# Examining Mechanisms of Pyrethroid Resistance in Eggs of Two Populations of the Chagas' Disease Vector *Triatoma infestans* (Hemiptera: Reduviidae)

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**ABSTRACT** Chagas disease is a zoonosis transmitted to man by blood-sucking triatomine bugs found in the Americas. *Triatoma infestans* (Klug, 1834) is the main vector of Chagas' disease in Argentina. The control of this illness relies heavily on vector control through the use of insecticide. However, resistance to pyrethroid insecticides associated with ineffective field treatments has been increasingly reported in *T. infestans* from Argentina and Bolivia. There are few reports on the expression and causes of resistance in eggs of resistant populations, and even fewer studies on insecticide resistance throughout embryonic development. In this study, we explore the biochemical and molecular mechanisms potentially associated with the deltamethrin resistance assessed in the developing eggs of the Argentinean (Campo Largo) and Bolivian (Entre Ríos) *T. infestans* populations.

We found measurable activity of monooxigenases and pyrethroid esterases throughout embryonic development. The pyrethroid esterase activity grew steadily throughout development in all the studied populations and was highest in eggs 12 d old. Mean enzyme activity increased from 13.6 to 16.3 and 22.2 picomol 7-hydroxycoumarin/min (7-OHC) in eggs of 4-, 7-, and 12 d old from the susceptible reference bug colony. Mean activity of resistant populations increased from 16.0 to 25.9 picomol 7-OHC/min in eggs of 4- to 12 d old in Entre Ríos population, and from 15.9 to 28.9 picomol 7-OHC/min in Campo Largo population. Molecular analysis of susceptible and resistant developing eggs detected L1014F mutation in both resistant populations, but no L925I mutation was found in any of the studied populations.

Higher esterase activity and L1014F presence justify the resistance to pyrethroid throughout developing eggs of both studied *T. infestans* populations. The description of resistance profiles including resistance mechanisms involved will allow a rational design of campaigns for the control of Chagas disease transmission.

**KEY WORDS** *Triatoma infestans*, resistance, pyrethroid, egg, immature

Chagas disease is a zoonosis due to the flagellated protozoan parasite *Trypanosoma cruzi* Chagas. It is transmitted to man by infected feces of blood-sucking triatomine bugs found in the Americas. *Triatoma infestans* Klug is the main vector of Chagas' disease in Argentina. In this country, as across Latin America, the control of this illness relies heavily on vector control through the use of residual insecticide spraying. However, the success of these control methods is threatened by resistance to insecticides. Resistance to pyrethroid insecticides associated with ineffective field treatments has been increasingly reported in *T. infestans* from Argentina and Bolivia (Picollo et al. 2005, Germano et al. 2010, Lardeux et al. 2010, Gurevitz et al. 2012).

According to the toxicological and biochemical characteristics of the pyrethroid resistance populations three resistant profiles have been detected in *T. infestans* (Germano et al. 2012). The resistance profiles were identified as Ti-R1, Ti-R2, and Ti-R3, corresponding to the Argentinean Chaco (Acambuco, Salta), Bolivian Chaco (Entre Ríos, Tarija), and Bolivian Andean Valleys (Mataral). The resistance pattern observed in Ti-R1 (high deltamethrin resistance in nymphs and 12-d-old eggs) is different to that of Ti-R2 (high and medium deltamethrin resistance in nymphs and 12-d-old eggs, respectively, low fipronil resistance). It is also different to that of Ti-R3 (low deltamethrin resistance in nymphs and 12-d-old eggs, medium fipronil resistance). Moreover, deltamethrin resistance observed in Ti-R1 are caused by metabolic mechanisms (pyrethroid esterases), which are absent in the other resistance profiles. Increased activity of these enzymes was also reported for nymphs of other field populations collected in the same locality used to determine the Ti-R1 profile (Santo-Orihuela et al. 2008). Recently, the presence of a resistance-conferring mutation (L1014F) was identified in a pyrethroid resistant population (Madrejones)

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from the TiR1-resistant area from Argentina (Fabro et al. 2012), and another point mutation associated with pyrethroid resistance (L925I) was reported in a small locality from the Gran Chaco region of Argentina (Mala; Capriotti et al. 2014).

