Analysing deltamethrin susceptibility and pyrethroid esterase activity variations in sylvatic and domestic *Triatoma infestans* at the embryonic stage

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The aim of the present work was to study the deltamethrin susceptibility of eggs from Triatoma infestans *populations and the contribution of pyrethroid esterases to deltamethrin degradation. Insects were collected from sylvatic areas, including Veinte de Octubre and Kirus-Mayu (Bolivia) and from domiciliary areas, including El Palmar (Bolivia) and La Pista (Argentina). Deltamethrin susceptibility was determined by dose-response bioassays. Serial dilutions of deltamethrin (0.0005-1 mg/mL) were topically applied to 12-day-old eggs. Samples from El Palmar had the highest lethal dose ratio (LDR) value (44.90) compared to the susceptible reference strain (NFS), whereas the Veinte de Octubre samples had the lowest value (0.50). Pyrethroid esterases were evaluated using 7-coumaryl permethrate (7-CP) on individually homogenised eggs from each population and from NFS. The El Palmar and La Pista samples contained 40.11 and 36.64 pmol/min/mg protein, respectively, and these values were statistically similar to NFS (34.92 pmol/min/mg protein) and different from Kirus-Mayu and Veinte de Octubre (27.49 and 22.69 pmol/min/mg protein, respectively). The toxicological data indicate that the domestic populations were resistant to deltamethrin, but no statistical contribution of 7-CP esterases was observed. The sylvatic populations had similar LDR values to NFS, but lower 7-CP esterase activities. Moreover, this is the first study of the pyrethroid esterases on* T. infestans *eggs employing a specific substrate (7-CP).*

Key words: *Triatoma infestans* - egg - pyrethroid insecticides - pyrethroid esterases

Triatoma infestans (Klug 1834) (Hemiptera, Reduviidae, Triatominae) is the main vector of *Trypanosoma cruzi*, which is the parasite responsible for causing Chagas disease. At present, this endemic infection affects approximately nine million people in Argentina and Bolivia (Schofield et al. 2006). *T. infestans* is a haematophagous insect that originally lived in natural foci, but started to colonise houses several thousand years ago. Rural and rustic dwellings provide shelter and food sources for these insects, allowing for the formation of intradomiciliary colonies (Dias & Schofield 2007). This type of *T. infestans* colony has been successfully controlled in the southern cone of Latin America by spraying houses with pyrethroid insecticides (Dias et al. 2002, Schofield et al. 2006). However, several areas in the Gran Chaco of Argentina, Bolivia and Paraguay have been targeted with intensive vector control efforts without success (Gurtler et al. 2007). Several authors have demonstrated that insects from this region have developed resistance to pyrethroid insecticides (Picollo et al. 2005, Santo-Orihuela et al. 2008, Toloza et al. 2008, Germano et al. 2010, Lardeux et al. 2010, Santo-Orihuela & Picollo 2011).

Moreover, a study by Alarico et al. (2010) demonstrated deltamethrin-resistant *T. infestans* populations from this region were susceptible to a micro-encapsulated formulation containing organophosphate insecticides. The development of resistance in this and other regions has led investigators to evaluate the toxicity of non-pyrethroid insecticides against *T. infestans* (Carvajal et al. 2012). Most of these studies were conducted in *T. infestans* populations from intradomiciliary environments. In addition, some sylvatic populations studied by Lardeux et al. (2010) and Depickère et al. (2012) demonstrated deltamethrin toxicity based on toxicological analyses of topical insecticide applications. Roca-Acevedo et al. (2011) recently focused on the relevance of cytochrome P450 monooxygenases and pyrethroid esterases in first instar nymphs, while Santo-Orihuela et al. (2013) studied the detoxification activity of glutathione transferases on organophosphate insecticides applied to sylvatic *T. infestans* instar nymphs from Bolivia.

Previous studies have suggested that sylvatic populations are very important because of their possible role in resettling intradomiciliary environments. In addition, several reports have shown that these populations are more widely distributed than previously estimated (Noireau et al. 2005, Noireau 2009, Buitrago et al. 2010, Waleckx et al. 2012).

