Susceptibility of Sylvatic *Triatoma infestans* From Andeans Valleys of Bolivia to Deltamethrin and Fipronil

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ABSTRACT We describe the susceptibility to deltamethrin and fipronil of four sylvatic *Triatoma infestans* populations from the Andean valleys of Bolivia. Fifty percent lethal doses were determined from topical application of insecticide on first instars, and mortality was assessed after 24 h for deltamethrin and 48 h for fipronil. In comparison with a reference strain from Argentina, the Bolivian populations showed deltamethrin 50 percent lethal dose ratios ranging from 1.9 to 17.4. In the case of fipronil, an insecticide never used for control of *T. infestans*, the Bolivian populations showed even higher variation in toxic response, with relative susceptibilities ranging from 0.5 to 139.2. However, although the sylvatic *T. infestans* toxicological profiles differ from each other and from those of the domiciliary population studied in this work, there were no significant differences in the activities of P450 mono-oxygenases and pyrethroid esterases between the reference strain and the studied populations.

KEY WORDS *Triatoma infestans*, insecticide susceptibility, sylvatic populations

Chagas disease, because of infection with *Trypanosoma cruzi*, is endemic to the American continent, mainly transmitted to humans by blood-sucking triatomine bugs (Hemiptera, Reduviidae, Triatominae). In Argentina and Bolivia, ≈9 million people are currently infected (Schofield et al. 2006), and the main vector of *T. cruzi* is *Triatoma infestans* (Klug, 1834).

Domestic infestations of *T. infestans* are being successfully controlled in much of the Southern Cone of South America, by spraying infested dwellings with residual pyrethroid insecticides (Dias et al. 2002, Schofield et al. 2006). However, only limited success has been achieved in the Gran Chaco of Argentina, Bolivia, and Paraguay, even in areas under intensive vector control efforts (Gürtler et al. 2007). This region is thought to represent the origin of *T. infestans* and it is there where it shows highest genetic variability (Bargues et al. 2006, Torres-Pérez et al. 2011) and emerging resistance to pyrethroid insecticides (Gürtler et al. 2004, Picollo et al. 2005, Lardeux et al. 2010, Moncayo and Yanine 2006, Cecere et al. 2006,

Toloza et al. 2008, Santo Orihuela et al. 2008, Ceballos et al. 2009).

Characterization of the resistance developed north of Salta (Argentina) and south and center of Bolivia has shown important toxicological differences (Toloza et al. 2008, Germano et al. 2010). Populations near the Argentinian and Bolivian border had high levels of resistance to deltamethrin, but were susceptible to fipronil, a phenylpyrazole insecticide. Populations of the Andean areas of Bolivia are of particular interest because they show different patterns of resistance in eggs and first instars than in the north of Salta, showing intermediate levels of resistance to deltamethrin and very high levels to fipronil. (Toloza et al. 2008, Germano et al. 2010).

Research on resistance to insecticides in *T. infestans* has been focused on domiciliary populations, but recent reports provide evidence that sylvatic populations of *T. infestans* are much more widespread than previously thought (Noireau et al. 2005, Noireau 2009). The objective of the current study was to determine whether the susceptibility of sylvatic *T. infestans* populations from Bolivia is similar to that of domiciliary populations.

Materials and Methods

Insects. *T. infestans* were collected in 2007 from domicilliary (D) areas in the Department of Cochabama, Bolivia (Mataral–D), and from a nearby sylvatic (S) area ≈2 km distant (Mataral–S). Additional sylvatic bugs were collected in February 2008 from other

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Table 1.   Samples of sylvatic (S) and domiciliary (D) populations of *T. infestans* analyzed according to the collecting site in Bolivia

Site of collection	Location (city/province)	Latitude/Longitude	Altitude meters above sea level	No. of specimens collected in the field
NFS[a]	Susceptible reference strain	–	–	–
Mataral—D	Alquile/Cochabamba	18°35′44,08″ S/65°08′58,74″ W	1,750	10
Mataral—S	Alquile/Cochabamba	18°36,190 S/65°07,117 W	1,750	18
Kirus Mayu—S	Toro Toro/Potos	17°59,302 S/65°50,281 W	2,070	30
Ilicuni—S	Omereque/Cochabamba	18°09,502 S/64°51,943	1,580	10
20 de Octubre—S	Cochabamba/Cochabamba	17°29,057 S/66°06,741 W	2,596	32

[a] Susceptible reference strain.

sites in Cochabamba (Ilicuni–S and 20 de Octubre–S) and Potosi (Kirus-Mayu–S) (Table 1, Fig. 1).

