

Toxicity of pyrethroids and repellency of diethyltoluamide in two deltamethrin-resistant colonies of *Triatoma infestans* Klug, 1834 (Hemiptera: Reduviidae)

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The aim of the current investigation was to evaluate (a) the toxicity of three pyrethroids (deltamethrin, lambda-cyhalothrin, and tetramethrin); (b) the effect of these insecticides on the locomotor activity; and (c) the repellent effect of N,N-diethyl-m-toluamide (DEET) on two deltamethrin-resistant strains of Triatoma infestans from Argentina (El Chorro and La Toma), and one susceptible strain. The resistance ratios (RRs) obtained for the La Toma strain were: > 10,769, 50.7, and > 5.2 for deltamethrin, lambda-cyhalothrin, and tetramethrin respectively. The RRs for the El Chorro strain were: > 10,769, 85.8, and > 5.2 for deltamethrin, lambda-cyhalothrin, and tetramethrin respectively. The hyperactivity usually caused by the three pyrethroids was in both the deltamethrin-resistant strains compared to the susceptible reference strain. No differences were observed in the repellent effect of DEET between the three groups. These results indicate that the deltamethrin-resistant insects have a cross resistance to lambda-cyhalothrin and tetramethrin, and are also resistant to the first symptom of pyrethroid poisoning (hyperactivity). However, the sensorial process related to DEET repellency does not appear to be altered.

Key words: *Triatoma infestans* - blood-sucking insects - pyrethroid resistance - hyperactivity - repellency

Pyrethroid insecticides have been used for over 20 years to control the vectors of Chagas disease in Argentina and other Latin American countries (Zerba 1999). Over the last ten years, some resistance has been detected in Brazilian and Venezuelan triatomines (Vassena et al. 2000). Deltamethrin is commonly used for the chemical control of *Triatoma infestans* in several Latin American countries. In general, it is a very effective triatoinicide, as recently shown by Rojas de Arias et al. (2004).

Low pyrethroid resistance levels have also been found in several areas of Argentina (Vassena & Picollo 2003, González Audino et al. 2004). Lambda-cyhalothrin is also used, but less frequently. Tetramethrin is generally not used as it has not proven to be a very effective triatoinicide. However, due to its flushing-out effect on triatomines (Wood et al. 1993), it is widely used for monitoring the density of the triatoinine population (Pinchin et al. 1980).

In 2002, the Health Authorities for the Control of Vectors in Argentina reported failures in the chemical control of *T. infestans* in the surroundings of Salvador Mazza

(San Martín Department, Salta Province). In this region, deltamethrin has been the main factor of selective pressure. Similar problems were also observed at almost the same time in Yacuiba, a small city near Salvador Mazza, over the Bolivian border.

Several colonies, originated from insects collected in the Department of San Martín, have shown high deltamethrin resistance levels when this insecticide was topically applied in the laboratory (Picollo et al. 2005). Additional studies suggested that the resistance observed in these colonies was partly due to changes in the voltage-dependent sodium channels, the target site of pyrethroid insecticides. This mechanism is known as *kdr*.

We have previously proposed a hypothesis based on the *kdr* mechanism in order to explain the resistance of the triatoinine colonies of La Toma and El Chorro (Picollo et al. 2005). Changes in voltage-dependent sodium channels could be altering the normal function of the nervous system of these insects. For this reason, we were interested in studying the behavior of pyrethroid-resistant colonies exposed to an external stimulus that delivers a response controlled by the nervous system. For this, the hyperactivity produced by a sublethal dose of pyrethroids and repellency to the N,N-diethyl-m-toluamide (DEET) repellent (Khan 1977) were measured in susceptible and resistant *T. infestans* strains. Before determining the behavioral responses, the toxicity in *T. infestans* of the pyrethroids used in this study were assessed. For this purpose, the effective concentrations to produce 50% mortality (EC50) for deltamethrin, lambda-cyhalothrin, and tetramethrin, all applied by contact on filter paper films, were obtained as well as the respective resistance ratios (RRs).

