Toxicological and biochemical analysis of the susceptibility of sylvatic *Triatoma infestans* from the Andean Valley of Bolivia to organophosphate insecticide

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To increase our knowledge of the natural susceptibility of Triatoma infestans *to an organophosphate insecticide, we performed toxicological and biochemical studies on three sylvatic populations from Bolivia and two populations from domestic dwellings from Bolivia and Argentina. Fifty-per-cent lethal doses (LD$_{50}$) were determined based on the topical application of fenitrothion on first instar nymphs and mortality was assessed at 24 h. Both type of populations exhibited LD$_{50}$ ratios significantly higher than 1 with a range of the values (1.42-2.47); the maximum value were found in a sylvatic (-S) population, Veinte de Octubre-S. Samples were biochemically analysed using a glutathione S-transferase activity assay. The highest significant activity was obtained for Veinte de Octubre-S and the lowest activity was obtained for the reference population (102.69 and 54.23 pmol per minute per mg of protein respectively). Two out of the three sylvatic populations (Veinte de Octubre-S and Kirus Mayu-S) exhibited significantly higher glutathione S-transferase activity than that of the reference population. Based on this analysis of the natural susceptibility of this organism to organophosphate insecticides, continental and focal surveys of organophosphate susceptibility should be conducted to evaluate the evolution and distribution of this phenomenon.*

Key words: sylvatic - *Triatoma infestans* - organophosphate - insecticide - glutathione transferases

Chagas disease is an endemic infection of the South American continent caused by *Trypanosoma cruzi*. This flagellated parasite is transmitted to humans and animals by various blood-sucking triatomine insects (Hemiptera: Reduviidae: Triatominae). In Argentina and Bolivia, approximately nine million people are currently infected with this disease (Schofield et al. 2006) and the main vector of *T. cruzi* is *Triatoma infestans* (Klug, 1834). Houses were first colonised by the vectors that live in natural foci several thousand years ago. Rural and rustic dwellings provide Triatominae with shelter and food sources, allowing the formation of intradomiciliary colonies (Dias & Schofield 2007). Intradomiciliary colonies of *T. infestans* are successfully controlled in numerous areas of the Southern Cone of South America by the spraying of houses with pyrethroid insecticides (Zerba 1999, Dias et al. 2002, Schofield et al. 2006).

However, several areas in the Gran Chaco of Argentina, Bolivia and Paraguay have been targeted in an intensive vector control effort without success (Gürtler et al. 2007). Several authors have shown that insects from this area have developed resistance to pyrethroid insecticides (Audino et al. 2004, Picollo et al. 2005, Santo-Orihuela et al. 2008, Toloza et al. 2008, Germano et al. 2010, 2012, Lardeux et al. 2010, Santo-Orihuela & Picollo 2011). However, an important study by Alarico et al. (2010) demonstrated that intradomiciliary and peridomiciliary populations of *T. infestans* from the Bolivian Chaco region were susceptible to a micro-encapsulated formulation containing organophosphate active ingredients.

In recent years, the susceptibility of some sylvatic populations of *T. infestans* to insecticides has been studied by Lardeux et al. (2010); in addition, Depickère et al. (2012) demonstrated deltamethrin toxicity in 12 natural insect populations. These studies focused on the toxicological analysis of topical insecticide application, whereas Roca Acevedo et al. (2011) recently evaluated the role of cytochrome P450 monooxygenases and pyrethroid esterases in sylvatic *T. infestans*. These sylvatic populations are of great importance due to their possible role in the resettlement of intradomiciliary structures and 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). Organophosphate insecticides were previously used to control *T. infestans* in South American campaigns, but have been replaced by pyrethroid insecticides because of their safety in vertebrates and effectiveness in controlling insects (Zerba 1999). Nevertheless, the development of pyrethroid resistance in *T. infestans* in several places in Argentina has led to reintroduction of organophosphate insecticides. The organophosphate insecticides fenitrothion and malathion have been used to control deltamethrin-resistant populations in several localities in the Provinces of Salta and Chaco, Argentina

(Picollo et al. 2005, Gurevitz et al. 2012). Glutathione transferases (GSTs) comprise a diverse family of enzymes that play important roles in conferring insecticide resistance. Elevated GST activity has been associated with resistance to all major classes of insecticides (Enayati et al. 2005). The role of GSTs in the degradation of the organophosphorus compounds malathion, parathion and fenitrothion has been demonstrated (Wood et al. 1986, Sívori et al. 1999). Considering the increasing relevance of sylvatic *T. infestans* and that relatively few studies have reported on their susceptibility to insecticides, we chose to study the toxic response of sylvatic *T. infestans* populations to a relevant organophosphate insecticide and the relationship between this activity and glutathione S-transferases.