The sylvatic *T. infestans* were collected from rock-piles using mouse-baited sticky traps (Noireau et al. 1999). They were reared in Bolivia, and eggs of the descendent populations were transported to the laboratory in Argentina, where further generations were bred.

For comparison, we used a susceptible reference strain (NFS) derived from a domestic population collected in December 2004, from Santiago del Estero, Argentina, in an area where insects have since been successfully controlled with deltamethrin.

For the susceptibility tests, first instars of each population were kept in enclosed boxes (30 × 30 × 30 cm) at 28 ± 1°C, 50–60% RH, and a photoperiod of 12:12 (L:D) h. A pigeon was weekly provided as a blood meal source (WHO 1994).

Chemicals. Technical grade deltamethrin (99.0%) and fipronil (95.5%) were obtained from Ehrestorfer (Augsburg, Germany). Analytical grade acetone was purchased from J. T. Baker (Edo. de Mex., Mexico). The 7-ethoxycoumarin (7-EC) and 7-hydroxycoumarin (7-OHC) (umbelliferone) were purchased from Sigma-Aldrich (St. Louis, MO). The *cis-trans*

(43.8% *cis;* 56.2% *trans*)-permethrinic acid was supplied by Chemotecnica (Buenos Aires, Argentina), and thionyl chloride (Cl_2SO; 99%) and triethylamine (+99%) were purchased from Aldrich Chemical (Milwaukee, WI). The 7-coumaryl permethrate (7-CP) was synthesized in our laboratory by the method of Santo Orihuela et al. (2006).

Topical Application Bioassays. Lethal doses were determined according to the World Health Organization protocol (WHO 1994). *T. infestans* first instars (5–7 d old; mean weight 1.3 ± 0.2 mg), unfed since emergence, were selected for the toxicity tests. Bioassays consisted of topical application on the dorsal abdomen with 0.2 μl of the insecticide diluted in acetone, using a 10 μl Hamilton syringe with automatic dispenser. Control groups received only pure acetone.

Three replicates of at least four doses in a range that produced between 10 and 100% mortality were conducted. Mortality was evaluated after 24 h by placing the insects at the center of a circular filter paper, 11 cm diameter; only those nymphs able to walk to the border of the filter paper were considered alive.

Statistical Analysis. Mortality data were analyzed using the POLO Plus software (LeOra Software 1987).
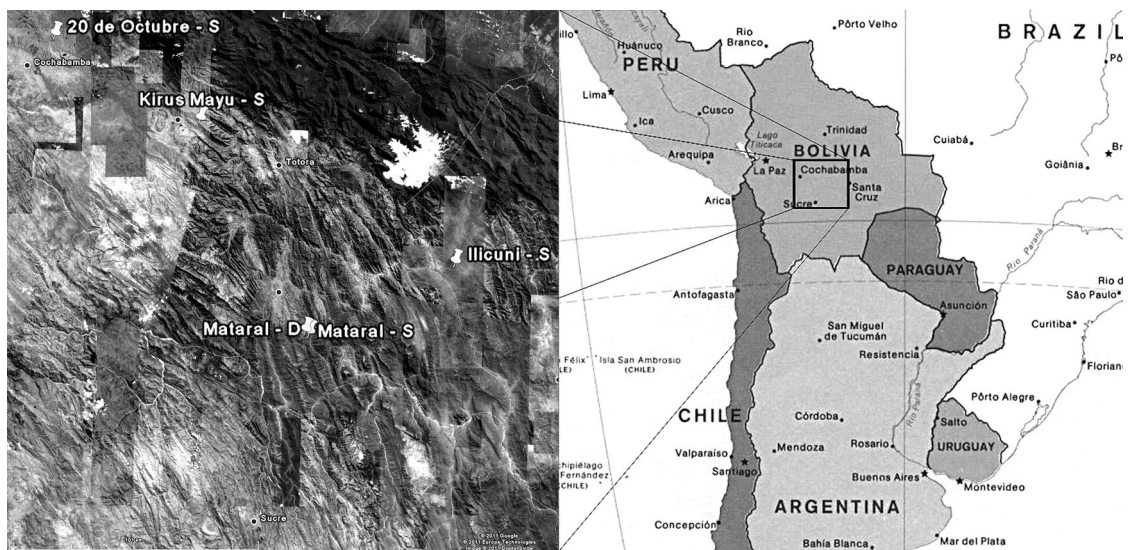