Most insecticide resistance studies were performed in insect adult and nymph stages. There are few reports on the expression and causes of resistance in eggs of resistant populations, and even fewer studies on insecticide resistance throughout embryonic development. Mougabure Cueto et al. (2008) reported high permethrin resistance in late development eggs of three head louse populations (Pediculus humanus capitis De Geer) whose pyrethroid resistance had been shown in adults and nymphs. By application of diagnostic concentrations, Rodríguez et al. (2011) assessed resistance to several insecticides in eggs and neonate larvae of Cydia pomonella (L.) (Lepidoptera: Tortricidae) field populations from three areas of apple production in Spain. These authors studied the enzymatic detoxification systems were involved in the resistance in neonate larvae, but not in eggs. Differences in pyrethroid susceptibility were reported for younger and older eggs of the spotted tent form leaf miner Phyllonorycter blancardella (Marshall & Pree, 1986). Eggs treated 0–2 d postoviposition were more susceptible than 4–6-d-old eggs for susceptible and resistant strains. In a recent work, we reported that deltamethrin resistance was expressed throughout embryonic development of T. infestans from two resistant areas of Argentina (Campo Largo) and Bolivia (Entre Ríos) (Roca-Acevedo et al. 2013). The resistant ratios estimated for the Argentinean population were as high as 1,144, 1,193, and 822 in eggs of 4-, 7-, and 12-d-old, while for the Bolivian population were 21, 15, and 39, respectively.

In this study, we explore the biochemical and molecular mechanisms potentially associated with the deltamethrin resistance assessed in the developing eggs of the Argentinean (Campo Largo) and Bolivian (Entre Ríos) T. infestans populations. For this purpose, we analyzed the activity of detoxifying enzymes (P450-monooxigenases and pyrethroid-esterases) and the presence of point mutations in the para-type sodium channel of these deltamethrin-resistant populations.

## Materials and Methods

**Insects.** Field populations of T. infestans were collected in 2009 from infested houses of endemic areas in northern Argentina (Campo Largo, 22° 0′19.32″ S, 63°56′2.24″ O) and southern Bolivia (Entre Ríos 21° 17′44.07″ S, 63° 56′54.36″ O). Both populations have demonstrated high levels of deltamethrin resistance during the embryonic and postembryonic development. The resistant ratios estimated for the Argentinean population were 1,144, 1,193, and 822 in eggs of 4-, 7-, and 12 d old, and 1,108 in neonate larvae. For the Bolivian population, estimated resistance levels were 21, 15, and 39 in eggs of 4-, 7-, and 12 d old, and 173 in neonate larvae (Roca-Acevedo et al. 2013).

An insecticide-susceptible colony of T. infestans was obtained from descendants of the insects provided by

the laboratory of the Coordinación Nacional de Control de Vectores (Punilla, Córdoba).

The insects were kept at $28 \pm 2°$C with a relative humidity of 50–70% and photoperiod of 12:12 (L: D) h. All insects used in this study were weekly fed on pigeons.

**Chemicals.** 7-Ethoxycoumarin (7-EC) and 7-OHC (umbelliferone) were from Sigma-Aldrich (St. Louis, MO). Cis-trans (43.8% cis; 56.2% trans)-permethrinic acid was supplied from Chemotecnica (Buenos Aires, Argentina), and thionyl chloride ($Cl_2SO$; 99%) and triethylamina (99%) were purchased from Aldrich Chemical (Milwaukee, WI). 7-Coumaryl permethrate (7-CP) was synthesized in our laboratory according to the method described by Santo-Orihuela et al. (2006).

**Biochemical Assays.** For biochemical studies, eggs of 4-, 7- and 12 d old were selected based on the external morphological characteristics, as described by Picollo Villar et al. (1979). Briefly, 4-d-old eggs were white with a mild depression in the center; 7-d-old eggs were slightly more orange, with the posterior section clearer and two reddish eyespots, 12-d-old eggs were orange with dark eye spots.