MATERIALS AND METHODS

Insects - *T. infestans* were collected in 2008 from domiciliary (-D) and sylvatic (-S) areas in the Department of Cochabama, Bolivia (Mataral-D, Illicuni-S and Veinte de Octubre-S), the Department of Potosi, Bo-

Fig. 1: map showing locations from where populations of *Triatoma infestans* were collected. -D: domiciliary; -S: sylvatic.

livia (Kirus Mayu-S) [as previously described by Roca Acevedo et al. (2011)] and the Department of Tarija, Bolivia (El Palmar-D) (Fig. 1, Table I).

Sylvatic *T. infestans* were captured from rock piles using mouse-baited sticky traps (Noireau et al. 1999) and reared in Bolivia; eggs of the descendent populations were transported to the laboratory in Argentina where subsequent generations were bred. A susceptible reference strain (NFS) was derived from a domestic population captured in 2004 in Santiago del Estero, Argentina, in an area where insects had successfully been controlled using the pyrethroid insecticide deltamethrin. Laboratory tests with NFS showed no significant differences in insecticide susceptibility against the previously used reference strain CIPEIN. Insects of each population were reared in boxes at 28ºC and 50-60% relative humidity (RH) and a photoperiod of 12:12 (L:D) h was used. A pigeon was provided weekly to cover the blood requirements of the insects (WHO 1994).

Chemicals - Technical grade fenitrothion (99%) was obtained from Ehrestorfer (Augsburg, Germany). Analytical grade acetone was purchased from JT Baker (San Pedro Xalostoc, state of Mexico, Mexico) and analytical grade acetonitrile was obtained from SINTORGAN-SACIF, Argentina. 1-chloro-2,4-dinitrobenzene (CDNB) (97%) and reduced L-glutathione (GSH) were obtained from Sigma-Aldrich Co, St. Louis, USA.

Topical application bioassays - Serial dilutions of the insecticide fenitrothion were prepared in acetone and topically applied using a 10-µL Hamilton micro-syringe equipped with a repeating dispenser. Each first instar nymph was treated with 0.2 µL of solution on the dorsal surface of the abdomen (WHO 1994). The total dose ranged from 2-100 ng per insect.

At least 10 insects were used per dose and per replicate. A minimum of four doses, giving 0% and 100% mortality, were used for each treatment. Each experiment was replicated at least three times. Control groups received only pure acetone. Treated and control insects were placed onto filter paper discs. Insects were housed in an environmental chamber (Lab-Line Instruments, Melrose Park, IL) at 28 ± 1ºC and 55 ± 5% RH under a photoperiod of 12:12 (L:D) h. Mortality was recorded at 24 h after treatment. The criterion for mortality was
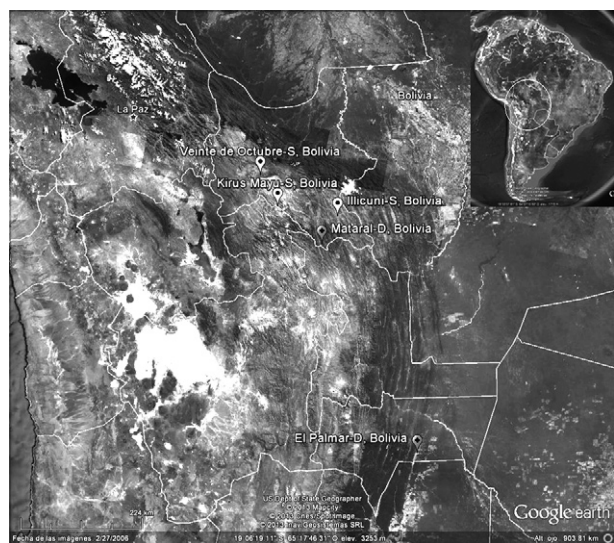
TABLE I

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

Site of collection	Location (city/province)	Latitude/longitude	Altitude (meters above sea level)
Illicuni-S	Omereque/Cochabamba	18º09'S/64º52'W	1.580
Kirus Mayu-S	Toro Toro/Potosí	17º59'S/65º50'W	2.070
Veinte de Octubre-S	Cochabamba/Cochabamba	17º29'S/66º07'W	2.596
El Palmar-D	El Palmar/Tarija	17º02'S/64º12'W	610
Mataral-D	Aiquile/Cochabamba	18º35'S/65º08'W	1.750
NFS	Susceptible reference strain	-	-

the inability of the nymphs to walk from the centre to the border of an 11-cm paper disc (Vassena et al. 2000, Picollo et al. 2005).

