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Contribution of general esterases to pyrethroid resistant *Triatoma infestans* (Hemiptera: Reduviidae) from Argentina and Bolivia

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Abstract. The objective of this work was the study of the relative contribution of general esterases: α-napthyl acetate (α-NA) and p-nitro phenyl acetate (p-NPA) in pyrethroid-resistant *T. infestans* from these locations. We used deltamethrin-resistant populations previously collected in two areas in Argentina (Salta and La Rioja) and one area in Bolivia (Tarija). In this study, frequency histograms of α-NA and p-NPA esterase activities, exhibited similar patterns between Bolivian population and the susceptible counterpart. In contrast, Argentinean field populations showed higher proportion of increased enzyme activity individuals. These results clearly demonstrated that resistances in Argentinean and Bolivian field populations are based on different mechanisms, suggesting that enzyme-based pyrethroid resistance in this species has multiple origins.

Keywords: *Triatoma infestans*; Pyrethroid resistance; α-naphtyl acetate esterases; p-nitrophenyl acetate esterases.

Resumen. El objetivo de este trabajo fue el estudio de la contribución relativa de esterasas generales: α-naftil acetato (α-NA) y p-nitro fenil acetato (p-NPA) a la resistencia a piretroides de *T. infestans* provenientes de esas localidades. Se evaluaron poblaciones resistentes a deltametrina previamente recolectadas en dos áreas de Argentina (Salta y La Rioja) y un área en Bolivia (Tarija). En este estudio, los histogramas de frecuencias de actividades de α-NA y p-NPA esterasas mostraron perfiles similares entre la población boliviana y la población susceptible. Por el contrario, en las poblaciones argentinas de campo se observó una elevada proporción de individuos con actividad enzimática incrementada. Estos resultados demuestran claramente que la resistencia en poblaciones argentinas y bolivianas de campo se origina a partir de distintos mecanismos, sugiriendo que la resistencia a piretroides basada en la acción de enzimas presentan múltiples orígenes en estas especies.

Palabras clave: *Triatoma infestans*; Resistencia a piretroides; α-naftil acetato esterasas; p-nitro fenil acetato esterasas.

INTRODUCTION

In Latin America, Chagas disease is currently the most important parasitic disease and it represents a major health and social problem. About 9 million people are infected by the causative agent of this disease, *Tripanosoma cruzi* (Kinetoplastida; Trypanosomatidae) (Schofield *et al.* 2006; Gurtler *et al.* 2007).

The main vector in Argentina and Bolivia is a blood-sucking insect, *Triatoma infestans* (Klug) (Hemiptera: Reduviidae). In Argentina, this vector has been controlled using pyrethroid insecticides for 30 years. As a result, resistance to pyrethroids has been detected in some areas of Argentina since 1997 (Vassena and Picollo 2003; González Audino *et al.* 2004). In 2002, high resistance to pyrethroid insecticides was associated with ineffective field treatments against *T. infestans* in northern Argentina (Picollo *et al.* 2005).

Resistance to pyrethroids in different species of insects has been found to be associated with elevated monooxygenases and esterases (Wilkinson 1983; Oppenoorth 1985; Santo Orihuela *et al.* 2008). These enzymes produce

rapid degradation of pirethroyd insecticides to their nontoxic compounds (Karunaratne 1998; González Audino et al. 2004; Hemingway et al. 2004). Resistance associated with increased metabolism in *T. infestans* was found in a deltamethrin-resistant field population from Salta, Argentina (González Audino *et al*. 2004). This study demonstrated a correlation between elevated monooxygenase activity, determined on individual abdomens through ethoxycoumarin-o-deethylase (ECOD) activity, and deltamethrin resistance. Moreover, the study showed a significant difference in non specific esterase activity measured as phenyl-thioacetate and α-naphtyl acetate activities between the susceptible and Salta populations. Later, a new fluorescent substrate for examining pyrethroid-cleaving esterases was reported by Santo Orihuela *et al.* (2006a; 2006b) and esterase and monooxygenase microplate assays were used for evaluating the frequency distribution of enzyme activities among resistant populations of *T. infestans* from two regions in Argentina and one in Yacuiba, Bolivia (Santo Orihuela *et al*. 2008).

Additionally, other methods to evaluate the contribution of esterases to pyrethroid resistance are based on non specific substrates as α-naphtyl acetate (α-NA) and para-nitro phenyl acetate (p-NPA). These substrates showed important additional information in the evaluation of resistance profiles. (Fontan and Zerba 1984; González Audino *et al*. 2004).

