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A methodology based on insecticide impregnated filter paper for monitoring resistance to deltamethrin in *Triatoma infestans* field populations

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Abstract. The domiciliary presence of *Triatoma infestans* (Klug) (Hemiptera: Reduviidae) after control interventions was reported in recent years. Toxicological studies showed high levels of resistance to pyrethroids suggesting resistance as one of the main causes of deficient control. The aim of the present study was to develop a protocol to test resistance to deltamethrin in *T. infestans* collected from the field by discriminate concentration. To evaluate field insects, the effect of age (early vs. later) and nutritional state (starved vs. fed) on the deltamethrin susceptibility of each developmental stage was studied. Topical and insecticide impregnated paper bioassays were used. Using the impregnated paper, the susceptibility to deltamethrin was not affected by the age of the stadium and the nutritional states, and varied with the post-exposure time and with the different developmental stages. A discriminant concentration of deltamethrin (0.36% w/v) impregnated in filter paper was established for all developmental stages. Finally, the methodology and the discriminant concentration were evaluated in the laboratory showing high sensitivity in the discrimination of resistance. The present study developed a methodology of exposure to insecticide impregnated papers and proposes a protocol to test *T. infestans* in field populations with the aim to detect early evolution of resistance to deltamethrin.

Key words. *Triatoma infestans*, bioassays, impregnated papers, insecticide resistance, resistance monitoring.

Introduction

Chagas disease is a chronic parasitic infection, and vectorial transmission is restricted to America (WHO, 2015). As no effective treatment is available for the chronic forms of the disease, vector chemical control represents the best way to reduce the incidence of disease (Zerba, 1999). After application of pyrethroids insecticides in dwellings and peri-domiciliary areas, intergovernmental control programmes reduced the geographic range and infestation prevalence of major triatomine vectors leading to the interruption of transmission mediated by *Triatoma infestans* in some countries of the Southern Cone (Brazil, Chile, Uruguay and provinces/departments from Argentina, Paraguay, Bolivia, and Peru), as well as decreasing infestations with *T. dimidiate* and the distribution of *Rhodnius prolixus* in Central America (Schofield *et al.*, 2006; Gürtler, 2009; Salvatella *et al.*, 2014). However, the domiciliary presence of *T. infestans* after control interventions has repeatedly been reported in recent years (Mougabure-Cueto & Picollo, 2015). Toxicological studies on insects, mainly from northern Argentina and southern of Bolivia, showed high levels of resistance to pyrethroids suggesting the evolution of resistance as one of the main causes

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of deficient control (Picollo *et al.*, 2005; Toloza *et al.*, 2008; Germano *et al.*, 2010, 2012, 2013; Lardeux *et al.*, 2010; Gomez *et al.*, 2014).

Insecticide resistance is a natural selection process that results from the differential toxic action of the insecticide on individuals exhibiting genetic variation for toxicological susceptibility (Roush & McKenzie, 1987). As the emergence of resistant individuals is unpredictable, a resistance management strategy should be implemented oriented to detect the resistance at the lowest possible level and to avoid the selection process continuing (Mougabure-Cueto & Picollo, 2015). Toxicological monitoring is the follow up in time of insecticide susceptibility in pest populations that are subject to chemical control and represent the basis of that strategy. Monitoring on a large geographical scale is usually carried out in two phases. In the first phase, groups of insects of each population are exposed to a dose/concentration that theoretically kills 100% of susceptible individuals (i.e. discriminant dose/concentration). The population is considered susceptible if 100% mortality (or tolerated survival below a certain threshold) is recorded and ends the study. By contrast, the population is considered resistant if survival (or survival above a certain threshold) is recorded and in the second phase a dose–response study is performed, and the resistance level is determined (ffrench-Constant & Roush, 1990).

The protocol developed by the World Health Organization (WHO, 1994) was used in all resistance studies in *T. infestans* from the 1990s (Mougabure-Cueto & Picollo, 2015). This protocol establishes the topical bioassay as the methodology of exposure to the insecticide and requires standardized insects under laboratory conditions, equipment and trained technicians that make it difficult to carry out monitoring at regional level working in field or laboratories without adequate infrastructure. Consequently, it would be useful to have a bioassay and monitoring logistics which are easier to implement and are suitable for field work. In addition to simplicity, monitoring would be expedited by a protocol that allows evaluation of insects collected from the field. It is recommended to carry out the bioassays on the descendants of the field insects standardized under controlled laboratory conditions, minimizing biological variation and ensuring that any differences in susceptibility are inherited and discard environmental effects. However, field insects can be used by implementing the strategy in two phases. Thus, the discriminant dose is applied on insects collected in the field and then, if there are survivors, the dose–response study is carried out with descendants from the field insects. The results of phase two determine whether the original survival was as a result of environmental factors (i.e. if the diminished susceptibility is not confirmed) or of heritable factors and is true resistance (i.e. if the diminished susceptibility is confirmed). However, field samples show individual variation in physiological conditions that may affect the insecticide susceptibility. Thus, a methodology that evaluates field insects should consider this variation, so it is necessary to study, with the proposed methodology, its influence on the toxicological response of insects.

Age and the nutritional state are two of the main factors determining the toxicological susceptibility as the processes associated with ontogeny or feeding/digestion affect toxicokinetic processes such as penetration, distribution and biotransformation (Busvine, 1971; Agosin, 1985; Bouvier *et al.*, 2002; Yu, 2015). *Triatoma infestans*, *T. sordida* and *Rhodnius prolixus* showed variation in susceptibility to insecticides between ages, i.e. differences both between and within of development stages. Overall, the fifth and the first instar nymph showed the lowest and highest susceptibility respectively (Correa & Schiavi, 1954; Fox & Bayona, 1966; Zerba *et al.*, 1997), and young individuals were more susceptible than old insects within the same stadium going through a peak of greater tolerance in middle age (Alzogaray & Zerba, 1996; Mougabure-Cueto *et al.*, 2005; Pessoa *et al.*, 2015). By contrast, newly fed insects were more susceptible to DDT and pyrethroids than fasted insects and not recently fed insects in *T. infestans* (Fontán & Zerba, 1992; Alzogaray & Zerba, 1996).

Besides the topical bioassay, exposure to insecticides impregnated surfaces is a suitable methodology for crawling insects as triatomines. This exposure method was used in the early works on insecticide resistance after a WHO instructive from 1975 (Nelson, 1994) and was adapted for resistance monitoring to pyrethroids in *T. infestans* collected from the field by WHO in 1994 (WHO, 1994). The WHO protocol describes the technical details for preparation of solutions and impregnated filter papers and proposes the exposure of only fifth instar nymphs to a discriminant concentration that is not specified nor provides a reference to the variation in the internal state of insects collected from the field. Lardeux *et al.* (2010) attempted to improve the WHO protocol and determined the deltamethrin susceptibility in impregnated filter papers for each immature instar proposing a discriminate concentration. However they did not assess adults (i.e. the stage more frequently collected) and did not consider the physiological variation found in the field. Thus, the aim of this research was to develop a bioassay and to propose a protocol to test susceptibility/resistance to deltamethrin in *T. infestans* using a discriminate concentration based on insecticide impregnated filter papers. To evaluate insects collected from the field, the effect of age and nutritional state on the deltamethrin susceptibility was studied with the methodology proposed and a discriminant concentration was determined.