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MATERIALS AND METHODS

Biological material - Three different strains of *T. infestans* first instar nymphs were used: an insecticide susceptible strain reared in our laboratory since 1975 (Cipein strain), and two strains came from individuals collected in the localities of El Chorro and La Toma, Department of San Martín (Salta Province, Argentina), during 2004 (El Chorro and La Toma strains). The insects were reared at 28°C and, in all the experiments, they were 1-5 days old and had been starved since hatching.

Chemicals - The technical grade insecticides used for the bioassays were: deltamethrin (Bayer, Buenos Aires, Argentina), lambda-cyhalothrin (Syngenta, Basel, Switzerland), and tetramethrin (Sumitomo Chemical Co., Osaka, Japan). DEET was 97% pure (Aldrich, Milwaukee, WI), and the acetone was of analytical grade (Merck, Darmstadt, Germany).

Effective concentration 50% - EC50 is the concentration of a compound necessary to produce a certain effect on 50% of the population of test animal species. To estimate this parameter, 4 cm diameter circles of Whatman No.1 filter paper (Whatman International Ltd., Maidstone, England) were treated with pyrethroid solutions in acetone. After allowing the solvent to evaporate for 30 min, the circles were placed on the floor of plastic containers (4 cm diameter × 4 cm high). Groups of ten nymphs were added to each container; they were covered with plastic tops and placed in a chamber with a constant temperature of 28°C and 60-90% RH. The control groups were exposed to filter papers treated only with acetone. The effects of the insecticide (incoordination and knock down) were recorded 24 h later. The incoordination has been previously defined as the incapacity of the insects to leave a filter paper disc (15 cm in diameter) (Alzogaray & Zerba 1996). At least four different concentrations of pyrethroid, ranging between 0.07 and 700 µg/cm², were used to estimate each EC50 value, except when otherwise is stated.

Each experiment was replicated a minimum of five times and the EC50 values were calculated using the probit method (Litchfield & Wilcoxon 1949). Differences between the values were considered significant ($P < 0.05$) when the respective 95% confidence limits did not overlap.

The resistance ratio (RR) values were calculated as the quotient between the EC50 of the field derived colony and the EC50 value of the susceptible strain (Cipein). The 95% confidence limits for each RR were calculated as described by Robertson and Preisler (1992).

Locomotor activity

Test arena - Evaluations were performed in a glass ring (2.5 cm high × 5 cm diameter). The test arena floor was covered with a rectangular piece (9 × 8 cm) of white No. 1 filter paper, pretreated as described below.

Recording equipment - A closed-circuit black and white video camera (VC 1910, Sanyo Electrical Co., Tokio, Japan) was placed 15 cm above the center of the test arena. An image analyzer (Videomex V, Columbus, OH) converted the analogue signal input from the video camera to digital data. The resolution was 256 × 192 pixels and the acquisi-

tion and processing speed was 30 frames s⁻¹. In the monitor, the video signal colors are inverted and therefore white objects appeared to be black and vice versa. Thus, the presence of insects in the arena was determined by a visual contrast between the individuals (white) and arena background (dark) and scored as the number of *on* pixels. The locomotor activity was recorded using the Multiple Zones Motion Monitor for Videomex software, which records the movement of multiple objects in one area. Each set of data was imported and handled in a personal computer.

Experimental procedure and data analysis - Filter papers were treated with 0.5 ml of pyrethroid acetone solutions or with acetone only (control), 30 min before starting each experiment in order to allow the solvent to evaporate. After previous screenings, the pyrethroid concentrations chosen were 7 µg/cm² for deltamethrin and tetramethrin, and 0.7 µg/cm² for lambda-cyhalothrin. The floor of the test arena was covered with the treated filter paper, and a group of three nymphs was placed on it. A 14 W fluorescent tube (Sylvania, São Paulo, Brazil) was placed beneath the plane of the test arena in order to illuminate it. In this way, the shadows from the camera and the nymphs were eliminated, obtaining a much better resolution. Nymphs movement was recorded for 30 min.