The possible development of resistance during the egg stage may contribute to failed control measures and should not be underestimated. Earlier studies demonstrated differences in the expression of resistance in eggs from pyrethroid-resistant populations (Toloza et al.

2008). These authors evaluated the resistance of more developed (12-day-old) eggs compared to other insecticide effect studies of *T. infestans* eggs (Toloza et al. 2008, Visciarelli et al. 2011).

However, no investigators have analysed the pyrethroid susceptibility of eggs from sylvatic *T. infestans* populations, despite their possible importance in the recolonisation of intradomiciliary areas. Moreover, no previous studies on the contribution of pyrethroid esterases to pyrethroid susceptibility have been conducted in *T. infestans* eggs.

The aim of the present work was to study the susceptibility of eggs to deltamethrin and the contribution of pyrethroid esterases to deltamethrin degradation in sylvatic and domestic *T. infestans* populations.

MATERIALS AND METHODS

Insects - *T. infestans* were captured from domiciliary (-D) and sylvatic (-S) areas of Bolivia (department of Cochabamba, Viente de Octubre-S, department of Potosí, Kirus-Mayu-S and department of Tarija, El Palmar-D) and from Argentina (Salta province, La Pista-D) in 2009. Detailed geographic locations of the captured populations are shown in Table I.

Sylvatic *T. infestans* were captured from rock piles using mouse-baited sticky traps (Noireau et al. 1999). The insects were then reared in Bolivia. Eggs from the descendent populations were transported to the Research Center and Insect Pests (CIPEIN), Buenos Aires, Argentina, where subsequent generations were bred.

A susceptible reference strain (NFS) was derived from a domestic population captured in 2004 from Santiago del Estero, Argentina, in an area where insects were later successfully controlled with the pyrethroid insecticide deltamethrin. The insects from each population were reared in boxes at 28ºC, 50-60% relative humidity (RH) and a photoperiod of 12:12 (L:D) h. One pigeon was provided each week to meet insect blood requirements (WHO 1994).

Chemicals - Technical grade deltamethrin (99%) was purchased from Ehrestorfer (Augsburg, Germany). Analytical grade acetone was purchased from JT Baker (Estado de Mexico, Mexico). 7-hydroxycoumarin (7-OHC) (umbelliferone) was purchased from Sigma-Aldrich Co (St. Louis, MO, USA). Permethrinic acid [(1R, S)-*cis*-

(98.7% *cis*, 0.9% *trans*)] was supplied by Chemotecnica (Buenos Aires, Argentina) and the thionyl chloride (Cl_2SO, 99%) and triethylamine (99%) were purchased from Aldrich Chemical (Milwaukee, WI, USA). 7-coumaryl permethrate (7-CP) was synthesised in the CIPEIN using the method of Santo-Orihuela et al. (2006).

Topical application bioassays - Twelve-day-old second generation (F2) eggs were collected from adult rearing boxes and selected according to external morphological characteristics (orange coloured eggs and dark eye spots) as described by de Villar et al. (1979).

Groups of at least 10 eggs per concentration were fixed to a microscope slide with double-sided adhesive tape. Egg treatments were conducted individually using topical applications to the operculum of 0.2 µL of serial deltamethrin dilutions in acetone using a 10 µL Hamilton syringe according to the method reported by Picollo et al. (1976). Final concentrations ranged from 0.0005-1 mg/mL deltamethrin diluted in acetone. At least four different concentrations producing between 10-90% mortality were used for each test. Each concentration, including the acetone only control (without insecticide), was replicated three times.

Following the topical application, eggs were incubated in a rearing cabinet (FOC-225E, Velp Scientifica, Milan, Italy) at 28ºC, 50% RH and a photoperiod of 12:12 (L:D) h. The estimated time for hatching control eggs under these laboratory conditions was 15 days. Mortality data were recorded six days after the topical application (3 days after the control eggs hatched).