Fig. 1.   Map showing study sites where *T. infestans* samples were collected. The populations collected in Bolivia were as follows: Mataral (domiciliary and sylvatic), Ilicuni (sylvatic), 20 de Octubre (sylvatic), and Kirus Mayu (sylvatic).

Table 2. Toxicity of topically applied deltamethrin to *T. infestans* first instars of a susceptible reference strain, 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% CL)	LDR$_{50}$ (95% CL)	LD$_{95}$ ng/insect (95% CL)	LDR$_{95}$ (95% CL)
NFS[b]	120	3.10 ± 0.26	0.96	0.13 (0.11–0.15)	–	0.44 (0.58–0.36)	
Mataral–D[c]	186	1.11 ± 0.10	5.17	2.25 (0.28–4.80)	17.4 (11.88–25.43)	68.31 (24.82–2,220.54)	156.5 (78.548–311.70)
Mataral–S	90	1.55 ± 0.10	51.34	1.53 (0.53–3.34)	11.9 (9.43–14.93)	17.46 (6.52–385.42)	39.9 (5.08–312.96)
Kirus-Mayu–S	120	1.42 ± 0.08	34.72	0.95 (0.49–1.66)	7.4 (5.78–9.25)	13.82 (6.33–60.50)	31.6 (20.73–48.16)
20 de Octubre–S	120	1.50 ± 0.15	25.2	0.88 (0.08–1.84)	6.8 (4.98–9.26)	10.91 (4.59–393.10)	24.9 (15.95–38.93)
Ilicuni–S	120	1.33 ± 0.10	4.33	0.25 (0.14–0.39)	1.9 (1.44–2.56)	4.22 (2.09–13.82)	9.7 (5.74–16.25)

CL, confidence limit; LD$_{95}$, 95% lethal dose; LDR$_{50}$, 50% LDR; LDR$_{95}$, 95% LDR (calculated following Robertson et al. [2007]).

[a] Number of insects used for bioassays.

[b] Reference strain.

[c] Data from Toloza el al. 2008.

Dose-mortality data were subject to probit analysis (Litchfield and Wilcoxon 1994) to estimate the lethal dose (nanograms per insect) required to kill 50% of treated individuals (50% lethal dose [LD$_{50}$]). Lethal dose ratios (LDR) and 95% confidence limits of each population were calculated, as described by Robertson et al. (2007), by comparison of the dose-response curves between the studied populations and the reference strain. Studied populations were considered resistant if the LDR confidence limits did not include the number 1.

A nonparametric test (Kruskal-Wallis) was used to compare the enzymatic activities (P450 mono-oxygenases and pyrethroid esterases) of the studied populations.

Cytochrome P450 Mono-Oxygenase. Enzymatic activities were measured for individual abdomens using 7-EC-*O*-deethylation (Bouvier et al. 1998, González Audino et al. 2005). Fluorescence of 7-OHC was determined using a microplate fluorescence reader (Packard Fluorocount, Meriden, CT) with 400 nm excitation and 440 nm emission filters.