**Cytochrome P450 Monooxygenase.** Activity was measured using 7-EC as substrate, according to the direct fluorometric test method for individual abdomens of T. infestans previously reported (Santo-Orihuela et al. 2008). Fluorescence of 7-hidroxycoumarin (7-OHC) was determined using micro-plate fluorescence reader (Fluoroskan Ascent, Thermo Scientific, Helsinki, Finland)), with 390-nm excitation and 440-nm emission filters.

Each 4-, 7- and 12-d-old egg was cut into two halves by a cross cut made with entomological scissors (vannas 8,5 cm SRT, WPI, FL) under stereoscopic microscope (OLYMPUS SZ61). Each egg was placed individually into wells of a 96-well micro-plate containing 100 µl of 0.05 M phosphate buffer and 4 mM 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, micro-plates were centrifuged at $2,000 \times g$ for 30 s in a refrigerated centrifuge for micro-plates (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.

**Esterase Activity.** Esterase activity was determined by the hydrolysis of 7-Coumaryl (1R, S)-cis-trans-3-(2,2-dichlorovinyl)-2,2-imethylcyclopropanecarboxylate (7-CP), a fluorescent substrate appropriate for determining pyrethroid hydrolysis activity on individual insects (Santo-Orihuela et al. 2006). This substrate shows a structure very similar to permethrin insecticide and yield fluorescent products on hydrolysis. It was synthesized by reacting the (1R, S)-cis-trans-3-(2, 2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate acid chloride (1.5021 g) with 7-OHC (0.7565 g; 55°C, 48 h) using toluene as solvent and triethylamine as catalyst.

Reaction progress was monitored using thin-layer chromatography (TLC) with silica gel 60 F254

(Merck, Germany), 0.25 mm, and methylene chloride. Structural analysis was performed by gas chromatography–mass spectrometry on a Shimadzu MS-Q P5050A in electron impact (IE) mode; the samples were analyzed on a nonpolar column (15 m by 0.25 μm film thickness, DB5-MS, Agilent, Folsom, CA). The gas chromatography column was held at 70°C for 2 min, the temperature was programmed at 10°C/min to 280°C and held for 5 min; the carrier gas was helium with a head column pressure of $14 \times 10^3$ Pa. $^1$H NMR and $^{13}$C NMR analyses were performed in a Bruker Advance DPX 400 spectrometer using deuterochloroform as solvent.

Measurement of enzyme activity was performed by cooling each 4-, 7-, and 12-d-old eggs and homogenizing it in 190 μl of phosphate buffer (0.05 M, pH 7.2), using a bio-vortexer (Biospec, Bartlesville, OK), disposable plastic pestles and a 1.5-ml reaction tube. Reaction was initiated by adding 10 μl of 7-CP (3.5 mM, 2-methoxy ethanol) to 190 μl of each homogenate. Incubation was performed at 25°C for 30 min, at pH 7.2. Fluorescence was measured using a micro-plate fluorescence reader (Fluoroskan Ascent, Thermo Scientific, Vantaa, Finland), and results were analyzed with Ascent Software 2.6 (Thermo Scientific, Vantaa, Finland) and the Excel 2010 (Microsoft, Redmond, WA) software. Assays were conducted in black 96-well polystyrene flat-bottomed micro-titer plates (PerkinElmer Life and Analytical Sciences) at 25°C. Production of 7-OHC was monitored with excitation wavelength at 392 nm and emission at 440 nm, automatic instrument gain and light intensity. Activity was measured every 3 min for 30 min, with the assay being linear over this time (Santo Orihuela et al. 2013).

The relative fluorescence units were corrected for background hydrolysis and nonspecific substrate fluorescence and then transformed to picomol 7-OHC/min (activity units) using a calibration curve per replicate with dilutions of 7-OHC (15.42, 154.19, 308.38, 616.75, 1233.50 and 1850.25 total picomol per well).