Esterase activity - Pyrethroid esterase activity was determined by measuring the hydrolysis of 7-CP, a fluorescent substrate used to determine pyrethroid hydrolysis activity in individual insects (Santo-Orihuela et al. 2006, 2008). Eggs were collected and selected using the same criteria as those used in the bioassays. The eggs were cooled and each egg was homogenised in 220 µL of phosphate buffer (0.05 M, pH 7.2) using a plastic mortar and pestle. Because the 7-CP pyrethroid esterase method was conducted with the entire volume of each individual insect homogenate, these tests could not be replicated. The reaction was initiated by adding 10 µL of 7-CP (in 3.5 mM 2-methoxyethanol) to 190 µL of each homogenate. Incubation was conducted at 25ºC for 33

TABLE I

Sylvatic (-S) and domiciliary (-D) populations
of *Triatoma infestans* analysed according to the collecting site in Bolivia and Argentina

Site of collection	Location (locality/country)	Latitude/longitude
Kirus-Mayu-S	Toro Toro/Potosí/Bolivia	17º59'S 65º50'W
Veinte de Octubre-S	Cochabamba/Cochabamba/Bolivia	17º29'S 66º07'W
El Palmar-D	El Palmar/Tarija/Bolivia	17º02'S 64º12'W
La Pista-D	Salvador Mazza/Salta/Argentina	22º4'S 63º41'W
NFS	Susceptible reference strain/Argentina	-

min at pH 7.2. The fluorescence was measured in a Fluoroskan Ascent Microplate Fluorometer (Thermo Scientific, Helsinki, Finland) and the results were analysed with Ascent (Thermo Scientific) and Microsoft Excel 2010 (Microsoft). Assays were conducted in black 96-well polystyrene flat-bottomed microtitre plates (Perkin Elmer Life and Analytical Sciences) at 25ºC. 7-OHC production was monitored at an excitation wavelength of 390 nm and an emission wavelength of 440 nm; 7-CP pyrethroid esterase activity was measured 3 min for 33 min. The relative fluorescence units (RFU) were corrected for background hydrolysis and nonspecific substrate fluorescence and then transformed to picomoles/min (activity units) using one calibration curve per replicate with dilutions of 7-OHC (68.5, 342.69, 685.44 and 1370.8 total picomoles/well).

Insect protein concentrations were quantified with a protein kit (Total Protein Kit, Sigma) based on the Bradford (1976) assay. The absorbance of the wells was determined using a spectrophotometric microplate reader equipped with 340 nm, 405 nm, 415 nm, 540 nm, 595 nm and 655 nm wavelength filters (Model 680, Bio-Rad Laboratories, Inc). Microplate Manager Software v. 5.2.1 (Bio-Rad Laboratories, Inc) was used to collect, analyse and output absorbance data from the Bio-Rad microplate readers.

Statistical analysis - Mortality data were processed with POLO Plus (LeOra Software 1987). Data from each *T. infestans* population were corrected using Abbott's formula (Abbott 1987) and were then pooled and analysed based on probit analysis (Litchfield Jr & Wilcoxon 1949) to estimate the lethal dose (LD) (nanograms of insecticide per egg) that killed 50% of treated individuals (LD_{50}). LD ratios (LDRs) and 95% confidence intervals (CI) for each population were calculated according to Robertson et al. (2007) by comparing the dose-response curves between studied populations and the reference strain NFS. Studied populations were considered different from the reference strain according to the criteria of Robertson et al. (2007) (Russell et al. 1977). According to these criteria, the LDR 95% CI did not include the number 1.0.

The pyrethroid esterase activity values of eggs from different populations were expressed as picomoles of 7-OHC pmol/min/mg protein and these values were plotted on a scatter graph (Montella et al. 2007). Statistical analysis was performed using InStat v. 3.01 (Graphpad Software, San Diego, CA, USA). An ANOVA test was used to compare protein amounts among study populations and non-parametric Kruskal-Wallis and Dunn tests were used to compare 7-CP enzymatic activities per minute and per mg of protein among populations.

RESULTS

Figure and Table II show the toxicity of deltamethrin against 12-day-old *T. infestans* eggs from Bolivia and Argentina. The Bolivian population El Palmar-D showed the highest LD_{50} and LDR values (27.80 ng/egg and 44.90, respectively), whereas Veinte de Octubre-S had the lowest values (0.31 ng/egg and 0.50, respectively).