Determination of enzymatic activity - GST activity was measured using CDNB as a substrate as previously described by Habig et al. (1974); this method was adapted for use with nymph I homogenates. Briefly, the insects were cooled and each nymph was homogenised in 100 µL of phosphate buffer (pH 6.5, 0.1 M) using a plastic pestle and mortar. The reaction was begun by adding 10 µL of 0.05 M reduced GSH to 178 µL of homogenates placed into 96-well polystyrene flat-bottom microtitre plates. Two microlitres of 0.05 M CDNB in acetonitrile was then added and the mixture was incubated at 25ºC for 2 min. The reaction was kinetically evaluated by measuring the absorbance at 340 nm every 30 sec for 5 min, such that the assay was linear over the reported time. The well absorbances were determined using a spectrophotometric microplate reader equipped with 340, 405, 415, 540, 595 and 655-nm wavelength filters (Microplate reader, model 680, Bio-Rad Laboratories, Inc). Microplate Manager® software v. 5.2.1 (Bio-Rad Laboratories, Inc) was used to collect, analyse and record the absorbance data.

The activity measurements were corrected with sample blanks and transformed into nanomoles of obtained product (CDNB conjugated with GSH) per minute and per milligrams of protein using a molar extinction coefficient of $\Delta\varepsilon$ according to Habig et al. (1974)

The protein concentration of the insect homogenates was quantified using a protein assay (Total Protein Kit-Micro, Sigma®) based on the technique of Bradford (1976).

Statistical analysis - The mortality data were processed using POLO Plus (LeOra Software Company© 1987). Bioassay data from each *T. infestans* population were pooled and analysed based on probit analysis (Litchfield & Wilcoxon 1949) to estimate the lethal dose (LD) (nanograms per insect) that kills 50% of treated individuals (LD_{50}). LD ratios (LDRs) and 95% confidence intervals (CI) of each population were calculated

according to Robertson et al. (2007) by comparing the dose-response curves between studied populations and the reference strain. Studied populations were considered different from the reference strain if the LDR CIs did not include the number 1.

GST activity profiles from different populations were represented using a scatter plot (Montella et al. 2007). The non-parametric Kruskal-Wallis Test and Dunn's Multiple Comparisons Test were used to compare the values of enzymatic activity (GST) per minute and per milligram of protein among the populations.

RESULTS

The estimated toxicity data to fenitrothion for three samples of sylvatic *T. infestans* (Illicuni-S, Kirus Mayu-S and Veinte de Octubre-S), two samples of domestic *T. infestans* (El Palmar-D, Mataral-D) and one reference (NSF) population of *T. infestans* are listed in Table II. Among the sylvatic populations, the highest significant value of LD_{50} was 26.8 ng/insect (LDR = 2.47) for the Veinte de Octubre-S population and this value was also the highest among all examined populations. Among domestic populations, the values of LD_{50} were quite similar (17.4 and 15.3 ng/insect).

The enzymatic activities from all populations were corrected using the mean protein content of each population (from 51.9-105.4 µg of protein per insect).

A scatter plot is used to represent the GST activity profiles from different populations (Fig. 2); in this representation, the activity of each individual from every population is plotted. The mean glutathione transferase activities are listed in Table III. Veinte de Octubre-S exhibited the highest significant GST activity (102.69 pmol per minute and per milligram of protein) and NFS exhibited the lowest GST activity (54.23 pmol/min/mg of protein). The domestic populations El Palmar-D and Mataral-D and the sylvatic population Illicuni-S exhibited higher values of LDR than the NFS reference population, but no significant change in GST activity was observed. However, the sylvatic populations Veinte de Octubre-S and Kirus Mayu-S exhibited higher LDRs and GST activities than the NFS reference population.