Material and methods

Insects

Insects from the second generation of laboratory descendants from insects collected in the Calingasta Department (San Juan Province, Argentina) were used. Captured insects and their offspring were raised in the insectarium of the Centro de Referencia de Vectores (CeReVe) (Ministerio de Salud de la Nación Argentina) under controlled conditions (26 ± 1 °C, 50–70% RH, 12 : 12 LD photoperiod). A chicken was provided weekly as a bloodmeal source (WHO, 1994). The chickens were cared and handled in accordance with resolution 1047/2005 of the National Council of Scientific and Technical Research (CONICET) regarding the National Reference Ethical Framework for Biomedical Research with Laboratory, Farm, and Nature Collected Animals; and the National Law 14346 regarding Animal Welfare. The susceptibility to deltamethrin was studied for each instar nymph and males and females. Two physiological variables in two conditions each in all developmental stages

were studied: (a) age - early (3–5 days post-moult for nymphs I, II, III and IV, and 5–10 days post-moult for nymphs V and adults) and late (15–20 days post-moult for nymphs I, II and III; 20–30 days post-moult nymphs IV and 30–40 days for nymphs V and adults), and (b) nutritional status - starved (insects were fasted since eclosion) and fed (insects were fed the day before exposure to the insecticide). Thus, four experimental groups were conformed for each developmental stage: early age and starved early age and fed, late age and starved, late age and fed.

To evaluate the developed methodology, insects from two deltamethrin-resistant strains of *T. infestans* reared in the CeReVe and descendent from insects collected in the Chaco Province, Argentina, were used: Colonia Castelli strain, a low-resistant strain [resistance level = 3.06 (1.31–7.14)], and La Rinconada strain, a very high resistant strain (resistance level > 1000) (Fronza *et al.*, 2016).

Chemicals

Técnical grade deltamethrin (94.4% purity) (Sigma-Aldrich Co., St Louis, MO, U.S.A.), analytical grade acetone (Merk, Buenos Aires, Argentina), silicone oil (Tetrahedron - Laboratorio Andes, Mendoza, Argentina) and analytical grade chloroform (Dorwil, Química analítica, Buenos Aires, Argentina).

Topical bioassay

The development of a methodology to test susceptibility/resistance to insecticides should be performed with insects susceptible to those insecticides. Thus, the susceptibility of the San Juan strain was evaluated by topical application according to the World Health Organization protocol (WHO, 1994). A volume of 0.2 μL of deltamethrin diluted in acetone was applied on the dorsal abdomen of first instar nymphs (5–7 days old, mean weight 1.3 ± 0.2 mg) starved since hatching. The application was performed with a 10-μL micro-syringe with an automatic dispenser. Dose–response assays were carried out whereby groups of 10 insects were exposed to serial dilutions of insecticide (0.0001, 0.00025, 0.0005, 0.00075, 0.001 and 0.01 mg/mL), one concentration for group. Each concentration was replicated at least three times. The control group received only acetone. After exposure, insects were kept under controlled laboratory conditions for 24 h when mortality was evaluated. The criterion for mortality was the inability to walk from the centre to the border of a circular 7 cm diameter filter paper. Only those nymphs that were able to reach the filter paper border, with or without mechanical stimulation with forceps, were considered alive (Picollo *et al.*, 2005).

Exposure to insecticide impregnated filter papers

The exposure methodology was developed using circular filter paper (Qualitative Filter Paper 102 Moderate, 9 cm diameter; Xinxing, Zhejiang, China) on which was distributed 1 mL of deltamethrin diluted in a mixture of silicone oil (non-volatile solvent) and chloroform (volatile solvent) in proportion 1 : 3 (oil: chloroform). The papers were impregnated using a pipette and in a spiral form towards the centre ensuring a homogeneous distribution of the solution with the insecticide (WHO, 1994). Chloroform allows a homogenous distribution of solution on the paper and the oil improves the bioavailability and the absorption of the insecticide. The chloroform was allowed to evaporate during 24 h and then the insects were exposed to the impregnated papers during 1 h (i.e. the insects could walk on papers for 1 h). A plastic container (diameter: 9 cm; height: 7 cm) disposed on paper with its opening downwards was used to prevent insects from escaping the paper. Dose–response assays were carried out whereby groups of 10 insects were exposed to impregnated papers with serial dilutions of insecticide (0.3, 0.2, 0.1, 0.03 and 0.01% w/v deltamethrin in oil), each group in a paper with one concentration. Each concentration was replicated at least three times. Insects exposed to papers impregnated only with silicone oil and chloroform mixture were used as a control. At the end of the exposure, the insects were removed from the paper and were placed in plastic containers (diameter: 9 cm; height: 7 cm) which were kept in controlled laboratory conditions for 72 h. The mortality was registered at 24, 48 and 72 h post-exposure. The criterion for mortality was the inability to walk from the centre to the border of a circular 11 cm diameter filter paper. Only those nymphs that were able to reach the filter paper border, with or without mechanical stimulation with forceps, were considered alive (Picollo *et al.*, 2005). With this bioassay, the dose–response curves and susceptibility parameters for each experimental group defined above in each stage of development and every moment of registration of mortality were obtained.

For the two bioassays, treated insects of each developmental stage were observed for 5 min from the time in which the controls of each stage were able to walk and reach the edge of the paper.

Laboratory evaluation of methodology of exposure to insecticide impregnated filter paper

To evaluate the methodology developed, the percentage of mortality among groups of insects from each strain (i.e. reference, low resistant and high resistant strains) exposed to a discriminant concentration (DC) obtained in this study was compared. The bioassay was carried out according to a previous description, but the insects were exposed only to papers impregnated with DC. The insects of each strain (10 insects of each stage) were collected randomly simulating a field collection to obtain individuals that show random variation in the studied variables (age and nutritional state).