The egg protein content results and statistical analyses from the study populations are shown in Table III.

The sylvatic populations, Kirus-Mayu-S and Veinte de Octubre-S, had higher protein contents than the domestic populations, El Palmar-D and La Pista-D and the reference strain (NFS). For this reason, all 7-CP activity values were corrected according to the mean protein content of the respective population and expressed per mg of protein.

The domestic populations El Palmar-D and La Pista-D from Bolivia and Argentina, respectively, had 7-CP activity values (40.11 and 36.64 pmol/min/mg protein, respectively) similar to the values for reference strain NFS (34.92 pmol/min/mg protein) and were significantly different from the sylvatic populations Kirus-Mayu-S and Veinte de Octubre-S (27.49 and 22.69 pmol/min/mg protein, respectively). These data are shown with their standard deviations and statistical analyses in Table III.

DISCUSSION

This study was the first analysis of pyrethroid esterase activities in eggs from sylvatic *T. infestans* using a specific substrate, namely, 7-CP (Santo-Orihuela et al. 2006). Previous studies have demonstrated the importance of sylvatic *T. infestans* and its possible role in recolonising insecticide-treated houses (Noireau 2009, Waleckx et al. 2012).

Most insecticides used in control campaigns are directed at larval and adult stages, but eggs are also subject to selection pressure. Therefore, it is relevant to evaluate the susceptibility of sylvatic *T. infestans* eggs.

In a previous study, Roca-Acevedo et al. (2011) analysed the toxicological profile of sylvatic *T. infestans* and found a slight increase in the LD_{50} and LDR for deltamethrin in the first instars of sylvatic populations in contrast with the reference strain, but no statistical evidence of a detoxifying enzyme contribution was reported.