TABLE II

Toxicity of topically applied fenitrothion to *Triatoma infestans* first instar nymphs of a susceptible reference strain (NFS), sylvatic (-S) and domiciliary (-D) field populations collected from the Andean valleys of Bolivia

Population	n[a]	Slope ± SE	χ^2	LD_{50} (ng/insect) (95% CI)	LDR (95% CI)
Illicuni-S	78	1.36 ± 0.35	0.216	20.2 (12.0-43.0)	1.87 (1.05-3.35)
Kirus Mayu-S	90	5.60 ± 0.67	0.000	14.6 (13.4-15.8)	1.35 (1.11-1.63)
Veinte de Octubre-S	85	4.15 ± 1.29	0.214	26.8 (20.6-54.8)	2.47 (1.67-3.66)
El Palmar-D	76	2.01 ± 0.44	2.252	17.4 (11.6-30.4)	1.61 (1.01-2.58)
Mataral-D[a]	170	3.43 ± 0.47	3.79	15.3 (9.6-22.0)	1.42 (1.08-1.85)
NFS	80	2.07 ± 0.54	6.490	10.8 (4.0-26.6)	-

a: data from Toloza et al. (2008); CI: confidence interval; LD: lethal dose; LDR: LD ratios; SE: standard error.

DISCUSSION

The chemical control of domestic *T. infestans* is being successfully pursued in most of the Southern Cone countries (Chile, Uruguay and Brazil and in sections of Argentina, Bolivia and Paraguay) (Silveira 2002, Schofield et al. 2006).

The spraying of infested dwellings with pyrethroid insecticides was the main method used to control *T. cruzi* vectors. Although the reappearance of vectors in sprayed houses is usually the result of insects moving from another, unsprayed building nearby, re-infestation may also arise from insects that have survived the initial insecticide treatment or from insects that originated in the sylvatic environment (Moncayo & Silveira 2009, Noireau 2009).

In areas with successful control programs, reports of sylvatic species invading human dwellings have led researchers to focus on their original habitats (Noireau et al. 2000). Bolivia is the first country in which true sylvatic foci of *T. infestans* from Andean valleys have been reported (Torrico 1946, Bermudez et al. 1993, Buitrago et al. 2010) and a recent study has made new discoveries throughout the Bolivian Chaco (Waleckx et al. 2012). Moreover, foci of sylvatic *T. infestans* have been reported in Paraguay (Rolón et al. 2011) and in Argentina (Ceballos et al. 2009). These data provide evidence that wild populations of *T. infestans* are much more widespread than previously thought, drawing attention to the need for further research on this important and neglected issue, particularly with regard to the role that such wild populations may play in the process of recolonising insecticide-treated villages (Noireau 2009, Ceballos et al. 2011).

Based on genetic research, it has been concluded that *T. infestans* originated in Bolivia and it is there that the highest genetic variability exists (Bargues et al. 2006,

Cortez et al. 2010, Waleckx et al. 2011). This suggests that if natural resistance to insecticides were to develop, this would occur in Bolivia. The discovery of *T. infestans* foci with high tolerances to chemical control is, therefore, a clear warning sign (Dias & Schofield 2007).

It is necessary to analyse the biochemical and toxicological profile of wild insect populations to establish their natural susceptibility and the potential development of resistance because they may play a role in domiciliary re-infestation and colonisation. An initial toxicological assessment was performed by Lardeux et al. (2010), who studied a wild population from Chivisivi (Department of La Paz) that was captured in a rocky environment; this population exhibited a slightly higher LDR to deltamethrin compared to a susceptible reference strain (CIPEIN). Furthermore, Depickère et al. (2012) studied the susceptibility to deltamethrin by applying a discriminating dose (DD) to 12 sylvatic populations from Bolivia. In this work, the authors reported one wild resistant population from Potosi and two other populations from La Paz and Cochabamba that exhibited a slight decrease in the mortality rate at the DD. An initial analysis of the biochemical and toxicological profiles of the sylvatic populations with respect to pyrethroid and phenyl-pyrazole insecticides was carried out by Roca Acevedo et al. (2011). These authors found that natural populations exhibit slightly higher LDR or lower sensitivity for both types of studied insecticide than the reference population and the authors did not detect biochemical differences regarding P450-monooxygenases and pyrethroid esterases.

In the present work, no significant differences in LDRs between domestic and sylvatic populations were found; however, all populations were different from the reference NFS population and one of the sylvatic populations exhibited the highest LDRs.