Statistical analysis

Mortality data were corrected by the eventual mortality of controls using Abbott's formula (Abbott, 1925). Mortality data for each dose or concentration were used to estimate the dose–response (D-R) curves and the toxicological parameters Lethal Dose 50 (LD_{50}), Lethal Concentration 50 (LC_{50}) and

Lethal Concentration 99 (LC_{99}). The LD_x and LC_x indicate the dose and concentration, respectively, of an insecticide that is lethal to x% of the strain/population studied, and are indicators of the susceptibility of the strain to an insecticide applied. The LD_{50} or LC_{50} are considered the most reliable values to describe the susceptibility of an organism to a toxic because the D-R curve obtained by regression shows the 95% confidence interval (CI) narrower at this level of response (Yu, 2015). The CL_{99} is the reference value to determine the concentration that allows discriminating between susceptible and resistant strains, i.e. the discriminant concentration (DC). The D-R curves, DL_{50}s, CL_{50}s and CL_{99}s and 95% CIs were obtained through Probit regression analysis (Litchfield & Wilcoxon, 1949) using POLO-PC software (LeOra Software, 1987). The possible differences between susceptibilities of experimental groups or strains were evaluated through the Lethal Dose Ratio (LDR) described by Robertson *et al.* (2007). The LDR and its 95% CIs compare dose–response curves pairs and are based on estimates of each line (i.e. slope and its variance, intercept and its variance, and the slope-intercept covariance) and a coefficient according to the level of response in which the lines are compared. As the CL_{50} is the optimal value of susceptibility (see above), all comparisons were made at 50% mortality level. Thus, the LDR estimate the susceptibility ratio between the groups or strains compared. If group A is compared with group B, LDR = 1 (i.e. not differ significantly from 1) indicates that groups do not differ in their susceptibility, LDR < 1 (i.e. significantly smaller than 1) indicates that A is more susceptible than B, and LDR > 1 (i.e. significantly greater than 1) indicates that A is less susceptible than B. LDR is significantly different to 1 (i.e. greater or less) when the 95% CIs did not include the number one ($P < 0.05$) (Robertson *et al.*, 2007). In the context of resistance studies, LDR is named the resistance ratio (RR).

Results

Resistance status of the experimental strain

Topical bioassay results of the San Juan strain compared to the reference strain are presented in Table 1. The RR value and its 95% CIs show no significant differences between the susceptibilities demonstrating that the San Juan strain is not resistant to deltamethrin.

Deltamethrin susceptibility in T. infestans *through exposure to insecticide impregnated filter papers*

Susceptibility of each development stage in each physiological state and at the different post-exposure times. The exposition of insects to filter papers impregnated with deltamethrin allowed us to determine the susceptibility of each development stage of *T. infestans* in the different conditions evaluated. The susceptibility values (CL_{50}) and the slopes of doses-responses (D-R) curves for each stage in each age and nutritional state at 24, 48 and 72 h post-exposure are shown in Table 2. This table shows the pooled data from males and females as the adult stage. However, the possible difference between the toxicological response of males and females adults was previously evaluated in insects of early age both starved and fed and at the different post-exposition time. The lethal dose ratios (LDRs) and 95% CIs show no significant differences between the susceptibilities of males and females in any comparison allowing pooling data from both sexes ($P > 0.05$) (data not shown).

To determine the effect of age and the nutritional state on the susceptibility of each stage of *T. infestans* and the effect of post-exposure time on the mortality recording, the susceptibilities showed in Table 2 were compared between nutritional states, ages and post-exposure times.

Comparison between the nutritional states. The susceptibility ratios between starved and fed insects for each development stage in each age at different post-exposure times are shown in Table 3. The LDRs and 95% CIs show not significant differences between starved and fed insects in 33 of the 36 comparisons. Only second instar in the early age at 72 h and adults in the later age at 48 and 72 h were significantly different with CIs close to 1.

Comparison between the ages. The susceptibility ratios between insects in an early and later age for each development stage in each nutritional state at different post-exposure times are shown in Table 4. The LDRs and 95% CIs showed not significant differences between early and later insects in 32 of the 36 comparisons. Only fed fifth instar at 72 h, starved the fifth instar at 48 and 72 h, and starved adults at 72 h were significantly different with CIs close to 1.

Comparison between the post-exposure times. The susceptibility ratios between the mortality registered at 24, 48 and 72 h post- exposure for each development stage in each nutritional state and each age are shown Table 5. Three relationships were determined: 24 vs. 48 h, 24 vs. 72 h, and 48 vs. 72 h. The LDRs and 95% CIs show significant differences between different post-exposure times in 52 of the 72 comparisons. The relationship 72/24 h was significantly different for all comparisons which indicate that the measured susceptibility decreases according to the time elapsed since the end of the exposure.

Susceptibility of development stages with ages and nutritional states grouped. The analysis of results shown in the Tables 3–5 allows a determination of the effects of the variables studied on the susceptibility of *T. infestans*. Most of the susceptibilities compared both to age and nutritional state, (i.e. 33 and 32, respectively, of 36) were not significantly different, and the few comparisons significantly different showed LDRs with CIs very close to significance criterion (i.e. close to 1). Thus, the susceptibility to deltamethrin of *T. infestans* determined through impregnated papers was not affected by the age and the nutritional state of the insects exposed. By contrast, most of the susceptibility ratios between the different post-exposure times (i.e. 52

Table 1. Susceptibility values and resistance ratio to deltamethrin of San Juan strain of *T. infestans* determined by topical application.

Strain	n	χ^2	Slope ± SE	LD_{50} (ng/i) (95% CI)	RR (95% CI)
Reference∗	120	11.31	1.41 ± 0.35	0.18 (0.03–0.35)	-----
San Juan	280	34.18	2.97 ± 0.42	0.17 (0.13–0.26)	0.93 (0.42–2.03)

∗Data from Fronza *et al.* (2016).
LD_{50}, Lethal Dose 50%,%; RR, Resistence Ratio; ng/i, nanograms per insect; SE, estándar error; CI, confidence interval.

of 72) showed significant differences evidencing that the elapsed time between 24 and 72 h affects the mortality produced by the exposure to impregnated papers. Thus, to analyse the variation in susceptibility between the development stages, mortality data for each concentration of the two nutritional states and the two ages were pooled in each post-embryonic stage. Then, the D-R curves and the susceptibility values (CL_{50}) for each stage at each post-exposure time with pooled data were determined (Table 6). The new comparisons between post-exposure times with pooled data (data not shown) confirmed the results of Table 5, i.e. the CL_{50} of each development stage increases significantly with the time elapsed from the end of the exposure.

Comparison between the development stages. The susceptibility ratios between the development stages at 24, 48 and 72 h post-exposure, using the D-R curves from Table 6, are shown in Tables 7–9 respectively. The combined analysis of CL_{50}s from Table 6 and LDRs from Tables 7–9 allowed us to establish the order of susceptibility of the development stages at each post-exposure time. As a general toxicological pattern, the first instar was the most susceptible stage, and the fourth instar was the least susceptible stage at all post-exposure time evaluated. Other stages showed intermediate susceptibilities depending on the post-exposure time.