Our objective was the study and evaluation of the relative contribution of general esterases (α-NA and p-NPA esterases) in pyrethroid-resistant *T. infestans* from Argentina and Bolivia.

MATERIAL AND METHODS

Chemicals

The chemicals used were α-naphtyl acetate, α-naphtol, 4-nitrophenyl acetate, 4-nitrophenol, diazo Blue B (o-dianisidine tetrazotized) from Sigma Chemical Co. USA and proanalisis acetone from Merck, Argentina.

Insects

Insect sampling. Field populations of *T. Infestans* were collected in May 2005 from infested houses of three geographical regions in Argentina and Bolivia. The chosen regions represented areas previously identified as having pyrethroid resistant *T. infestans* based on the laboratory test, areas with reported ineffectiveness of the field application of pyrethroids, or both. The collection sites in each area are described below (*Figure 1*).

Figure 1. Map of Argentina showing study sites where *T. infestans* were collected. Area A: Salvador Mazza; Area B: Cuatro Esquinas; Area C: Tarija; Area D: Figueroa.

Area A. Salvador Mazza is located in San Martín Department (22º 03´ S, 63º 41´ W), Salta Province, Argentina. Resistance ratio (RRs) was 133.1 (Picollo *et al.* 2005). Samples from this area were provided by Mario Zaidemberg from the Ministry of Health of Argentina.

Area B. Cuatro Esquinas, San Martín Department (31º 48´ S, 65º 52´ W), this site is located to the south of La Rioja Province, Argentina, the RR was 14.1 (Vassena *et al.*, 2007). Samples from this area were provided by Cynthia Spillman from the Ministry of Health, Argentina.

Area C. Yacuiba is located in southern Bolivia (Tarija Department) (22º 00´ S 63º 40´ W). In 2003, serious levels of infestation after chemical control of field insects were reported by Abraham Jemio from the Ministry of Health and Sports, Bolivia. Later, high deltamethrin resistance was established in the laboratory (RR = 154.4) (Santo Orihuela *et al.* 2008).

All populations were resistant to deltamethrin. The Salta and Yacuiba populations showed high resistance ratios (RRs) to deltamethrin compared with the reference strain (RRs:

133.1 and 154.4 respectively). La Rioja population showed a lower RR to deltamethrin (RR: 14.09) (Santo Orihuela *et al.* 2008).
The laboratory reference strain was the susceptible CIPEIN (Centro de Investigaciones de Plagas e Insecticidas), which has been reared without insecticide exposition in our laboratory since 1975 (Picollo *et al.* 1976).
A field population collected in 2005 in an area without control failures or other indications of possible resistance was used as field reference strain. This population (RR: 1.6) was collected in the Figueroa Department, located in the Santiago del Estero Province (28º 26´S, 63ºC 33´ W) and provided by Raúl Stariolo from the Ministry of Health, Argentina (Santo Orihuela *et al.* 2008).
Field collected insects were reared in the laboratory in order to obtain enough number of individual for assays. The RRs values reported in 2005 were confirmed in 2007 against first nymphs of the second generation of field insects (Picollo *et al.* 2005). The enzymatic activity was assessed in 2007 against the second generation of insects (development time: about 1 year).
All populations were maintained in the laboratory at 28 ± 1ºC, 50% RH and a photoperiod of 12:12 (L:D) h. Bugs were fed weekly on pigeons. For all the experiments, laboratory-reared first instars (3-5 d old) and starved since eclosion (mean weight 1.2 ± 0.2 mg), were selected for the tests according to the World Health Organization protocol (WHO 1994). This protocol has been used in numerous works in this area (Vassena et al. 2000; González Audino *et al.* 2004; Sonoda *et al.* 2009).

Equipment
The absorbance of the wells was determined using a microplate reader spectrophotometric equipped with 340, 405, 415, 540, 595 and 655-nm wavelength filter (Microplate reader, model 680, Bio-Rad Laboratories, Inc.). The Microplate Manager® software v. 5.2.1 (Bio-Rad Laboratories, Inc.) was used to collect, to analyze, and to output absorbance data from Bio-Rad's microplate readers.