The Videomex-V software compares consecutive images and quantifies the number of pixels that change from *on* to *off*, and vice versa, as a measurement of insect motion (M). It also quantifies the number of *on* pixels as a measurement of the area (A) occupied by insects. To obtain independent values of the changes in insect position during the experiment, we calculated the values of locomotor activity (LA) = M/A, as defined by Alzogaray et al. (1997). Finally, we defined the parameter of locomotor activity variation (LAV) = LA(t) - LA(c), where LA(t) is the LA in insects exposed to the insecticide, and LA(c) is the LA in the control group. Each experiment was repeated five times. The results are expressed in units of pixels/area and were analyzed using one-way ANOVA followed by Tukey's *post hoc* test. The level of significance considered was 0.05.

Repellency

The test arena and recording equipment used were as previously described.

Experimental procedure and data analysis - Rectangular pieces of filter paper were cut into halves (Zones I and II). Zone I was treated with 0.25 ml of acetone, Zone II was treated with 0.25 ml of a DEET solution in acetone (7 or 70 µg/cm²). After the acetone evaporation (30 min), the filter paper halves were fitted together to make a single layer to cover the test arena floor. For the controls, both filter paper halves were treated only with acetone.

In order to determine the distribution of the nymphs on the test arena, the TV field image was divided into two zones using the Multiple Zones Motion Monitor for Videomex software. In each zone, the area (expressed in pixels) occupied by the nymphs during the experiment was recorded. The distribution of the nymphs was recorded for 30 min. The results were expressed as a repel-

lency coefficient (RC) = $(1 - A(II)/A(I)) \times 100$, where A(I) is the area occupied by insects in Zone I, and A(II) the area occupied in Zone II. When CR = 0, the distribution of the insects is random; as RC values increase, the repellent effect is higher. Each experiment was repeated four times, and the results were analyzed using one-way ANOVA. The level of significance considered was 0.05.

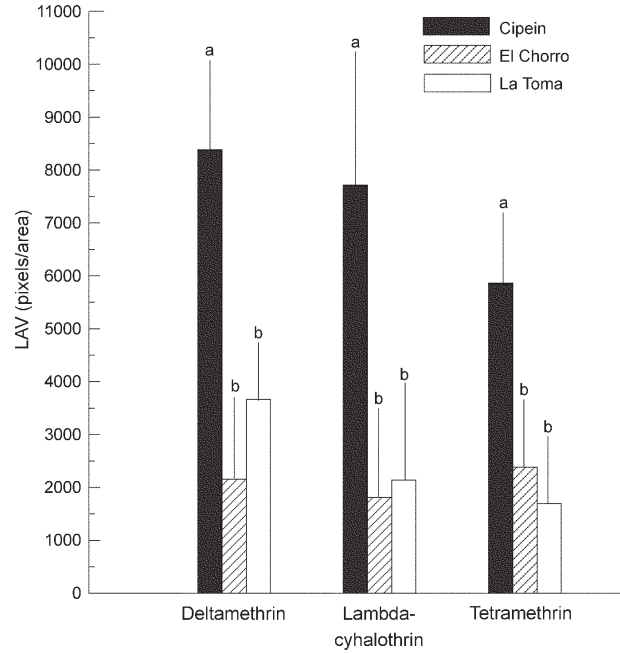
RESULTS

Table I shows the data of the toxicity of deltamethrin, lambda-cyhalothrin and tetramethrin for the El Chorro and La Toma colonies and for the reference susceptible strain (Cipein) of *T. infestans*. The EC50 values for deltamethrin and tetramethrin could not be calculated because the responses obtained using the maximum concentration were below 50% (the maximum concentration used in both cases was the acetone limit of solubility for these insecticides).

Insects from El Chorro and La Toma colonies showed an extremely high resistance to deltamethrin. The RRs estimated for this insecticide were > 10,769 in both colonies. The RR values for tetramethrin were also estimated and were > 5.2 in both colonies. The RRs for lambda-cyhalothrin were 85.5 for El Chorro and 50.7 for La Toma, indicating in both cases high levels of resistance.