Determination of discriminant concentration

All previous susceptibility ratios were obtained comparing D-R curves at the level of mortality 50%. However, the discriminant concentration (DC) for a pest, an insecticide and a methodology of exposure is based on the LC_{99} of that insecticide on the pest considered obtained by the bioassay used in the monitoring. Thus, the LC_{99} of each development stage and each post-exposure time were obtained from the D-R curves presented in Table 6. Because D-R curves of the development stages were not parallels (see slopes in Table 6), the order of the susceptibilities between stages at 99% mortality level was different from the order at 50% level. To obtain the DC, the LC_{99}s from different stages were grouped in a way to test the largest number of stages with the least number of doses. Due the DC is generally defined as the LC_{99} or $2xCL_{99}$, the criterion for grouping development stages under a single DC was that this DC lies within the interval CL_{99}-$2xCL_{99}$ of each development stage of the group. For example, a single DC equal to 0.36% w/v was obtained at 24 h post-exposure time. This DC is twice the lowest CL_{99} (0.18% for adults) and equal to the highest CL_{99} (0.36% for third instar nymphs) at 24 h post-exposure, with the rest of the stages showing intermediate CL_{99}s. Therefore, filter papers impregnated with 0.36% w/v of deltamethrin in oil evaluate resistance to deltamethrin in all developmental stages at 24 h post-exposure. In the same way, two DC in two forms of grouping were obtained at 48 h: 0.9% (nymphs I, II, III, IV) and 0.3% (nymph V, adult); or 0.9% (nymphs I, II) and 0.6% (nymphs III, IV, V, adult). Finally, two or three DC were obtained at 72 h: 1.5% (nymph I, II, III) and 0.43% (nymph IV, V and adult), or 1.5% (nymphs I, II, III), 0.43% (nymph IV) and 0.36% (nymph V, adult); this last grouping optimizes the determination of the DC for field insects. Thus, the concentration of 0.36% is proposed as single and optimal DC for determination of resistance to deltamethrin in *T. infestans* collected in the field. The DC proposed would evaluate all developmental stages at 24 h after exposure and also fifth instar nymphs and adults (i.e. the stages most commonly caught in the field) again at 72 h. Survival of some stage at 24 h or of fifth instar or adults at 72 h is indicative of the possible evolution of resistance to deltamethrin; by contrast, 100% of mortality in both registration times indicates no resistance (see discussion).

Laboratory evaluation of methodology of exposure to insecticide impregnated filter papers

The percentages of mortality at 24 and 72 h post-exposure of the colonies San Juan (susceptible), Colonia Castelli (low resistance) and La Rinconada (high resistance) exposed to papers impregnated with the DC proposed are shown in Table 10. The susceptible colony showed 100% of mortality in all stages at the two times while different survival percentages were observed for the two resistant colonies. According to resistance levels, La Rinconada showed a higher survival than Colonia Castelli. These results show that the DC proposed to discriminate between susceptible and resistant insects. In addition, the discrimination of a colony with a very low resistance level and Cl 95% close to 1 [GR = 3.06 (1.31–7.14)] evidence the high sensitivity of the methodology and DC developed.

Discussion

This study developed a methodology of exposure to insecticide impregnated filter papers and proposed a protocol to carry out toxicological monitoring on *T. infestans* populations using field-collected insects with the aim to detect early evolution of resistance to deltamthrin. Using this methodology, the present research showed that the age and nutritional states do not affect the susceptibility to deltamethrin of *T. infestans*,

Table 2. Susceptibility to deltamethrin of each post-embryonic stage of *T. infestans* in every age and nutritional state at each time after exposure to insecticide impregnated papers.

		Time (h)	Early age				Later age			
			n	χ^2	Slope ± SE	LC_{50} (% w/v) (CI)	n	χ^2	Slope ± SE	LC_{50} (% w/v) (CI)
Nymph I	Fed	24	127	34.02	1.63 ± 0.31	0.019 (0.003–0.037)∗	83	17.08	2.87 ± 0.56	0.033 (0.014–0.061)∗
		48		49.20	1.33 ± 0.25	0.050 (0.016–0.114)†		20.74	2.76 ± 0.49	0.049 (0.020–0.096)∗
		72		54.32	1.20 ± 0.25	0.061‡		28.99	2.31 ± 0.42	0.084 (0.039–0.184)†
	Starved	24	115	24.41	1.86 ± 0.37	0.017 (0.004–0.031)∗	83	4.48	3.01 ± 0.75	0.022 (0.013–0.031)∗
		48		21.95	1.56 ± 0.28	0.044 (0.020–0.081)∗		18.77	1.97 ± 0.45	0.030 (0.003–0.053)†
		72		21.57	1.72 ± 0.28	0.057 (0.031–0.103)∗		17.63	1.60 ± 0.36	0.052 (0.018–0.098)†
Nymph II	Fed	24	84	35.33	1.68 ± 0.36	0.042‡	116	16.03	3.45 ± 0.56	0.041 (0.030–0.056)∗
		48		38.81	2.15 ± 0.41	0.076‡		36.78	3.07 ± 0.47	0.074 (0.044–0.125)∗
		72		39.90	3.72 ± 0.72	0.108‡		44.39	2.59 ± 0.38	0.089 (0.047–0.173)∗
	Starved	24	85	19.96	1.78 ± 0.37	0.060 (0.020–0.120)†	120	19.67	2.45 ± 0.36	0.036 (0.023–0.054)∗
		48		14.04	1.56 ± 0.38	0.129 (0.06–0.367)†		36.88	2,20 ± 0.35	0.095 (0.052–0.193)∗
		72		15.07	2.75 ± 0.65	0.163 (0.108–0.340)†		45.44	2.14 ± 0.35	0.116 (0.059–0.328)∗
Nymoh III	Fed	24	159	21.15	3.00 ± 0.39	0.051 (0.039–0.064)∗	117	18.73	2.71 ± 0.40	0.060 (0.041–0.087)∗
		48		26.56	3.21 ± 0.40	0.079 (0.061–0.101)∗		77.51	3.07 ± 0.46	0.091 (0.043–0.183)†
		72		43.14	2.77 ± 0.37	0.106 (0.074–0.151)∗		100.45	2.73 ± 0.44	0.129‡
	Starved	24	156	20.59	2.51 ± 0.34	0.049 (0.037–0.064)∗	117	8.82	2.94 ± 0.44	0.051 (0.038–0.066)∗
		48		21.25	1.87 ± 0.29	0.078 (0.055–0.110)∗		62.64	3.06 ± 0.47	0.086 (0.045–0.152) †
		72		33.77	1.82 ± 0.30	0.114 (0.074–0.196)∗		27.17	3.61 ± 0.63	0.127 (0.083–0.187)∗
Nymph IV	Fed	24	160	10.84	3.60 ± 0.30	0.060 (0.049–0.073)∗	86	8.31	2.63 ± 0.54	0.054 (0.032–0.074)∗
		48		31.91	3.54 ± 0.47	0.103 (0.075–0.133)∗		9.99	4.27 ± 0.80	0.101 (0.072–0.128)∗
		72		179.41	4.27 ± 0.57	0.135‡		4.37	9.69 ± 1.66	0.140 (0.123–0.158)∗
	Starved	24	156	16.14	3.27 ± 0.44	0.064 (0.050–0.079)∗	86	4.52	2.91 ± 0.57	0.057 (0.036–0.077)∗
		48		27.45	3.08 ± 0.42	0.110 (0.082–0.144)∗		12.15	3.41 ± 0.62	0.084 (0.052–0.116)∗
		72		61.47	3.31 ± 0.46	0.135 (0.085–0.208)∗		43.68	4.53 ± 0.84	0.110‡
Nymph V	Fed	24	215	28.68	2.87 ± 0.34	0.048 (0.036–0.060)∗	162	11.73	4.15 ± 0.61	0.045 (0.036–0.055)∗
		48		39.00	4.05 ± 0.45	0.083 (0.066–0.101)∗		28.03	3.90 ± 0.51	0.071 (0.052–0.090)∗
		72		261.80	5.42 ± 0.66	0.113‡		33.02	3.97 ± 0.52	0.083 (0.060–0.106)∗
	Starved	24	217	25.63	3.54 ± 0.39	0.059 (0.048–0.070)∗	164	6.62	3.74 ± 0.50	0.053 (0.042–0.064)∗
		48		102.84	5.70 ± 0.79	0.101 (0.067–0.131)∗		13.65	3.73 ± 0.48	0.074 (0.060–0.087)∗
		72		2853.7	6.26 ± 0.73	0.1–0.2§		28.59	4.15 ± 0.55	0.096 (0.073–0.119)∗
Adult	Fed	24	168	9.29	3.59 ± 0.46	0.047 (0.039–0.057)∗	107	15.46	4.57 ± 0.99	0.061 (0.039–0.079)∗
		48		30.79	4.37 ± 0.54	0.089 (0.072–0.108)∗		0.062	8.28 ± 1.51	0.105 (0.088–0.118)) *
		72		60.49	4.41 ± 0.71	0.135 (0.097–0.177)∗		10.88	9.87 ± 1.70	0.125 (0.110–0.140)∗
	Starved	24	172	32.13	3.94 ± 0.53	0.042 (0.032–0.053)∗	110	24.65	2.90 ± 0.69	0.035 (0.009–0.057)∗
		48		46.56	3.38 ± 0.42	0.081 (0.059–0.106)∗		14.84	3.45 ± 0.67	0.056 (0.035–0.074)∗
		72		243.69	5.17 ± 0.76	0.132‡		18.54	3.80 ± 0.68	0.077 (0.052–0.098)∗