Esterases
The activity of α-naphtyl acetate esterases was measured using α-naphtyl acetate as substrate. The α-naphtol generated by enzymatic hydrolysis was mixed with o-dianisidine tetrazotizade and the formed diazo-compound was detected by espectrophotometry (600 nm) (Gomori 1953; van Asperen 1962).
Briefly, each nymph I was homogenized individually in 250 µl of phosphate buffer (KH_2PO_4/ K_2HPO_4) pH 7.2, 0.05 M. Volumes of 50 µl of homogenates were individually placed into 96-well microplates containing 50 µl of α-naphtyl acetate. The mix was incubated at 20ºC for 10 minutes and then, 50 µl of o-tetrazotized dianisidine (5 mg/ml) were added. The absorbances were measured after a second incubation of 2 minutes at 20ºC.
Another substrate used to determinate esterase activity was p-nitrophenyl acetate (p-NPA). This method is based on the kinetic spectrophotometric measure of p-nitrophenoxide (400 nm), product of hydrolysis of this substrate (Wallace *et al.* 1988).
First nymphs were homogenized in 250 µl of buffer Na_2HPO_4 / NaH_2PO_4, 0.05 M, pH 7.2. A volume of 133 µl of homogenate was incubated with 3 µl of 61 mM of p-NPA. The absorbance was measured each 30 seconds for 10 minutes.
The α-naphtyl acetate and p-nitrophenyl acetate esterases activities of individuals were based on standard curves performed with commercial α-naphtol and 4-nitrophenol in concentrations of 34.75; 17.38; 8.69; 4.34; 2.17 nanomoles per well and 45.98; 16.33; 5.11; 1.70 nanomoles per well, respectively. These curves were performed per assay and each concentration of standard was made per duplicate. Activities were all corrected for background hydrolysis and expressed as nanomoles of hydrolyzed substrate per minute per insect.
Protein concentration in insects was quantified using a protein kit (Total Protein Kit, Sigma®) based on the technique of Bradford (1976). The number of nymphs I used was between 10 and 20 individual for each studied population.

Statistical analysis
The Kruskall-Wallis test was used to compare the amount of protein among studied populations. The values of enzymatic activity were compared among populations also using Kruskall-Wallis test. The biochemical data were plotted as the percentage of individuals responding within a particular range of values of enzyme activity (Sokal and Rohlf 1980). Frecuency profiles for α-naphtyl acetate and p-nitrophenyl acetate esterases activities from individual insects were analyzed for general

esterases. To compare histograms for different populations a reasonable and discretionary threshold containing the majority (80%) of insects was established for the susceptible strain, and the percentage of insects over the threshold was calculated for each enzymatic activity and each population according to previous works about monooxigenases and esterases insect activities (Picollo *et al.* 2005; Santo Orihuela *et al.* 2008; Barrios *et al.* 2010).

RESULTS

The amount of protein calculated was not different among studied populations and susceptible strain (Kruskall-Wallis Test), thus the esterases activity (nanomoles per minute) was expressed per insect (*Table 1*).

Table 2 shows the mean enzymatic activities and statistical values for the studied populations.

The frequency distribution of esterases activities between resistant (Salvador Mazza, Cuatro Esquinas and Tarija) and susceptible (CIPEIN and Figueroa) *T. infestans* populations, based on individual assays, are shown in *figures 2 and 3*.

The α-napthyl acetate activity for the laboratory reference strain (CIPEIN) and the susceptible field population (Figueroa) showed the minor values compared to the S. Mazza and Cuatro Esquinas resistant field populations. The percentage of individuals with activities over the susceptible activity threshold, 1.7 nanomoles per minute per insect, was 12.2 and 10.4% for susceptible populations, and 44.2 and 93.1% for the resistant ones. In contrast, the resistant Tarija population exhibited lower levels of activity relative susceptible ones (3.6% of insects over the susceptible activity thereshold). (*Figure 2*)

Table 1. Means, standard deviations of protein content and number of insects (n) for studied populations

Strain / Population	n	Mean protein content ± standard deviation (µg per insect)
Cipein	26	94.37 (± 28.88) [a]
Figueroa	19	84.96 (± 20.95) [a]
Salvador Mazza	22	109.14 (± 30.69) [a]
Cuatro Esquinas	17	110.14 (± 21.35) [a]
Tarija	12	88.67 (± 16.24) [a]

Means of protein content followed by the same letter are not significantly different (P>0.05). Kruskal-Wallis Statistic KW = 7.479.