The Figure shows the effect of deltamethrin, lambda-cyhalothrin, and tetramethrin exposure on the locomotor activity of *T. infestans* nymphs. In all cases, the LAV induced by pyrethroid exposure was significantly lower for the resistant individuals compared to those of the Cipein susceptible strain ($P < 0.05$). This result suggests that these colonies also have a certain resistance to the hyperactivity phenomenon.

The RCs for 70 µg/cm² DEET in El Chorro, La Toma, and Cipein, are summarized in Table II. In all cases, the RCs reached values close to 100. These results indicate an evident repellent effect of DEET, even in pyrethroid-



Hyperactivity produced by pyrethroid exposure on first instar nymphs from a susceptible and two pyrethroid-resistant strains of *Triatoma infestans*. LAV: locomotor activity variation; defined as LAV = LA(t) - LA(c), where LA(t) is the locomotor activity of the insects exposed to the insecticide, and LA(c) is the locomotor activity in the control group. Different letters indicate significant differences ($P < 0.05$, one-way ANOVA).

resistant individuals. No significant differences among the RCs were observed ($P > 0.05$). Similar results were obtained when insects were exposed to a tenfold decreased concentration of DEET (7 µg/cm²) (data not shown).

TABLE I
Toxicity of three pyrethroids on first instar nymphs from a susceptible and two pyrethroid-resistant strains of *Triatoma infestans*

Strain	Slope ± SE	EC50 (µg/cm ²)	Confidence intervals 95%	Resistance ratios (CI 95%) (contact application)	Resistance ratios ^a (topical application)
Deltamethrin					
El Chorro		> 1,400		> 10,769	99.0
La Toma		> 1,400		> 10,769	86.9
Cipein	4.0 ± 0.3	0.1	0.1 - 0.3		
Lambda-cyhalothrin					
El Chorro	1.5 ± 0.1	23.2 ^a	17.0 - 32.9	85.8 (56.1 - 131.1)	288.7
La Toma	1.7 ± 0.1	14.4 ^a	10.9 - 19.8	50.7 (34.1 - 75.4)	141.7
Cipein	6.3 ± 1.3	0.3 ^b	0.2 - 0.3		
Tetramethrin					
El Chorro		> 1,400		> 5.2	
La Toma		> 1,400		> 5.2	
Cipein	1.6 ± 0.6	270.0	133.6 - 404.9		

Resistance ratios: defined as LD50 *fd* / LD50 *is*, where *fd* is field-derived strain, and *is* is the insecticide-susceptible strain. Values followed by the same letter are not significantly different ($P < 0.05$, based on non overlapping of the IC 95 %). *a*: data in this column are from Picollo et al. (2005).

TABLE II

Repellency coefficients of N,N-diethyl-m-toluamide on first instar nymphs from a susceptible and two pyrethroid-resistant strains of *Triatoma infestans*

	RC ± SE
El Chorro	99,7 ± 0,21 ^a
La Toma	99,5 ± 0,50 ^a
Cipein	99,9 ± 0,06 ^a

RC: repellency coefficient; defined as $RC = (1 - AII/AI) \times 100$, where AII is the area occupied by insects in Zone II treated with DEET and AI is the area occupied by insects in Zone I, treated only with acetone. *a*: values followed by the same letter are not significantly different ($P > 0.05$, one-way ANOVA).

DISCUSSION

Previous studies performed in our laboratory showed that, when insecticides were topically applied, the RR values for *T. infestans* colonies from the Salvador Mazza area (Department of San Martín, Salta Province) ranged from 55.5 to 133.1 (Picollo et al. 2005). It is possible that the development of resistance towards deltamethrin and other pyrethroids in *T. infestans* from Salvador Mazza is due to the continuous exposure of these insects to the pyrethroids used in vector control programs. As several disease vectors are endemic to this region of the Salta Province, it is usually submitted to a number of insecticide application programs for the control of triatomines as well as other insects.

Knock down - ED50 and EC50 values obtained in laboratory bioassays are affected by a number of factors, such as the vehicle used, environmental temperature, and application methods. The EC50 values obtained in this work allowed us to calculate values of RR and to compare them with RR values obtained by topical application in our laboratory (Picollo et al. 2005).

