and standard errors (SE). N = number of insects used per assay. The different letters (*a*–*c*) are significantly different ($P < 0.05$) [Kruskal–Wallis (KW) and Dunn's multiple comparison test]. 7-CP KW statistic = 35.71; P450KW statistic = 17.59.