In the present study, the eggs from domestic populations showed higher LDRs than the reference strain, indicating the development of embryonic resistance in these Bolivian and Argentine populations. These toxicological findings for eggs were in accordance with instar results demonstrated by Germano et al. (2010). These data indicate that eggs from domestic populations were resistant to deltamethrin, but no statistical contribution from 7-CP esterases was observed. However, deltamethrin resistance may be attributed to a reduced nerve sensitivity caused by a change in the action site (i.e., kdr and sodium channels) (Soderlund & Knipple 2003, Fabro et al. 2012). The studied sylvatic populations showed LD_{50} and LDR values similar to the reference strain, but with higher protein contents and lower pyrethroid esterase activities; the increased protein content may be caused by differences in food sources and hosts under sylvatic conditions (Noireau et al. 2005, Cortez et al. 2007, Alvarado-Otegui et al. 2012). The toxicological pattern of sylvatic eggs in this study was very similar to the pattern of sylvatic instars described by Roca-Acevedo et al. (2011). The eggs in the present study had decreased 7-CP esterase activity in contrast with the reference strain and domestic population and a lower LDR than instars from previous studies (Roca-Acevedo et al. 2011). Toloza et al. (2008) demonstrated the existence of deltamethrin-
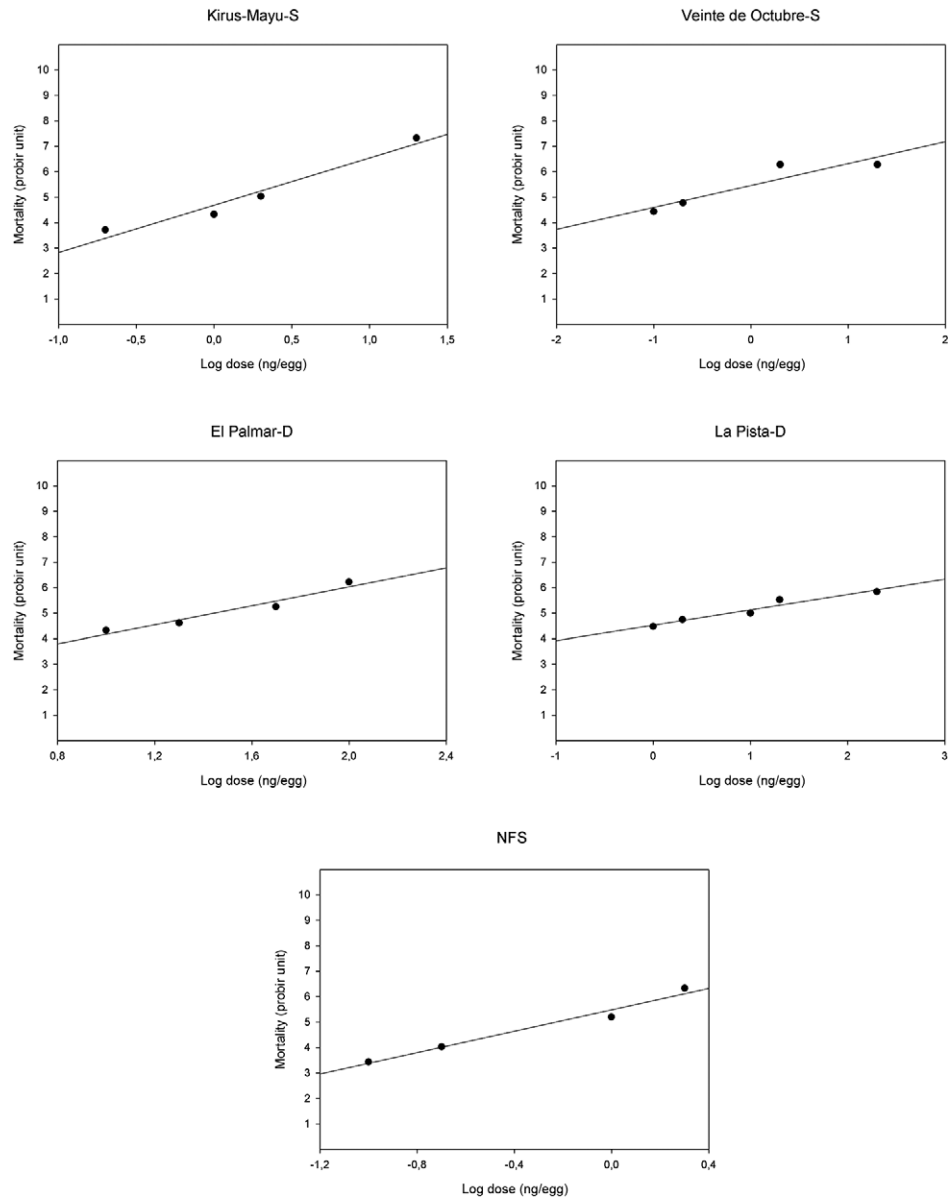
Graphics show mortality to deltamethrin (probit units) of eggs vs. logarithm of applied doses (nanograms per egg) for all studied *Triatoma infestans* populations and reference strain (NFS).

susceptible eggs and resistant first instar nymphs in domestic *T. infestans* populations from Sucre and Mataral (Bolivia). These authors attributed this phenomenon to an insecticide selection response to different field insecticide exposures and/or biological variations between Bolivian and Argentinean populations. Conversely, these sylvatic population findings might be explained by probable contact between sylvatic and domestic populations in neighbouring areas (Depickère et al. 2012).

One of the sylvatic populations studied in this work (Kirus-Mayu-S) showed a slight increase in LDR, suggesting a lower susceptibility to deltamethrin. This finding may be explained by the existence of naturally decreased susceptibility by the development of resistance resulting from exposure to insecticides used in crops

and vector control campaigns or by contact and probable gene flow between geographically close sylvatic and domestic resistant populations. Additionally, a combination of these hypotheses may be possible (Lardeux et al. 2010, Depickère et al. 2012, Santo-Orihuela et al. 2013).