EC50 is the toxicological parameter that is obtained when the concentration of insecticide applied is known but the exact amount of insecticide incorporated by each individual is not. This usually happens when insects are exposed to insecticides by making them walk over treated surfaces. ED50 values can be calculated when the exact amount of insecticide received by each insect is known. This is possible with topical application, in which a known volume of an insecticide solution is delivered directly onto the surface of the insect's body.

The RRs of lambda-cyhalothrin obtained with topical application bioassays were 288.7 and 141.7 for El Chorro and La Toma respectively (Picollo et al. 2005). The RR values calculated by the exposure to filter papers treated with the same insecticide were 85.5 for El Chorro and 50.7 for LA TOMA.

Scharf et al. (1995), found similar results in a chlorpyrifos- and cypermethrin-resistant strain of *Blattella germanica*. The RR values ranged between 5.2 and 32.3 when resistant insects were topically treated with clorpyrifos and cypermethrin, while RRs ranged between 1.5 and 5.3 when obtained by exposure to surfaces treated with the same insecticides. Another study, in which a

pyrethroid-resistant strain of *B. germanica* was topically treated with deltamethrin, reported RR values between 17.7 and 42.4, while the RRs obtained using the exposure method were between 2.2 and 22 (Wi Choo et al. 2000).

The differences in the RRs calculated from data obtained by various application methods could be due to certain features of each method that influence the penetration of the insecticide to the insect body, or its bioavailability. Scott et al. (1986) demonstrated that insecticides applied by filter paper exposure elicit their action mainly on the peripheral nervous system, more specifically at the neuromuscular junction level. However, topically applied insecticides reach the central nervous system rapidly via the hemolymph (Soderlund 1979).

Field conditions are better reproduced in the laboratory using the filter paper method, which is also easier to perform, although it requires higher amounts of insecticide solutions. For this reason, when the resistance levels are high, the solubility limit of the insecticide in the solvent could be a limiting factor to obtain finite EC50 values via this method. We encountered this difficulty with both deltamethrin and tetramethrin, as the maximum concentration used, which was close to their acetone solubility limit, did not produce any observable symptoms in resistant insects. It was therefore not possible to calculate the respective EC50 values for the resistant colonies.

A similar situation was reported by Vassena et al. (2003), who were not able to determine the EC50 values of permethrin using the filter paper method in a pyrethroid-resistant strain of *Pediculus humanus capitis*. The maximum concentration tested, allowed by the insecticide's solubility, was not enough to produce responses greater than 50% in the resistant head lice.

When a solid insecticide with a melting point significantly higher than the environmental temperature, i.e. deltamethrin, is applied as an acetone solution, it crystallizes in the treated filter paper. It has previously been reported that the crystallization of solid insecticides reduces their toxicity to different insect species (Busvine 1971). Also, the physicochemical properties of each molecule or the particular features of each application method, could be influencing the results by affecting the bioavailability of the insecticides. The toxicity of the same molecule assayed with the same insect species can differ according to the application method.

Concerning tetramethrin toxicity in *T. infestans*, we observed that this pyrethroid compound has a poor triatomocidal activity (Casabé et al. 1988). El Chorro and La Toma colonies showed cross resistance to tetramethrin, which has not been used for the control of *T. infestans* in the field. It was not possible to determine a finite RR value for this pyrethroid using the filter paper method because of its acetone solubility limit.

Locomotor activity - The first observable symptom in insects treated with pyrethroids is an increase in their locomotor activity (Gammon 1978, Miller & Adams 1982, Benoit et al. 1985, Alzogaray et al. 1997). Poisoned insects suffer a number of symptoms such as incoordination, tetanization, prostration and death, as the process of intoxication proceeds (Gammon 1978). Tetanization is associated

to a knock down effect, defined as a rapid and reversible paralysis, commonly caused by pyrethroids.

There is a clear difference of opinions concerning the mechanism underlying the sublethal effects of pyrethroids, particularly on whether knock down, hyperactivity, and death are manifestations of the same process of intoxication (Ruigt 1985). An important point in this discussion is the location of the target sites for the sublethal effects in the insects' nervous system. It is still not clear whether these effects occur mainly in the central or peripheral nervous system.