First-instar abdomens were placed individually into wells of a 96-well microplate containing 100 μl of 0.05 M phosphate buffer and 4 mM 7-EC. The reaction was stopped after incubation (4 h at 30°C) by adding 100 μl of glycine buffer (10^{-4} M), pH 10.4. 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 previous to incubation were used for blanks. The relative fluorescence units were all corrected for background hydrolysis, nonspecific fluorescence of substrate, and transformed to picomoles per minute (activity units) by using a calibration curve per replicate with dilutions of 7-OHC (60.19, 117.50, 172.50, and 224.33 total pmol/well).

Esterase Activity. Esterase activity was determined by the hydrolysis of 7-CP, a new fluorescent substrate appropriate for determining pyrethroid hydrolysis activity on individual insects (Santo Orihuela et al. 2006). For this, the insects were cooled and each nymph was homogenized in 220 μl of phosphate buffer (pH 7.2, 0.05 M) using a plastic mortar and pestle. Reaction was initiated by adding 10 μl of 7-CP (3.5 mM, 2-methoxy ethanol) to 190 μl of each homoge-

nate. Incubation was performed at 25°C for 33 min, at pH 7.2. The fluorescence was measured using a microplate fluorescence reader (Packard Fluorocount), and results were analyzed with Fluorocount and Excel 2000 software. Assays were conducted at 25°C in black 96-well polystyrene flat-bottom microtiter plates (Packard, Meriden, CT). Production of 7-OHC was monitored with excitation wavelength at 400 nm and emission at 440 nm. Activity was measured each 3 min for 33 min, such that assay was linear over the reported time. The relative fluorescence units were all corrected for background hydrolysis, nonspecific fluorescence of substrate, and transformed in picomoles per minute (activity units) using a calibration curve per duplicate with dilutions of 7-OHC (68.5, 342.69, 685.44, and 1370.8 total pmol/well).

Results

Toxicology. The sylvatic Mataral–S population showed a LD$_{50}$ similar to that of the domiciliary insects (Mataral–D) from the same area. Kirus-Mayu–S and 20 de Octubre–S populations showed LD$_{50}$ lower than Mataral insects, but higher than those previously reported for susceptible populations (Table 2).

Susceptibility values (LD$_{50}$ and 95% lethal dose), slopes of regression lines, and LDR to fipronil in the studied population are shown in Table 3. Mataral–D, Mataral–S, and Kirus-Mayu–S populations showed LDR (50%) between 23.4 and 139.2, values that differ significantly from 1, indicating that those populations were resistant to fipronil. The Ilicuni–S population showed a lower level of resistance, whereas the 20 de Octubre–S population showed susceptibility to fipronil.

As the slopes of dose-response curve of the studied populations were steeper than that of the reference strain, the LDR calculated at 95% mortality levels were higher than those calculated at 50% mortality. Because the toxicity values at 50% mortality have the lowest statistical error, the discussion of results is based on LDRs obtained at that level.

We had observed that sylvatic *T. infestans* tend to be larger than domiciliary *T. infestans*, but even expressing the deltamethrin and fipronil activity as ng insec-

Table 3. Toxicity of topically applied fipronil to *T. infestans* first instars of a susceptible reference strain, 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% CL)	LDR_{50} (95% CL)	LD_{95} ng/insect (95% CL)	LDR_{95} (95% CL)
NFS[b]	90	1.15 ± 0.11	11.55	2.12 (1.6–3.2)	–	56.86 (31.14–131.01)	–
Mataral—D	90	0.75 ± 0.09	3.98	296.0 (160.2–414.8)	139.2 (77.05–251.46)	4.62×10^4 (1.83×10^4–16.7×10^4)	819.1 (169.72–3,952.90)
Mataral—S	120	0.49 ± 0.05	13.43	49.8 (10.2–256.0)	23.4 (12.07–45.54)	10.62×10^4 (0.34×10^4–3.32×10^9)	1,900.9 (324.98–11,118.72)
Kirus-Mayu—S	120	0.78 ± 0.06	8.16	96.86 (32.0–332.0)	45.6 (26.67–77.80)	1.23×10^4 (0.31×10^4–12.6×10^4)	217.7 (72.41–654.43)
20 de Octubre-S	90	0.53 ± 0.09	5.05	1.0 (0.5–2.1)	0.5 (0.21–1.13)	1,294.9 (350.45–7,927.64)	23.2 (2.27–237.97)
Illicuni—S	90	1.16 ± 0.14	0.96	11.8 (8.6–17.0)	5.5 (3.39–8.97)	304 (140–1,048)	5.4 (1.65–17.41)