The biochemical data were plotted as the individual pyrethroid-esterase activities of 4-, 7-, and 12-d-old eggs from the three studied populations. Nonparametric Kruskal–Wallis analysis of variance was used to compare the values of enzymatic activity per minute among the days of development and the studied populations.

**Molecular Analysis of Susceptible and Resistant Developing Eggs.** We analyzed two point mutations in the para-type sodium channel that were previously reported, as associated with pyrethroid resistance in adults of *T. infestans* (L1014F and L925I mutations; Fabro et al. 2012, Capriotti et al. 2014). The methodologies described by these authors were adapted to different embryonic stages. Genomic DNA was extracted from groups of 10 eggs from each susceptible (S) and resistant (R) population with a commercial kit following the manufacturer's instructions (Promega, Madison, WI). Fragments of the sodium channel gene were PCR amplified using pooled genome from S or R *T. infestans* as templates.

The presence of L1014 mutation was analyzed with the PCR amplification of specific alleles (PASA) assay (Fabro et al. 2012), which shows the presence or absence of the mutation in an agarose gel.

The presence of L925I mutation was analyzed according to Capriotti et al. (2014), which shows the presence or absence of the mutation in an agarose gel after an enzymatic digestion.

## Results

**Synthesis of Substrate.** Cis-trans-7-CP was synthesized in this work as specific substrates of pyrethroid esterase enzymes. Total yields ranged from 50 to 70%.

**Biochemical Assays.** A significant increase in the level of pyrethroid esterase was observed during embryo development in reference and both resistant populations. Moreover, a significant increase of pyrethroid-esterase activity was observed in both resistant populations compared with the susceptible reference (Fig. 1).

Mean enzyme activity increased from 13.6 to 16.3 and 22.2 picomol 7-OHC/min in eggs of 4-, 7-, and 12-d-old from the reference population. Mean activity of resistant populations increased from 16.0 to 25.9 picomol 7-OHC/min in eggs of 4- to 12-d-old in Entre Ríos population, and from 15.9 to 28.9 picomol 7-OHC/min in Campo Largo population (Table 1). Statistical analysis showed significant increase in the activity of pyrethroid-esterase enzymes throughout embryonic development in both resistant populations compared with the reference population. The highest value of enzyme activity was found in 12-d-old eggs of Campo Largo (28.9 picomol 7-OHC/min) when compared to the susceptible population. However, no significant difference was detected between the two resistant populations throughout embryonic development (Table 1).

For cytochrome P-450 monooxigenase there were no significant differences in the embryonic development of the studied populations, except between the 4- and 7 d olds of the Entre Ríos population. The mean enzyme activity was 12.9–13.7 and 17.2 picomol 7-OHC/min in 4-, 7-, and 12-d-old eggs of the susceptible reference. Moreover, no significant increase in monooxigenase activity was observed in resistant populations compared with the susceptible reference (Table 2).

**Knockdown resistance (kdr) Assays.** Sequence analysis of susceptible and resistant eggs reveals that all the sequenced clones from the susceptible strain displayed the wild-type sequence (476 bp). On the contrary, in all the sequenced clones of eggs corresponding to Entre Ríos and Campo Largo-resistant populations, a mutation consisting of a nucleotidic substitution of an adenine (t**t**a) to a thymine (t**t**t), leucine to phenylalanine, was present (130 bp; Fig. 2). This mutation has been identified in several insect species, including *T. infestans*, as an L1014F kdr mutation (Fabro et al. 2012).