Based on the identification of different toxicological profiles according to different geographic areas for sylvatic and domestic *T. infestans* (Roca-Acevedo et al. 2011, Germano et al. 2012), it is possible to geographically define wild or domestic toxicological profiles. Although previous studies have suggested that the Andean valley in Bolivia represents the centre of origin and dispersal for *T. infestans* (Bargues et al. 2006, Cortez et al. 2010), an ancestral toxicological profile from that area may be unlikely. If this profile existed, it would be locat-

TABLE II

Toxicity of deltamethrin against *Triatoma infestans* 12-days-old eggs from Bolivia and Argentina

Population	n	Slope ± SE	χ2	df	LD_{50} (ng per egg) (95% CI)	LDR (95% CI)
Kirus-Mayu-S	107	1.79 ± 0.48	1.62	2	1.90 (1.25-3.36)	3.02 (1.76-5.21)
Veinte de Octubre-S	74	0.92 ± 0.29	1.61	2	0.31 (0.06-0.94)	0.50 (0.15-1.60)
El Palmar-D	88	1.84 ± 0.45	0.95	2	27.80 (16.00-44.80)	44.90 (25.60-77.20)
La Pista-D	80	0.76 ± 0.20	2.56	4	6.80 (2.69-24.00)	8.10 (1.02-26.37)
NFS	88	2.07 ± 0.35	1.26	2	0.61 (0.43-0.91)	-

CI: confidence interval [calculated following Robertson et al. (2007)]; -D: domestic population; df: degrees of freedom; LD: lethal dose; LDR: LD ratios; -S: sylvatic population; SE: standard error; χ2: chi-square. χ2 for probit analysis tests goodness-of-fit, in this case the values (responses) predicted by the statistical model adequately fit the data observed in the bioassay ($p > 0.05$).

TABLE III

Means and standard deviations (SD) of protein content and activities of 7-coumaryl permethrate (7-CP) esterases

Strain/population	n^a	Mean protein content ± SD (μg per insect)	n^b	7-CP activity ± SD (pmol/min/mg protein)
Kirus-Mayu-S	26	400.82 (± 197.86)[d]	22	27.49 (± 9.83)[c,d]
Veinte de Octubre-S	43	350.83 (± 131.85)[d]	43	22.69 (± 9.41)[c]
El Palmar-D	26	289.91 (± 156.75)[c]	28	40.11 (± 10.74)[e]
La Pista-D	35	223.22 (± 96.16)[c]	35	36.64 (± 14.93)[e]
NFS	24	265.26 (± 118.42)[c]	32	34.92 (± 13.67)[d,e]

a: number of eggs used from studied populations in protein assay; *b*: number of eggs used from studied populations in 7-CP activity determination. Means of protein content followed by different letters (*c-e*) are significantly different ($p < 0.05$). Tukey-Kramer: F = 3.71. Values of activities of 7-CP esterases followed by different letters (*c-e*) are significantly different ($p < 0.05$) [Kruskal Wallis (KW) and Dunn's Multiple Comparisons Test]. KW statistic = 42.45. SD: standard deviation.

ed in completely sylvatic areas, far from human activities. To date, the studied sylvatic populations have not been situated far from human influence and these populations may be under insecticide pressure or in contact with domestic *T. infestans* during their life cycle.

Although nonspecific esterases and pyrethroid esterases are likely to play a physiological role during the embryonic development of *T. infestans*, no studies have analysed this topic. Moreover, few reports have determined the occurrence of cholinesterases during egg neurogenesis in *T. infestans* and *Triatoma patagonica* (de Villar et al. 1979, Visciarelli et al. 2011). de Villar et al. (1980) and Wood et al. (1984) analysed the effects of parathion on cholinesterases and eserine-resistant esterases and the influence of parathion, malathion and fenitrothion on carboxylesterases in the developing embryo of *T. infestans*, respectively. These authors reported daily increases in enzymatic activities during embryonic development. For this reason, 7-CP activity comparisons among studied populations and the reference strain were conducted on the same day of embryonic development (12-day-old eggs).

Further studies in *T. infestans* eggs and nymphs should be performed to clarify the relevance of these geographically different wild and domestic toxicological and biochemical profiles in the development of resistance to pyrethroid and non-pyrethroid insecticides. These data might help to improve the effectiveness of chemical controls when applied directly to eggs, nymphs and adults.

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