∗95% confidence interval.
†90% confidence interval.
‡Confidence interval not obtained.
§LC_{50} not obtained, the dose range indicated would contain the LC_{50}.
LC_{50}, Lethal Concentration 50%; SE, standard error; CI, confidence interval; % w/v, % weight per volume of deltamethrin in silicone oil.

that the mortality varies with the post-exposure time, and that the different developmental stages of *T. infestans* show different deltamethrin susceptibility. A discriminant concentration of deltamethrin impregnated in filter paper was established for all developmental stages of *T. infestans*. Finally, the methodology and the discriminant concentration were evaluated in the laboratory showing a high sensitivity in the discrimination of resistance.

Different variants of the methodology of papers impregnated with insecticides were applied for studies of insecticide resistance in triatomines. Early studies during the 1970s followed the first instructions of WHO by which fifth instar nymphs collected in the field were exposed to strips of papers impregnated with insecticides located inside test tubes with different exposure times and mortality registration time depending on the authors, species and insecticides (WHO, 1963; Fox & Bayona, 1966; Gonzalez-Valdivieso *et al.*, 1971; Cockburn, 1972; Nocerino, 1975; Nelson, 1994). After the introduction of pyrethroid insecticides, and due to the lack of standardization in exposure time, mortality registration time, impregnation of papers and individuals evaluated, the WHO described a new protocol of insecticide impregnated filter papers for *T. infestans* (WHO, 1994) and *R. prolixus* (WHO, 2001). This protocol described the impregnation of circular filter papers with insecticides dissolved in a mixture of acetone and oil (1 : 4) (e.g. olive oil for organophosphorus and carbamates and silicone oils for

Table 3. Susceptibility ratios to deltamethrin between starved and fed *T. infestans* for each post-embryonic stage in each age at different times after exposure to impregnated papers.

	LDR Starved/Fed (95% CI)					
	Early age			Later age		
Stage	24 h	48 h	72 h	24 h	48 h	72 h
N I	0.92 (0.47–1.81)	0.89 (0.49–1.60)	0.99 (0.59–1.52)	0.68 (0.43–1.07)	0.62 (0.35–1.09)	0.62 (0.35–1.09)
N II	1.42 (0.76–2.64)	1.72 (0.98–3.05)	1.52∗(1.05–2.20)	0.89 (0.60–1.31)	1.27 (0.86–1.89)	1.31 (0.87–1.98)
N III	0.98 (0.71–1.35)	0.99 (0.70–1.39)	1.15 (0.80–1.67)	0.84 (0.58–1.23)	0.94 (0.66–1.34)	0.98 (0.69–1.39)
N IV	1.06 (0.79–1.40)	1.07 (0.83–1.38)	1.00 (0.79–1.26)	1.06 (0.96–1.18)	0.84 (0.59–1.18)	0.79∗(0.62–1.00)
N V	1.23 (0.94–1.61)	1.22 (1.01–1.49)	1.15 (0.79–1.26)	1.17 (0.89–1.55)	1.04 (0.80–1.34)	1.16 (0.91–1.47)
Adult	0.89 (0.69–1.14)	0.91 (0.72–1.15)	0.98 (0.80–1.19)	0.57 (0.31–1.06)	0.56∗(0.38–0.81)	0.62∗(0.47–0.83)

∗LDR significantly different to 1.
N, instar nymph; LDR, Lethal dose ratio (estimates the susceptibility ratio); CI, confidence interval.

Table 4. Susceptibility ratios to deltamethrin between *T. infestans* in early and late ages for each post-embryonic stage in each nutritional state at different times after exposure to impregnated papers.