The results of the assay for the detection of L925I mutation in eggs are shown in Fig. 3. According to bibliography, when the mutant allele is present, the
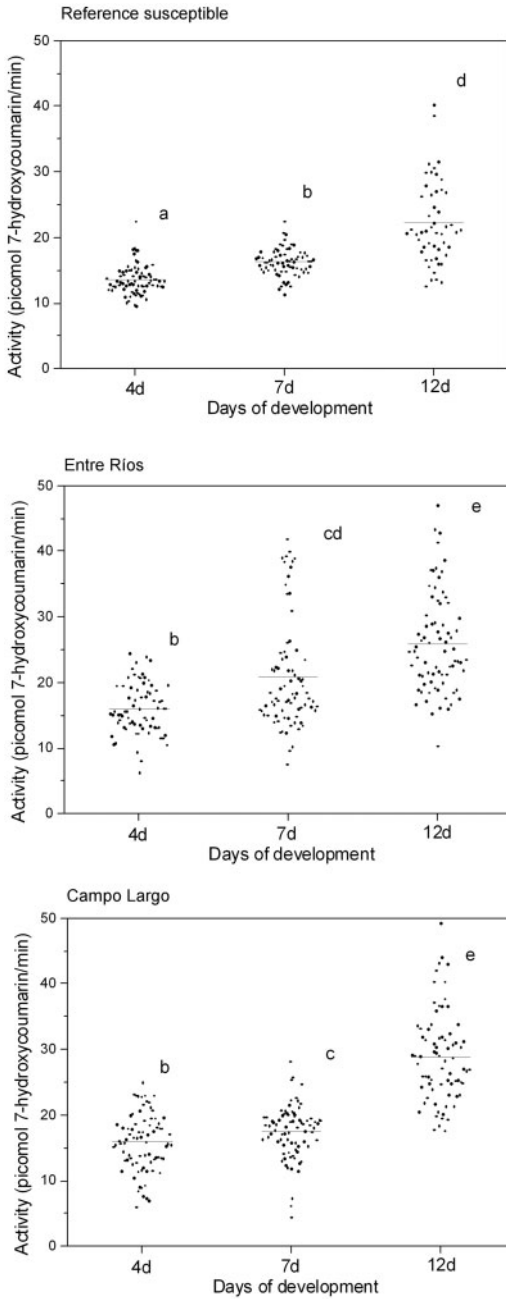
**Fig. 1.** Levels of pyrethroid esterase activity measured in developing eggs of susceptible and resistant *T. infestans* populations. Spots represent each individual activity (picomol 7-OHC/min). Horizontal line represents mean value. Different letters indicate statistically significant differences ($P < 0.05$).

**Table 1. Comparison of average values for pyrethroid-esterase activity between 4-, 7-, and 12-d-old eggs of susceptible and pyrethroid-resistant *T. infestans***

| Population | Age (days) | $n$ | Means (D.E) |
|---|---|---|---|
| Reference | 4 | 77 | 13.60a (2.20) |
| | 7 | 70 | 16.27b (2.03) |
| | 12 | 51 | 22.21d (6.22) |
| Campo Largo | 4 | 79 | 16.04b (4.24) |
| | 7 | 77 | 17.98c (3.34) |
| | 12 | 76 | 28.87e (6.80) |
| Entre Ríos | 4 | 71 | 16.17b (3.73) |
| | 7 | 74 | 21.44cd (8.15) |
| | 12 | 75 | 26.14e (7.33) |

Activity was measured using 7-Coumaryl permethrate (7-CP) as substrate in individual assays using 4-, 7-, or 12 d old of susceptible and resistant populations. Enzyme activity expressed as picomol 7-OHC/min.

Means with different letter are significantly different ($P < 0.05$).

**Table 2. Comparison of average values for P-450-monooxigenase activity between 4d-, 7d-, and 12-d-old eggs of susceptible and pyrethroid-resistant *T. infestans***

| Population | Age (days) | Mean activities (D.E.) |
|---|---|---|
| Reference | 4 | 12.91ab (9.41) |
| | 7 | 13.71ab (12.49) |
| | 12 | 17.21bc (15.00) |
| Campo Largo | 4 | 15.30abc (15.94) |
| | 7 | 20.65c (13.26) |
| | 12 | 14.02bc (9.80) |
| Entre Ríos | 4 | 12.17a (16.17) |
| | 7 | 15.76bc (15.19) |
| | 12 | 13.75ab (13.73) |

Activity was measured using 7-EC as substrate in individual assays using 4-, 7-, or 12 d old of susceptible and resistant populations. Enzyme activity expressed as picomol 7-OHC/min.