CL, confidence limit; LD_{95}, 95% lethal dose; LDR; LDR_{50}, 50% LDR; LDR_{95}, 95% LDR (calculated following Robertson et al. [2007]).
[a] Number of insects used for bioassays.
[b] Reference strain.

ticide per insect weight unit (Table 4), their lower susceptibility is apparent.

The frequency distribution of 7-OHC activities among domiciliary and sylvatic *T. infestans* populations is shown in Fig. 2. The frequency distribution of pyrethroid esterase activity is shown in Fig. 3. In these histograms, vertical lines are marked at values of 1.44 pmol/min for mono-oxygenases and 30.7 for pyrethroid esterase activity, which represent the corresponding threshold of enzyme activity containing the majority of insects (>75%) in susceptible populations.

There are no significant differences between the domiciliary, sylvatic, and reference populations in pyrethroid esterase and mono-oxygenase activity levels.

Discussion

This work presents the first study on toxicological profiles of sylvatic *T. infestans.* Those profiles included the susceptibility values to deltamethrin and fipronil, and the activities of detoxifying enzymes (pyrethroid esterases and mono-oxygenases), which were compared with those of a domiciliary population from the same Bolivian area and a reference colony. The toxicological profiles were different both among the Bolivian populations and between reference and Bolivian populations. All populations except one (20 de Octubre–S) were less susceptible than the NFS reference colony for the two insecticides, with the susceptibility to fipronil less than to deltamethrin. The domiciliary population, Mataral–D, was the least susceptible to both insecticides, followed by Mataral–S and Kirus-Mayu–S, whereas the 20 de Octubre–S and Illicuni–S populations were the most susceptible to both insecticides. These differences in the susceptibilities could not be explained by the activities of detoxifying enzymes given that there was no association between enzymatic activities and the susceptibility ratios. These comparative results support the idea that *T. infestans* from different geographic areas have different toxicological profiles determining differences in their susceptibility to insecticides (Germano et al. 2010).

The populations collected from sylvatic habitats had not been targeted by chemical control interventions. In addition, current evidence does not support a consistent flow of Andean *T. infestans* between sylvatic refuges and domestic habitats (Richer et al. 2007). In consequence, the toxicological profiles shown by sylvatic populations are unlikely to result from selection pressure with insecticide or gene flow from controlled areas, but those profiles would represent wild toxicological phenotypes. As those wild profiles were different, this study demonstrates that *T. infestans* from different geographic areas have different toxicological profiles.

As the Andean valleys in Bolivia are believed to represent the center of origin and dispersal of *T. infestans* (Bargues et al. 2006, Cortez et al. 2010), it is possible to speculate that the toxicological profiles of the sylvatic populations from Cochabamba could represent the ancestral toxicological profile of *T. infestans.* Nevertheless, phylogenetic evidence does not unequivocally support the hypothesized Andean origin

Table 4. Toxicity of deltamethrin against sylvatic (S) and domiciliary (D) populations of *T. infestans* from Andean valleys of Bolivia