	LDR early age/later age (95% CI)					
	Fed			Starved		
Stage	24 h	48 h	72 h	24 h	48 h	72 h
N I	0.57 (0.32–1.02)	1.03 (0.60–1.76)	0.72 (0.46–1.13)	0.77 (0.43–1.40)	1.48 (0.80–2.74)	1.11 (0.61–2.01)
N II	1.03 (0.61–1.74)	1.01 (0.66–2.53)	1.22 (0.85–1.74)	1.65 (0.99–2.76)	1.37 (0.79–2.38)	1.41 (0.92–2.15)
N III	0.84 (0.59–1.19)	0.87 (0.63–1.20)	0.82 (0.58–1.16)	0.97 (0.68–1.38)	0.91 (0.63–1.32)	0.97 (0.66–1.41)
N IV	1.12 (0.74–1.71)	1.02 (0.77–1.34)	0.97 (0.80–1.18)	1.12 (0.74–1.67)	1.31 (0.94–1.82)	1.22 (0.94–1.60)
N V	1.05 (0.79–1.41)	1.17 (0.93–1.49)	1.36∗(1.11–1.68)	1.10 (0.85–1.43)	1.37∗(1.11–1.70)	1.35∗(1.14–1.58)
Adult	0.77 (0.54–1.11)	0.89 (0.72–1.11)	1.09 (0.90–1.31)	1.20 (0.68–2.10)	1.46 (1.00–2.14)	1.71∗(1.28–2.29)

∗LDR significantly different to 1.
N, instar nymph; LDR, Lethal dose ratio (estimates the susceptibility ratio); CI, confidence interval.

pyrethroids) and the exposition of fifth instar nymphs from the field for 1 h to a discriminant concentration (DC) recording mortality at 72 h after exposure. However, the document does not propose a value of DC but that the concentration should be obtained previously by the researcher with the exposure methodology described in the protocol using standardized fifth instar (15–20 days post-moult and fasted by 7 days for *T. infestans*) of a reference susceptible colony. To extend the WHO protocol for the evaluation of all developmental stages of *T. infestans* collected in the field, Lardeux *et al.* (2010) determined the susceptibility to deltamethrin impregnated in filter papers of all instars nymphs, but not adults, from a deltamethrin susceptible colony at 24 h post-exposure and proposed a determinant concentration (0.3% w/v of deltamethrin in silicone oil) for all instars nymphs although overestimated for first instar. However, none of the reviewed protocols and studies on *T. infestans* determined the susceptibility of adults, and none considered the possible physiological differences that would present the insects from the field. The present study determined for the first time in *T. infestans* the susceptibility to deltamethrin (and to any insecticides) for all post-embryonic stages in different physiological conditions and measured at different post-exposure times. Using the exposure to insecticide-impregnated papers, this study complements the WHO protocol and the previous work by Lardeux *et al.* (2010). The proposed CD (0.36%) is similar to CD determined by Lardeux *et al.* (2010) (0.3%) and allowed us to evaluate for resistance to deltamethrin in a field sample of *T. infestans* conformed by any post-embryonic stage at different ages and nutritional states.

The effect of the nutritional state on susceptibility to insecticides was demonstrated in several insect species (Busvine, 1971; Agosin, 1985; Yu, 2015). However, there are not many studies in triatomines. Fontán & Zerba (1992) showed by topical application higher susceptibility to organochlorine insecticide DDT in second instar nymphs of *T. infestans* recently fed compared to fasted nymphs. Alzogaray & Zerba (1996) studied by topical application the toxicity of pyrethroids in first instar nymphs of *T. infestans* in different nutritional states and showed that deltamethrin was more toxic for fasted insects whereas cis-permethrin was more toxic for recently fed insects. The authors of both studies awarded the higher susceptibility of the fed insects on one hand to the increase of penetration rate of insecticide due to the changes in the composition and properties of integument occurring after feeding, and on the other to the exposure to the toxicant of the intersegmental membranes, more permeable to insecticides, of the distended abdomens of fed insects. Another factor associated with feeding should be particularly considered in blood-sucking insects which ingest a large volume of blood in relation to its size (Lehane, 2005). This ingestion produces an increase in internal volume in which the insecticide would be diluted and distributed determining a lower

Table 5. Susceptibility ratios to deltamethrin between the mortality registered at 24, 48 and 72 h after exposure to impregnated papers for each post-embryonic stage of *T. infestans* in each nutritional state and each age.

	LDR 48 h/24 h (95% CI)				LDR 72 h/24 h (95% CI)				LDR 72 h/48 h (95% CI)			
	Fed		Starved		Fed		Starved		Fed		Starved	
Stage	EA	LA	EA	LA	EA	LA	EA	LA	EA	LA	EA	LA
N I	2.68*(1.40–5.15)	1.48 (0.93–2.35)	2.57*(1.40–4.74)	1.35 (0.74–2.47)	3.27*(1.84–5.82)	2.57*(1.6–4.10)	3.35*(1.85–6.04)	2.33*(1.28–4.24)	1.22 (0.71–2.08)	1.74*(1.10–2.74)	1.30 (0.76–2.23)	1.73 (0.89–3.38)
N II	1.77 (1.00–3.14)	1.81*(1.28–2.57)	2.15*(1.16–3.98)	2.60*(1.68–4.01)	2.54*(1.52–4.25)	2.16*(1.49–3.12)	2.72*(1.64–4.51)	3.19*(2.07–4.92)	1.44 (0.96–2.16)	1.19 (0.82–1.73)	1.27 (0.73–2.18)	1.23 (0.80–1.90)
N III	1.57*(1.17–2.09)	1.51*(1.04–2.16)	1.58*(1.09–2.29)	1.69*(1.18–2.41)	2.09*(1.56–2.81)	2.13*(1.45–3.14)	2.31*(1.58–3.39)	2.49*(1.78–3.48)	1.34*(1.01–1.77)	1.36 (0.94–1.98)	1.46 (0.98–2.18)	1.47*(1.06–2.05)
N IV	1.71*(1.32–2.21)	1.88*(1.22–2.89)	1.73*(1.30–2.30)	1.48 (0.95–2.29)	2.24*(1.75–2.86)	2.59*(1.75–3.85)	2.11*(1.61–2.78)	1.93*(1.29–2.88)	1.31*(1.04–1.65)	1.38*(1.08–1.77)	1.22 (0.94–1.58)	1.30 (0.93–1.83)
N V	1.75*(1.35–2.27)	1.57*(1.20–2.05)	1.73*(1.42–2.11)	1.30*(1.02–1.66)	2.37*(1.86–3.01)	1.83*(1.41–2.38)	2.21*(1.88–2.60)	1.81*(1.40–2.35)	1.11 (0.95–1.31)	1.17 (0.91–1.50)	1.28*(1.14–1.43)	1.39*(1.06–1.82)
Adult	1.88*(1.49–2.38)	1.63*(1.15–2.30)	1.94*(1.51–2.48)	1.59 (0.85–2.98)	2.85*(2.26–3.58)	2.03*(1.45–2.83)	3.14*(2.52–3.93)	2.20*(1.22–3.98)	1.51*(1.22–1.88)	1.25*(1.03–1.50)	1.63*(1.32–2.01)	1.39 (0.90–2.14)

*LDR significantly different to 1.