Means with different letter are significantly different ($P < 0.05$).
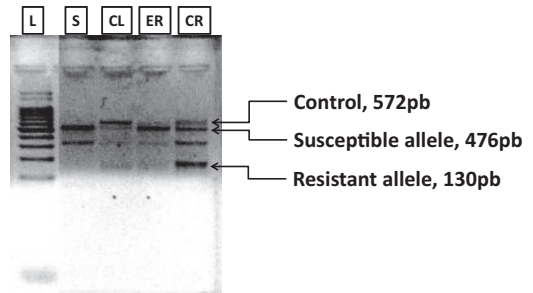


**Fig. 2.** PASA reaction products for the detection of resistant mutation in *T. infestans* eggs. The 572-bp control fragment is amplified in both reactions. The 476-bp fragment is selectively amplified when susceptible allele is present. The 130-bp fragment is amplified when the resistant allele is present. DNA ladder (L), susceptible reference population (S), Campo Largo (CL), Entre Ríos (ER), and control resistant with L1014F mutation (CR).

nucleotidic sequence changes from an adenine to a cytosine, which suppresses the restriction site for Sac I enzyme, then only one fragment is present in the agarose gel (572 bp). If the susceptible allele is present, two fragments will appear, although only the bigger one can be seen (521 bp + 51 bp). Moreover, different bands will be seen before and after digestion (Capriotti et al. 2014; Fig. 3).

According to the results, no mutation was observed at the 925 codon that is often associated with resistance in other insects and *T. infestans*.
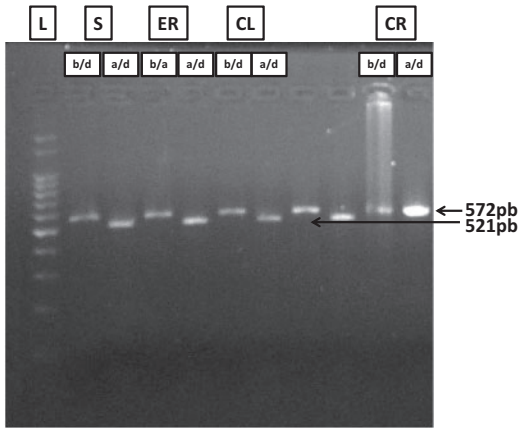
**Fig. 3.** L925I mutation gel results. First line from left to right: DNA Ladder (L), susceptible reference population (S), Entre Ríos (ER), Campo Largo (CL), and control resistant with L925I mutation (CR). Second line indicates before (b/d) and after (a/d) digestion; 521 pb band after digestion means L925I mutation is not present, 572 pb after digestion means mutation is present.

## Discussion

Resistance to pyrethroid insecticides was firstly identified in nymphs of *T. infestans* from three areas of Argentina and Bolivia (Picollo et al. 2005, Germano et al. 2012). Subsequently, it was found that the oldest eggs of those populations were also resistant (Toloza et al. 2008). Recently, it was shown that pyrethroid resistance extends to developing eggs (Roca-Acevedo et al. 2013, Santo Orihuela et al. 2013). The present study reports the first investigation on the potential resistance mechanisms throughout the embryonic development in two *T. infestans* field populations from the resistant areas of Argentina and Bolivia.

Esterases and cytochrome P450 monooxygenases are the major metabolic mechanisms responsible for pyrethroid resistance in insects (Yang et al. 2004). In this study, we found measurable activity of both degrading enzyme complexes throughout embryonic development.

The pyrethroid esterase activity grew steadily continuously throughout development in all the studied populations and was highest in 12-d-old eggs. These results are consistent with the lower susceptibility to deltamethrin reported for late eggs (12-d-old) compared with early (4 d old) and middle (7 d old) development eggs (Roca-Acevedo el at 2013). A decrease in susceptibility to various pyrethroid insecticides was also reported for eggs of the spotter tentiform leaf miner *P. blancardella* (Marshall and Pree 1986). Studies made on this moth demonstrated that eggs treated 0–2 d postoviposition were more susceptible than 4- to 6-d-old eggs.