Population	Weight (mg) (95% CL)	DL_{50} (ng/µg) (95% CL) deltamethrin	LDR	DL_{50} (ng/µg) (95% CL) fipronil	LDR	DL_{95} (ng/µg) (95% CL) deltamethrin	LDR	DL_{95} (ng/µg) (95% CL) fipronil	LDR
NFS[a]	1.31 (0.07)	0.10 (0.09–0.11)	–	1.52 (1.22–2.44)	–	0.34 (0.44–0.27)	–	43.40 (23.77–100)	–
Mataral–D	1.94 (0.22)	0.86 (0.14–2.47)	8.7	152.58 (82.58–213.81)	100.38	35.21 (5.72–1,144.61)	104.83	2.38×10^4 (9.43×10^3–8.60×10^4)	548.38
Mataral–S	2.31 (0.04)	0.66 (0.23–1.45)	6.70	21.56 (4.41–110.82)	14.18	7.56 (2.82–166.85)	22.24	4.60×10^4 (1.47×10^3–1.44×10^9)	1,059.90
Kirus-Mayu–S	2.19 (0.41)	0.43 (0.22–0.76)	4.38	44.24 (14.61–151.60)	29.10	6.31 (2.89–27.63)	18.56	5.61×10^3 (1.41×10^3–5.75×10^4)	129.26
20 de Octubre–S	2.33 (0.10)	0.37 (0.03–0.79)	3.81	0.43 (0.21–0.90)	0.28	4.68 (1.97–168.71)	13.76	555.75 (150.41–3,402.42)	12.81
Ilicumi–S	1.95 (0.05)	0.13 (0.07–0.20)	1.30	6.05 (4.41–8.72)	3.98	2.16 (1.07–7.09)	6.35	155.90 (71.80–537.44)	3.59

Activity expressed as ng of insecticide per insect weight unit (ng/µg). CL, confidence level.

[a] Susceptible reference strain.

of *T. infestans*, and instead suggests an origin in the Gran Chaco (Torres-Pérez et al. 2011). Future studies should be conducted to test those evolutionary and toxicological hypotheses.

The situation is different for the domiciliary population, which showed the lowest susceptibility to both deltamethrin and fipronil. This result is in accordance with Toloza et al. (2008) and Germano et al. (2010), who detected a low susceptibility to fipronil in domiciliary populations with moderate resistance to deltamethrin in the center and south of Bolivia. The toxicological profile of those populations can be interpreted in terms of resistance. As the pyrethroids alphacypermethrin and deltamethrin have been widely used for control of domestic *T. infestans* during the last decade, the evolution of pyrethroid resistance could be the consequence. However, Germano et al. (2010) discussed the possibility of cross-resistance between fipronil and lindane or dieldrin due to the early use of these compounds in Bolivia.

The higher resistance level to fipronil than that to deltamethrin in the domiciliary population of Mataral could be explained in terms of the alleged evolutionary relationship between domiciliary and sylvatic populations of *T. infestans* from that area. As yet there is no evidence for recolonization of sylvatic habitats from a domiciliary population, so the hypothesis that domiciliary *T. infestans* have derived from sylvatic populations is favored. The sylvatic populations from Mataral would express the wild toxicological profile of *T. infestans* from that area, as follows: low susceptibility to deltamethrin and fipronil, with lower fipronil than deltamethrin susceptibility. In this context, it is possible to hypothesize that the toxicological baseline of domiciliary Mataral *T. infestans* was the wild toxicological profile of Mataral. Similarly, because the sylvatic Andean *T. infestans* showed lower susceptibility than the reference colony from Argentina, it may be that the toxicological profile of the reference susceptible population could be representing a wild toxicological profile of *T. infestans* from Argentina. This would imply the existence of different wild profiles for Argentina and Bolivia.

Toxicological differences between Argentinean and Bolivian populations were demonstrated in previous studies. Toloza et al. (2008) showed that deltamethrin resistance is shown in nymphs from Yacuiba (Bolivia), but not in eggs, whereas both the eggs and nymphs from Salvador Mazza (Argentina) express resistance to this insecticide. Toloza et al. (2008) and Germano et al. (2010) detected fipronil resistance in Bolivia, but not in Argentina. Santo Orihuela et al. (2008), studying the same populations, showed that the esterase activity was increased only in the population of Salvador Mazza. Those differences could have resulted from selection pressure with insecticides on populations of *T. infestans* with different wild toxicological profiles.