However, this higher LDR in a sylvatic population might result from any one of at least three hypotheses according to Depickère et al. (2012): one possibility may
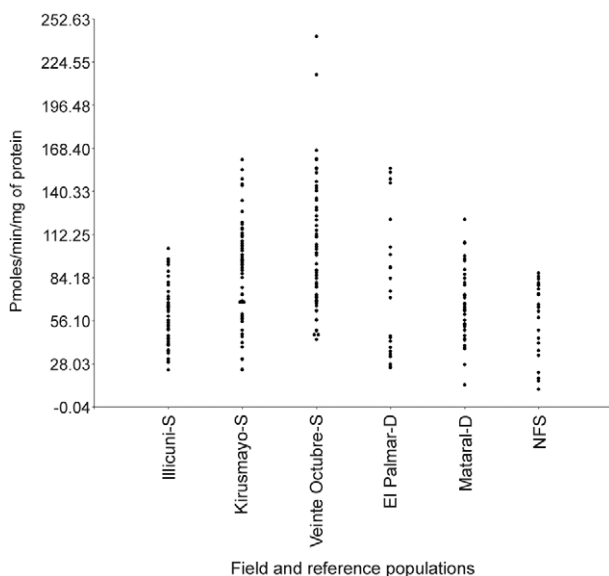

Fig. 2: scatter graph of glutathione transferases activity from different populations in comparison with the reference strain NFS. -D: domiciliary; -S: sylvatic.

TABLE III

Mean enzymatic activities of glutathione transferases (GST), standard deviations and total number of insect used for studied populations

Strain/population	n	GST activity (pmol/min/mg protein)
Illicuni-S	49	59.87 (±19.83)[a]
Kirus Mayu-S	60	87.13 (±33.76)[b,c]
Veinte de Octubre-S	63	102.69 (±41.55)[c]
El Palmar-D	22	76.98 (±45.32)[a,b]
Mataral-D	40	66.54 (±22.70)[a]
NFS	28	54.23 (±25.60)[a]

values in the same column followed by different letter are significantly different (p < 0.05) [Kruskal Wallis (= 70.26) and Dunn's Multiple Comparisons Test]. -D: domiciliary; -S: sylvatic.

be the existence of naturally decreased susceptibility in sylvatic populations, another cause could be the development of resistance resulting from exposure to insecticides used in farming and vector control campaigns, and a third possibility might be contact and probable exchange of genetic material between these sylvatic populations and the domestic resistant populations, which are geographically close. Although the presence of natural resistance in *T. infestans* has not been demonstrated (Lardeux et al. 2010, Roca Acevedo et al. 2011, Depickére et al. 2012), these explanations suggest that sylvatic populations, depending on their geographical location, may be exposed to insecticide pressures and/or connected to domiciliary populations that were treated with insecticides at some point in their life history. Therefore, further studies must be carried out to clarify and understand the possible origins of the reduced susceptibility to insecticides detected in the sylvatic populations of *T. infestans*.

Although no significant differences between populations were found in the toxicological analysis, the finding of increased enzymatic activities in some sylvatic populations such as Veinte Octubre-S and Kirus Mayu-S indicate a possible contribution to the reduction of sensitivity to this type of insecticide (Kostaropoulos et al. 2001). Alternately, no significant evidence of higher GST activities (compared to the reference population) was found in domiciliary populations that are resistant to other type of insecticides, such as pyrethroids.

Two out of the three studied sylvatic populations (Veinte de Octubre-S and Kirus Mayu-S) exhibited significant increase oftly higher GST activity than that observed in one domestic population and the reference populations, indicating that this parameter could be used as a possible indicator of reduced susceptibility; this finding also suggests future possible resistance against fenitrothion (Siegwart et al. 2011). This possibility is corroborated by previous findings related to the detoxification role of GSTs in *T. infestans* (Wood et al. 1986, Sívori et al. 1999).

Based on this analysis of natural susceptibility to organophosphate insecticides, continental and focal surveys of organophosphate susceptibility should be carried out to evaluate the evolution and distribution of this phenomenon. Moreover, the toxicological and biochemical profile of organophosphate insecticides should be analysed before this kind of insecticide is applied to pyrethroid-resistant populations of *T. infestans* to avoid the inappropriate use of insecticides and preserve human health and the environment.

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