Table 2. Mean enzymatic activities of α-naphtyl acetate (α-NA) and p-nitrophenyl acetate esterases, standard deviations and total number of insect used (n) for studied populations

Strain / Population	n	α-NA (nmoles/min)	n	p-NPA (nmoles/min)
Cipein	74	1.202 (±0.490) [a]	76	1.341 (±0.521) [a]
Figueroa	67	1.048 (±0.528) [ab]	29	1.440 (±0.614) [ab]
Salvador Mazza	68	1.658 (±0.561) [c]	54	1.962 (±0.898) [c]
Cuatro Esquinas	29	2.374 (±0.510) [d]	16	1.761 (±0.555) [bc]
Tarija	28	0.833 (±0.437) [b]	30	1.337 (±0.866) [a]

Values in the same column followed by different letter are significantly different (P<0.05) (Kruskal Wallis and Dunn´s Multiple Comparisons Test). KW=107.64 (α-NA) and 27.618 (p-NPA).

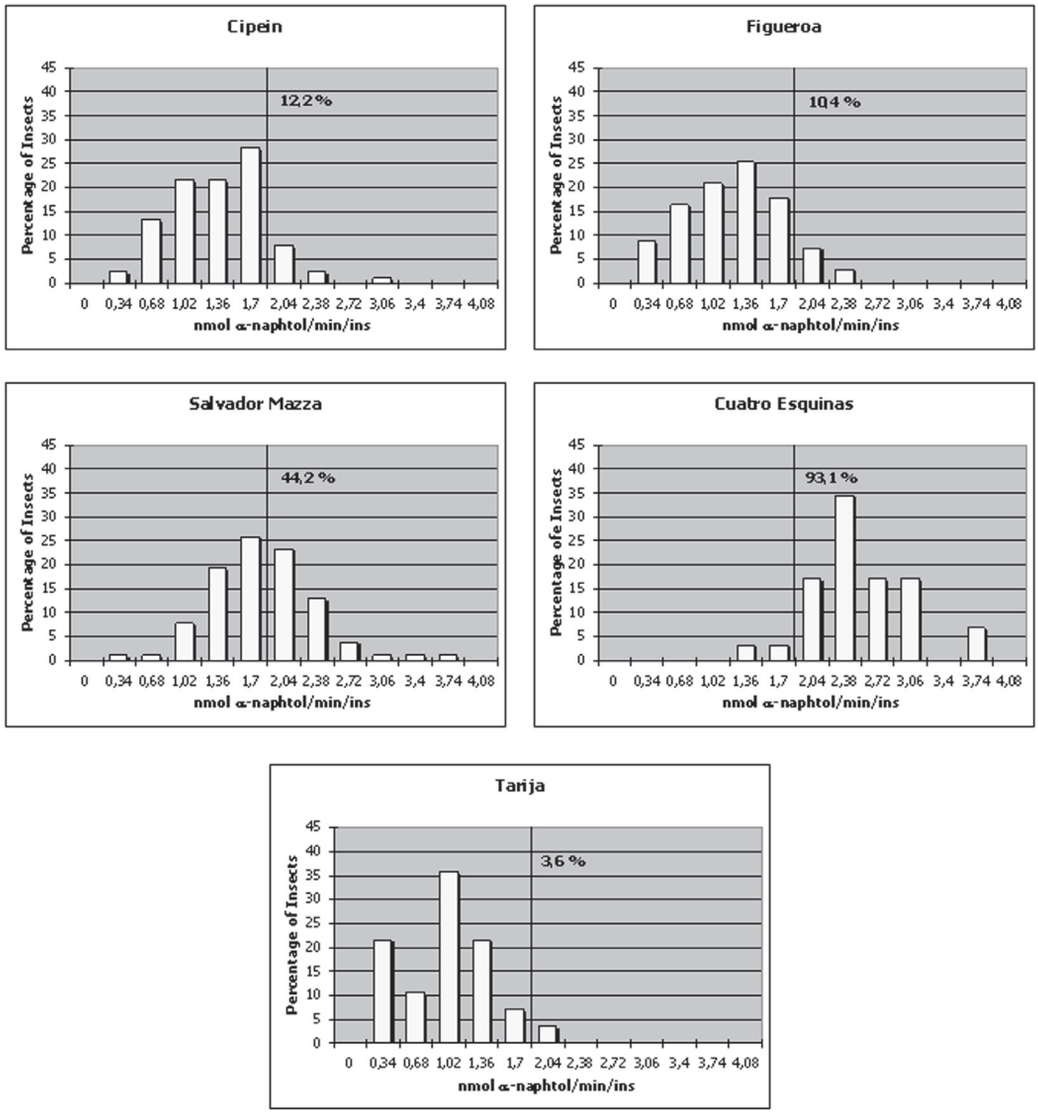

Figure 2. Histograms indicating the distribution of α-naphtyl acetate esterases activities exhibited by susceptible and resistant *T. infestans* in individual assays.