The susceptibility and biochemical differences may relate to the genetic structure of the *T. infestans* populations. Several studies confirmed that populations of *T. infestans* from Argentina and Bolivia are highly structured, both at macrogeographical and microgeo-
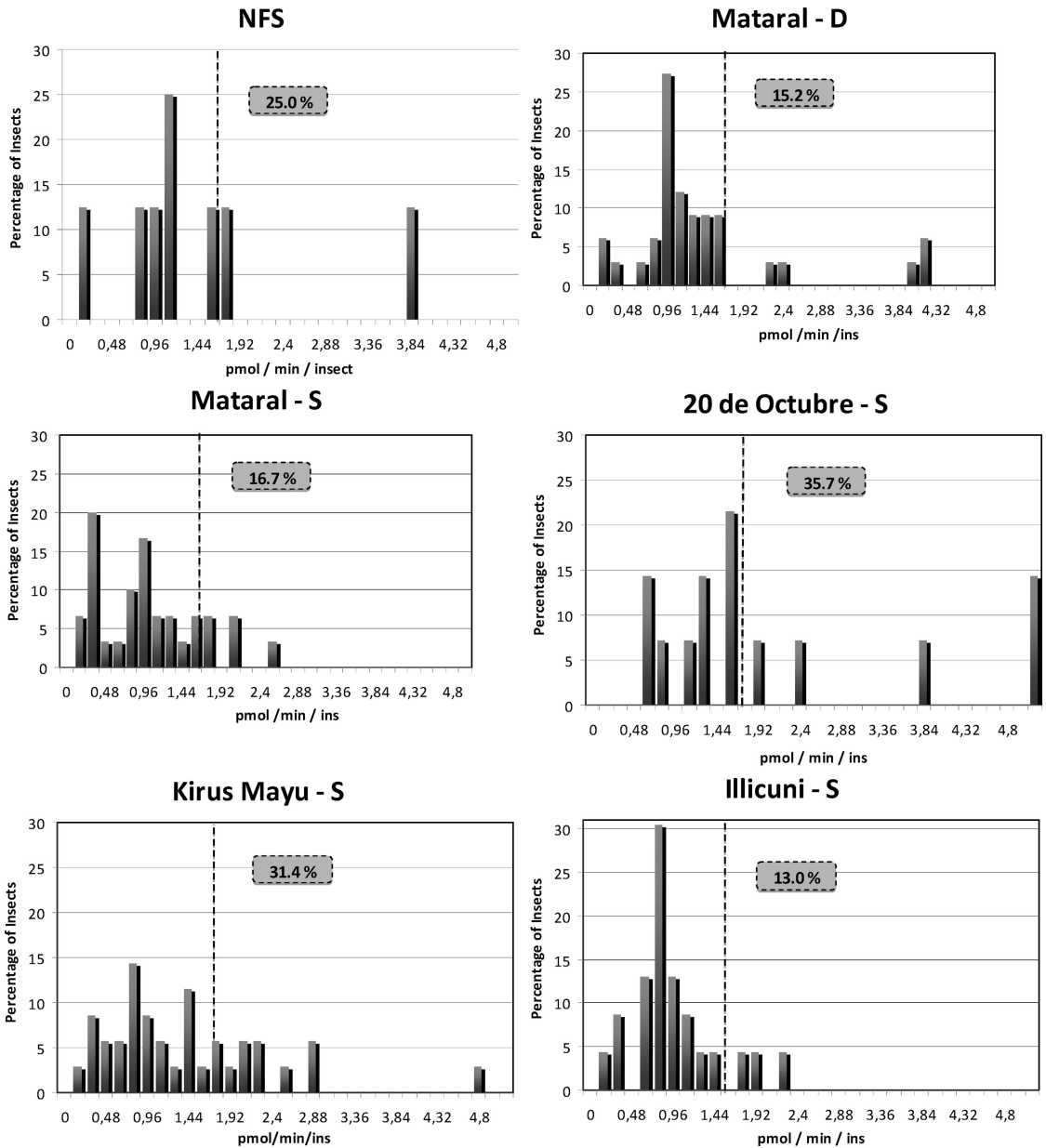
Fig. 2. Frequency distribution of P450 mono-oxygenase activity among sylvatic (S) and domiciliary (D) *T. infestans* from Andean valleys of Bolivia. Vertical lines are marked at values of 1.44 pmol/min, which represent the corresponding threshold of enzyme activity containing the majority of insects (>75%) in susceptible populations. Enzyme activity as picomoles of 7-OHC per minute.