N, instar nymph; LDR, Lethal dose ratio (estimates the susceptibility ratio); CI, confidence interval; EA, early age; LA, later age.

concentration of the toxicant at the site of action and therefore, only considering this factor, a lower susceptibility compared to fasted insects. Finally, biochemical interactions between food components and toxicokinetic or toxicodynamic processes can modify the effect of the insecticide (Busvine, 1971; Yu, 2015). For example, the effect of the diet on the activity of P450 enzymes was demonstrated by several authors (Agosin, 1985; Feyereisen, 1999; Petersen Brown *et al.*, 2005). Unlike aforementioned studies in *T. infestans*, the present study showed that feeding did not modify the susceptibility to deltamethrin. In the theoretical context discussed, the results presented could occur by (a) the three factors previously described results in a null effect of feeding on the insect susceptibility; and (b) the methodology used does not allow detecting the effects of these factors.

The effect of the age of individuals during each stadium on the susceptibility to insecticides was described for different insect species (Busvine, 1971; Hodgson, 1985; Roush & Tabashnik, 1990; Robertson *et al.*, 2007; Yu, 2015). However, few studies were carried out on triatomines. Alzogaray & Zerba (1996) studied the toxicity of deltamethrin and cis-permethrin on first instar nymph of *T. infestans* at different ages and showed the higher susceptibility in insects with 1 day in the stadium, the lower susceptibility after 1 week in the stadium and an intermediate toxic response 3 weeks after a moult. Mougabure-Cueto *et al.* (2005) showed a higher insecticide activity of 1-dodecanol applied topically on first nymphs of 1–3 h post-moult than on the same instar at 24–36 h post-moult, however, there was no difference when the 1-dodecanol was administrated by injection into the internal medium of the insects. Pessoa *et al.* (2015) reported that topical application of deltamethrin on first instar nymphs of *T. sordida* was more toxic at 1 and 5 days than at 3 days old. All these authors awarded the differences to the interaction between biochemical and physiological processes associated with ontogeny and the toxicokinetic and toxicodynamic processes that determine the susceptibility to the insecticides. For example, moulting and the development of new cuticle affect the penetration of the insecticide. Thus recently moulted insects show higher absorption of toxic and therefore higher susceptibility than insects older (Mougabure-Cueto *et al.*, 2005; Pessoa *et al.*, 2015). By contrast, the activity of detoxification enzymes may vary depending on the age (Agosin, 1985; Yu, 2015). Even more, the metabolism of molecules associated to ontogeny (e.g. hormones, cuticle components, etc.) can share enzymes with the metabolism of insecticides (Ruigt, 1985; Casida & Quistad, 1995; Chapman, 2013); thus, insects in developmental periods where these enzymes have higher activity would be less susceptible than insects in other periods. Alzogaray & Zerba (1996) suggested this kind of variation to explain the changes in toxicity of pyrethroids during the first stadium of *T. infestans*. In contrast to these studies, the present research did not show any variation in the susceptibility during each stadium suggesting newly that the methodology used does not detect the effects of age within each development stage.

The recovery of the effects of pyrethroids intoxication, depending on the time elapsed since exposure, is a phenomenon described by different methods in different insects (Soderlund *et al.*, 1983; Ruigt, 1985; Casida & Quistad, 1995), including triatomines (Casabé *et al.*, 1988, Alzogaray & Zerba, 1997). Casabé *et al.* (1988) reported recovery of adults of *T. infestans*

Table 6. Susceptibility to deltamethrin of each post-embryonic stage of *T. infestans* at each time after exposure to insecticide impregnated papers.

Time (h)	Stage	n	$\chi 2$	Slope ± SE	LC_{50} (% w/v) (95% CI)	LC_{99} (% w/v) (95% CI)
24	N I	484	78.25	2.01 ± 0.21	0.019 (0.013–0.025)	0.277 (0.169–0.625)
	N II	465	68.83	2.57 ± 0.21	0.038 (0.031–0.046)	0.306 (0.212–0.522)
	N III	637	70.61	2.83 ± 0.20	0.053 (0.047–0.060)	0.355 (0.279–0.483)
	N IV	568	40.76	3.14 ± 0.25	0.060 (0.052–0.067)	0.328 (0.265–0.432)
	N V	875	99.16	3.40 ± 0.21	0.051 (0.046–0.056)	0.248 (0.207–0.312)
	Adult	557	58.14	3.75 ± 0.29	0.044 (0.039–0.049)	0.182 (0.150–0.236)
48	N I	484	107.91	1.79 ± 0.16	0.043 (0.030–0.058)	0.858 (0.449–2.603)
	N II	465	130.54	2.32 ± 0.20	0.087 (0.066–0.116)	0.877 (0.496–2.365)
	N III	637	146.42	2.72 ± 0.19	0.083 (0.070–0.099)	0.6 (0.419–1.018)
	N IV	568	84.71	3.46 ± 0.26	0.102 (0.089–0.116)	0.482 (0.377–0.681)
	N V	875	157.14	4.13 ± 0.25	0.082 (0.073–0.090)	0.303 (0.252–0.387)
	Adult	557	118.72	4.06 ± 0.28	0.079 (0.069–0.088)	0.294 (0.242–0.381)
72	N I	484	106.49	1.78 ± 0.16	0.070 (0.052–0.094)	1.412 (0.699–4.815)
	N II	465	237.61	2.66 ± 0.24	0.121 (0.086–0.177)	0.908 (0.463–4.19)
	N III	637	186.51	2.50 ± 0.19	0.115 (0.093–0.142)	0.977 (0.610–2.070)
	N IV	568	457.73	4.40 ± 0.34	0.126 (0.094–0.162)	0.426 (0.285–1.118)
	N V	875	423.89	4.68 ± 0.30	0.107 (0.090–0.123)	0.336 (0.259–0.523)
	Adult	557	375.79	4.86 ± 0.38	0.118 (0.098–0.137)	0.356 (0.274–0.575)

N, instar nymph; LC_{50}, Lethal Concentration 50%; LC_{99}, Lethal Concentration 99%; SE, standard error; CI, confidence interval; DC, Discriminate Concentration; % w/v, % weight per volume of deltamethrin in silicone oil.

Table 7. Susceptibility ratios to deltamethrin between post-embryonic stages of *T. infestans* at 24 h after exposure to impregnated papers.