Burt and Goodchild (1971) observed a close correlation between changes in the amount of spontaneous activity in the sixth abdominal ganglion of cockroaches treated with pyrethrin I and the course of poisoning. They observed that the giant fibers only began to show abnormalities when the insects had already been postrated for 2 h and thus concluded that the sublethal effects was possibly associated with damage to ganglia in the central nervous system.

However, other authors have observed that sublethal effects, particularly knockdown, result from the excessive stimulatory effect of pyrethroids on sensory nerves and organs located close to the surface, causing a disruption of the normal information flow towards the central nervous system.

On the other hand, hyperactivity seems to be caused by the repetitive firing of motor neurons elicited by pyrethroids (Adams & Miller 1979). These results suggest that hyperactivity and knockdown are produced by effects on both the peripheral sensory system and the motor nervous system.

Hyperactivity induced by pyrethroids produces involuntary insect movements and it is the cause of the insecticide's flushing out effect observed in triatomines (Wood et al. 1993). Tetramethrin, formulated as an aerosol, is used in the field to assist the capture of triatomine bugs during surveys monitoring the insect population (Pinchin et al. 1980). In this research work we found that the hyperactivity elicited by sublethal concentrations of pyrethroids on the resistant individuals was lower than the hyperactivity observed in the susceptible ones.

Previous results from our laboratory suggest that the main mechanism of resistance in the insect colonies we have studied could be a reduction in their nervous sensibility, a mechanism known as *kdr* (Picollo et al. 2005). An increase in enzymatic activity would be a minor contributor to the resistant phenotype.

Due to its speed, the phenomenon of hyperactivity would not be affected by the degradative metabolism. It can therefore be considered that the resistance to hyperactivity observed in El Chorro and La Toma colonies is more related to changes in nerve membranes than with an increase in the activity of detoxicant enzymes.

Repellency - Enzymes, receptors and ionic channels with a determined physiological function, are usually the targets of insecticides. The effect of the insecticide consists in altering this function. When the resistance is due to changes in the target site, the resistant individuals carry enzymes, receptors and ionic channels that differ from

those of the susceptible individuals. It is interesting to ask whether these differences affect their normal functionality and thus influence the fitness of the resistant insects in an environment free from the insecticide pressure (McKenzie 1996).

Foster et al. (1999) studied the response to alarm pheromones, an important component of aphid fitness, in a controlled laboratory bioassay using a large number of clones of *Myzus persicae* with various combinations of *kdr* and esterase based insecticide resistance. *Kdr*-homozygous and heterozygous genotypes showed noticeably lower levels of disturbance responses after the exposure to the alarm pheromone, (E)- β -farnesene, compared to aphids without *kdr*. As voltage-dependent sodium channels have an important role in the transmission of nerve impulse, changes in these channels possibly interfere with the normal decoding of nervous signals produced, for example, by sensory stimuli.

Bearing this in mind, we wanted to know whether changes present in deltamethrin-resistant triatominae from El Chorro and La Toma colonies could interfere in the normal response to sensory stimuli. To test this hypothesis we exposed resistant and susceptible insects to DEET repellent and measured the insect response.

El Chorro and La Toma insects showed a clear repellency response towards DEET. Besides, the response was not significantly different from that elicited by the repellent in susceptible individuals. If the nervous system of the resistant insects has modifications conferring resistance, our results suggest that they do not affect the repellent action of DEET.

In conclusion, the results obtained in this work using the filter paper method to measure toxicological responses confirm previous results obtained in our laboratory on the high resistance to pyrethroids in El Chorro and La Toma *T. infestans* colonies. These resistant colonies showed a decreased response to the hyperactivity produced by sublethal concentrations of pyrethroid compounds but a normal response to the repellency produced by DEET. The high RR values obtained by exposure to treated filter paper and the resistance to the pyrethroid-induced hyperactivity are consistent with a possible *kdr* mechanism in the pyrethroid-resistant *T. infestans* arising from La Toma and El Chorro colonies. Further investigations to confirm this hypothesis are currently being undertaken in our laboratory.

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