graphical levels (Pérez de Rosas et al. 2007, 2008; Marcet et al. 2008). Furthermore, recent research demonstrated that Bolivian and Argentinian populations are part of different haplotype clusters (Monteiro et al. 1999, Piccinali et al. 2009). In toxicological bioassays, the slopes of the dose-response relationships are indicators of the population's phenotypic variation, e.g., high slopes indicate little phenotypic variation. If environmental variation were constant, the slope would be an indicator of genetic variation, with high slopes related to low genetic variation, and this would occur in populations fully susceptible or fully resistant. By contrast, populations in process of selection would have greater genetic variation, and therefore the slopes will be less steep (Chilcutt and Tabashnik 1995). This relationship seems to be observed in the susceptible population because they show higher slope values to both deltamethrin and fipronil. Field populations show a lower slope (except for Ilicuni–S and fipronil), suggesting greater genetic variability.
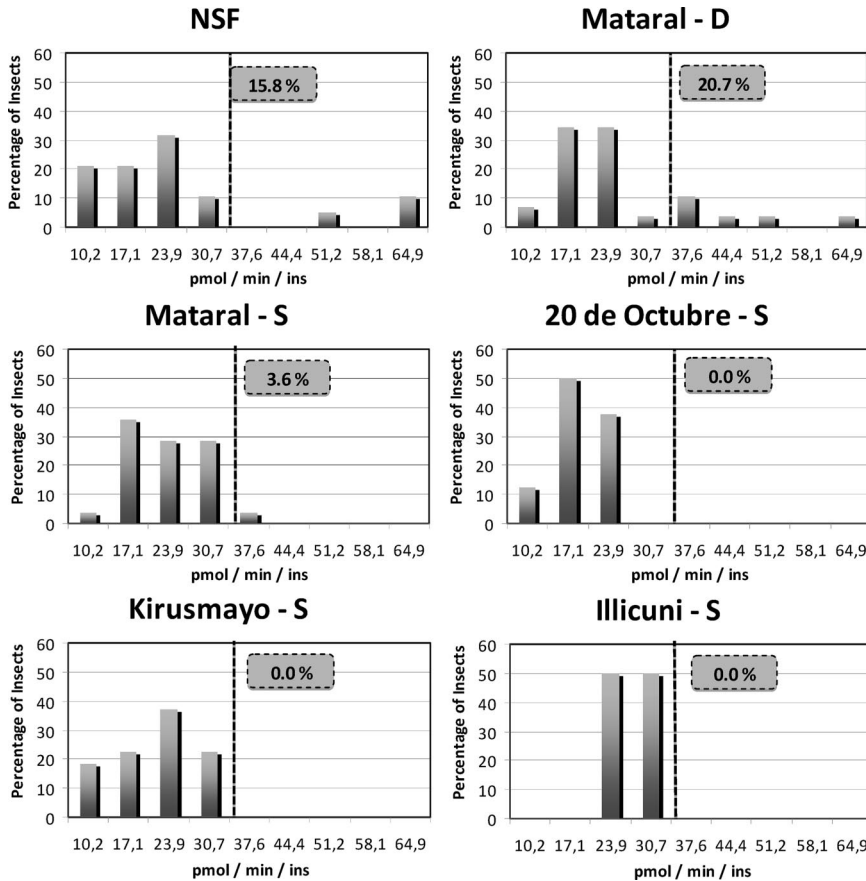
Fig. 3. Frequency distribution of pyrethroid esterase activity among sylvatic (S) and domiciliary (D) *T. infestans* from Andean valleys of Bolivia. Vertical lines are marked at values of 30.7 pmol/min, which represent the corresponding threshold of enzyme activity containing the majority of insects (>75%) in susceptible populations. Enzyme activity as picomoles of 7-OHC per minute.

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