The p-nitrophenyl acetate activity distribution also separated the resistant S. Mazza and Cuatro Esquinas populations from the susceptible CIPEIN and Figueroa and the resistant Tarija. The percentage of individuals showing activities over the susceptible threshold (1.86 nanomoles per minute and per insect) was 13.2 and 20.7% for susceptible populations, and 50 and 43.8% for S. Mazza and Cuatro Esquinas resistant ones. The resistant Tarija exhibited a similar pattern of p-NPA activity to those of susceptible insects (16.7% of individuals showing activity values over the threshold) (*Figure 3*).

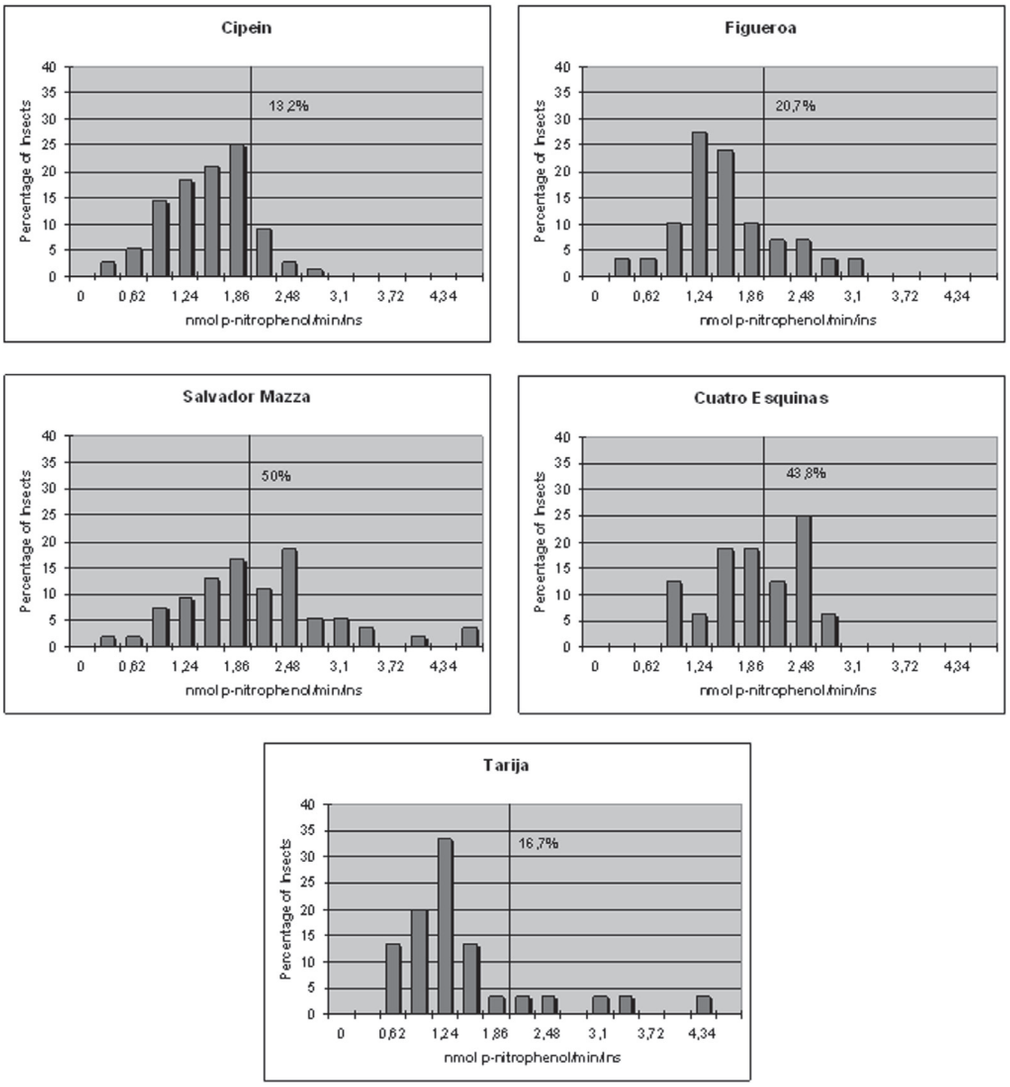

Figure 3. Histograms indicating the distribution of p-nitrophenyl acetate esterases activities exhibited by susceptible and resistant *T. infestans* in individual assays.