The increased level of pyrethroid-esterase activity found in eggs of Entre Ríos y Campo Largo populations suggested that metabolic resistance is involved in the resistance in both field populations. Instead, similar activity of monooxygenases in susceptible and resistant populations indicates that there are no correlation between the activity of these enzymes and resistance to deltamethrin in eggs.

Similarly, a previous study made on first nymphs demonstrated the contribution of esterases (and not monooxigenases) in pyrethroid resistance in *T. infestans* from two geographical regions in Argentina (Salta and La Rioja; Santo Orihuela et al. 2008). In that study, an increase in the percentage of insects with higher pyrethroid-esterase activity was found in first nymphs of the Argentinean populations, but unexpectedly, a lower pyrethroid esterase activity compared with the reference strain was shown in a Bolivian population (Yacuiba). Later, these authors reported a slight but not significant difference in the activity of pyrethroid esterases in old eggs (12-d-old) of a deltamethrin-resistant population of Bolivia (El Palmar; Santo-Orihuela et al. 2013).

Our studies on developing eggs indicate that metabolic resistance mediated by esterases is involved in the resistance to pyrethroids demonstrated throughout the embryonic development of both the Argentinean (Campo Largo) and the Bolivian (Entre Ríos) population.

The mechanism of resistance observed in developing eggs was also explored through the analysis of resistance-conferring mutations. Pyrethroids exert their insecticidal action on the insect nervous system by modifying the normal function of voltage-gated sodium channels in the membrane of excitable cells. Kdr is the reduction in the sensitivity to pyrethroids caused by point mutations in the sodium channel gene (Soderlund, 2008). This mechanism of resistance has been reported in several insect pests of economic and sanitary interest (Soderlund and Knipple, 2003), and recently, L1014F and L925I mutation associated with pyrethroid resistance has been reported for triatomines (Fabro et al. 2012, Capriotti et al. 2014). In this study, we detected the L1014F mutation in both resistant populations, but no L925I kdr mutation was found in any of the studied populations. These findings are consistent with previous studies that reported the presence of the L1014 mutation in a field population from the same geographical area to the populations herein studied.

In fact, three resistant profiles were described for *T. infestans* populations of different geographical areas according to toxicological characteristics (Germano et al. 2012). The resistance profiles were identified as Ti-R1, Ti-R2, and Ti-R3, corresponding to the Argentinean Acambuco, and the Bolivians Entre Ríos and Mataral, as described in the introduction.

The two populations used in this study were collected in the area of the profile T1R1, which also belongs to the population on which the L1014F mutation has been identified (Fabro et al. 2012). In contrast, the L925I mutation was reported for *T. infestans* from a new pyrethroid-resistant area (other than those where the resistance profiles were described) in the Province of Chaco, Argentina (Germano et al. 2013). The L925I has been found only in hemipteran species: the silverleaf whitefly *Bemisia tabaci* (Gennadius)

(Morin et al. 2002), the common bed bug *Cimex lectularius* L. (Yoon et al. 2008), and the greenhouse whitefly *Trialeurodes vaporariorum* Westwood (Karatolos 2012). In the three species, populations carrying L925I mutation present levels of resistance >100, as is the case in *T. infestans* from Argentina.

Higher esterase activity and L1014F presence justify the resistance to pyrethroid in developing eggs of both studied *T. infestans* populations, but does not explain the difference in the resistance ratios estimated for the Argentinean population (1,144, 1,193, and 822 in eggs of 4-, 7-, and 12 d old) compared with the Bolivian population (21, 15, and 39). Probably, the highest level of resistance of Campo Largo is because of a higher proportion of resistant individuals in the population. Also, other mechanisms of resistance such as lower rate of penetration through cuticle will be checked. The description of resistance profiles including resistance mechanisms involved will allow a rational design of campaigns for the control of Chagas' Disease transmission.

## Acknowledgments

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