DISCUSSION

Despite prolonged and intensive control campaigns against Chagas disease vectors, few studies have been reported on the possible development of insecticide resistance and changes in the susceptibility of the main vector, *Triatoma infestans*.

Currently, there is very little literature related to resistance to pyrethroid insecticides and the failure of control in the field. As well as works that provide evidence of the possible mechanisms involved in resistance.

Recently, high resistance to pyrethroid insecticides has been associated with ineffective field treatments against *Triatoma infestans* (Klug) (Hemiptera: Reduviidae) in Argentina and Bolivia (Picollo *et al.* 2005). Samples were collected from two areas in Argentina (Salta and La Rioja) and one area in Bolivia (Tarija), (Santo Orihuela *et al.* 2008) and they were subjected to toxicological and biochemical assays. All populations were resistant to deltamethrin. The Salta and Yacuiba populations showed high resistance ratios (RRs) to deltamethrin compared with the reference strain (RRs: 133.1 and 154.4 respectively). Otherwise, the La Rioja population showed a lower RR to deltamethrin (RR: 14.09). That work also reported that measured activity of P450 monooxygenase in individual

insects (based on ethoxycoumarine-O deethylase), tended to be higher in the deltamethrin-resistant populations, but the differences were not statistically significant. Activity of specific esterases determined by the hydrolysis of 7-coumaryl permethrate demonstrated an increase in the percentage of insects with higher esterase activity in Salta and La Rioja populations. Unexpectedly, the Yacuiba population showed lower pyrethroid esterase activity than the reference strain. These results demonstrated different contribution of specific esterases to pyrethroid resistance between *T. infestans* from Argentina and Bolivia, and suggested independent evolution of resistance in both areas.

In the present study, the resistant populations from Argentina (S. Mazza and Cuatro Esquinas) exhibited significantly higher levels of α-NA and p-NPA esterases relative to the susceptible counterpart. In contrast, the resistant population from Bolivia (Tarija) exhibited frequency histograms of α-NA and p-NFA esterase activities similar to the susceptible populations. These results suggest that α-NA and p-NFA esterases are involved in the pyrethroid resistant of Salvador Mazza and Cuatro Esquinas, but not in Tarija. These data clearly indicated different patterns of general esterase activity in Argentinean and Bolivian resistant populations, and suggested that resistances partly could be based on different mechanisms. Similarly, previous studies against a deltamethrin resistant population of *T. infestans* from Acambuco, Salta, Argentina were reported by Gonzalez Audino *et al.* (2004). In this work the authors studied the role of enhanced detoxification in a low resistant population (RR: 7.9). They found a significant difference in phenyltioacetate and α-NA esterase activity, and concluded that the degradative esterases play an important role in the resistance to deltamethrin in Salta colony.

The examination of elevated general esterase activity by simple assays has been commonly used to implicate the involvement of hydrolytic mechanisms in insecticide resistance (Soderlund and Bloomquist 1991). The individual assays using 96-well microplate format, allows both qualitative and quantitative measurement of enzymes activities from each insect and can provide an estimate of resistance frequency of field populations (Rose *et al.* 1995). Based on individual activity assays, Zhou *et al.* characterized differences in esterase activity between susceptible and methyl-parathion resistant population of the western corn rootworm *Diabrotica virgifera* (Le Conte) (Zhou *et al.* 2003). Using naphtolic esters as model substrates in single larva assays, the authors established differences in esterase activity between resistant and susceptible populations. They found that resistant populations were 7.3 and 5.8 fold higher than susceptible populations and the frequency histograms of esterase activity were clearly different between resistant and susceptible populations.

The different esterases patterns found in *T. infestans* from Argentina and Bolivia suggests that enzyme-based pyrethroid resistance in this species has multiple origins (Santo Orihuela *et al.* 2008).

Nevertheless, because nerve insensitivity (related to the presence of the kdr mutation) is also an important mechanism related to pyrethroid resistance, further studies on the kdr mutation should be carried out to clarify the relative contribution of each pyrethroid-associated mechanism in deltamethrin-resistant populations of *T. infestans.* Although the importance of this study and the number of research groups working on this issue, there is not information available yet about kdr mutation in triatomines.

This knowledge will provide rational bases for alternative strategies for the chemical control of pyrethroid resistant *T. infestans* field populations.

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