	LDR stage in row/stage in column				
	N I	N II	N III	N IV	N V
N II	1.96∗(1.47–2.62)				
N III	2.76∗(2.11–3.62)	1.41∗(1.13–1.75)			
N IV	3.08∗(2.37–4.00)	1.57∗(1.27–1.93)	1.12 (0.93–1.34)		
N V	2.62∗(2.04–3.37)	1.34∗(1.10–1.62)	0.95 (0.80–1.12)	0.85∗(0.73–0.99)	
Adult	2.25∗(1.74–2.92)	1.15 (0.94–1.41)	0.82∗(0.69–0.97)	0.73∗(0.62–0.86)	0.86 (0.74–1.00)

∗LDR significantly different to 1.
N, instar nymph.; LDR, Lethal dose ratio (estimates the susceptibility ratio).

topically treated with deltamethrin, cypermethrin and cyphenothrin; and Alzogaray & Zerba (1997) showed the reversion of knockdown of third instar nymphs of *T. infestans* intoxicated with deltamethrin and cis-permethrin. In general, these studies reveal that pyrethroids promote a knockdown effect that can have on the outcome the death or, on the contrary, the recovery of the individual. For an insecticide and an insect species, the outcome depends on the dose and the kinetics of the toxicokinetic and toxicodynamic processes. Briefly, during intoxication, the insects show a succession of symptoms which are a consequence of the interaction insecticide-site/s of action/s (Ruigt, 1985; Casida & Quistad, 1995; Alzogaray & Zerba, 1997). In addition, the toxicokinetic and toxicodynamic processes do not occur in a strict sequence; for example, the insecticide could have reached the site of action and continue its metabolism (Roush & Tabashnik, 1990; Hodgson & Levi, 1997). This explains the reversion of a symptom. For insecticides such as pyrethroids, whose site of action is located both in peripheral nerves and the central nervous system (Ruigt, 1985; Casida & Quistad, 1995), the intoxication symptoms begin shortly after exposure and presumably before the insecticide reaches the detoxification organs Thus, the effect recorded as 'mortality' at a time could be 'real mortality' or a reversible intoxication symptom, in the second case the recovery should be observed such as was shown for some individuals between 24 and 72 h post-exposure in this study. However, the endpoint recorded as mortality in any time can be used to test insecticide resistance due that is assumed that resistant insects show a shift of the dose–response curve in any post-exposure time.

As expected, both as shown in the extensive literature such as what can be inferred from knowledge of the developmental and toxicological processes (Roush & Tabashnik, 1990; Hodgson & Levi, 1997; Chapman, 2013; Yu, 2015), the present work showed differences between the susceptibility to deltamethrin of the stages of development. However, there was no relationship between susceptibility and ontogeny. In triatomines, this variation was reported both by topical application (Zerba *et al.*, 1997) as by exposure to impregnated papers (Correa & Schiavi, 1954; Fox & Bayona, 1966; Lardeux *et al.*, 2010), and none described an increasing or decreasing trend of the susceptibility depending on the post-embryonic development. The exposure of *T. infestans* and *R. prolixus* to different insecticides (e.g. lindane, dieldrin, malathion, fenthion, deltamethrin, β-cypermethrin) through both methodologies showed that the first instar nymph was the

Table 8. Susceptibility ratios to deltamethrin between post-embryonic stages of *T. infestans* at 48 h after exposure to impregnated papers.

	LDR stage in row/stage in column				
	N I	N II	N III	N IV	N V
N II	2.03∗(1.59–2.58)				
N III	1.94∗(1.53–2.44)	0.96 (0.80–1.15)			
N IV	2.74∗(1.90–2.98)	0.55∗(0.34–0.89)	1.23∗(1.05–1.44)		
N V	1.90∗(1.53–2.36)	0.94 (0.80–1.10)	0.98 (0.85–1.13)	0.80∗(0.70–0.91)	
Adult	1.82∗(1.46–2.27)	0.90 (0.76–1.06)	0.94 (0.81–1.09)	0.77∗(0.67–0.88)	0.96 (0.85–1.09)

∗LDR significantly different to 1.
N, instar nymph; LDR, Lethal dose ratio (estimates the susceptibility ratio).

Table 9. Susceptibility ratios to deltamethrin between post-embryonic stages of *T. infestans* at 72 h after exposure to impregnated papers.

	LDR stage in row/stage in column				
	N I	N II	N III	N IV	N V
N II	1.73∗(1.38–2.16)				
N III	1.64∗(1.33–2.03)	0.95 (0.79–1.15)			
N IV	1.80∗(1.49–2.19)	1.05 (0.88–1.24)	1.10 (0.95–1.28)		
N V	1.52∗(1.27–1.83)	0.88 (0.75–1.03)	0.93 (0.81–1.07)	0.85 (0.68–1.06)	
Adult	1.65∗(1.36–2.00)	0.96 (0.81–1.13)	1.01 (0.87–1.17)	0.92 (0.81–1.03)	1.08 (0.97–1.21)

∗LDR significantly different to 1.
N, instar nymph; LDR, Lethal dose ratio (estimates the susceptibility ratio).

most susceptible stage, the fifth instar nymph was the less susceptible stage, and the rest of the stages showed intermediate susceptibilities (Correa & Schiavi, 1954; Fox & Bayona, 1966; Zerba *et al.*, 1997). By contrast, the adults and fifth instar nymphs were the most susceptible and the most tolerant, respectively, to papers impregnated with DDT (Fox & Bayona, 1966). In agreement with the present study, *T. infestans* exposed to deltamethrin using a methodology similar to that used in this work showed the highest susceptibility in the first stadium and the lowest susceptibility in the fourth stadium (Lardeux *et al.*, 2010). The biochemical and physiological changes occurring during post-embryonic development can determine variation in the susceptibility between different stages of ontogeny. By contrast, this variation could be explained by the difference in size between the development stages. The bigger the individual, the greater surface area exposed to a toxicant, and consequently greater absorption of insecticide. However, larger insects have a lower surface/volume ratio that small insects, whereby the insecticide that entered the larger is distributed in a body volume whose increase is greater than the increase in surface compared to an individual smaller. The result is a lower concentration of the toxicant in the site of action, and therefore a higher tolerance in the individual larger (Busvine, 1971). Given that the size of the insect increases as the stages of ontogeny follow one another, geometric effects are not enough to explain the relative susceptibility between stages determined in the present study as it was not demonstrated the expected relationship between the toxic response and stage of development. Thus, the susceptibility of each stage of *T. infestans* appears to be mainly determined by the specific physiology and biochemistry of each.

The previous toxicological studies in *T. infesans* with deltamethrin and other pyrthroids were carried out through two bioassays, the topical application and exposure to impregnated filter papers. Briefly, the susceptibility of first instar nymphs by topical application ranged from 0.13 to 0.20 nanograms/insect (Picollo *et al.*, 2005; Germano *et al.*, 2010; Roca-Acevedo *et al.*, 2013) whereas by impregnated papers ranged from 0.03 to 0.04% w/v (Lardeux *et al.*, 2010). The different methodologies of exposure determine differences in the first toxicokinetic process (i.e. the penetration of insecticide) and, consequently, in the response measured (Busvine, 1971; ffrench-Constant & Roush, 1990). Topical exposure occurs by placing a small drop of insecticide solution on the dorsal cuticle of the abdomen of the insect, whereas exposure by impregnated papers occurs when the insect walks on the paper incorporating the insecticide and is through the cuticle of the tarsi and another body surface in contact with the paper (e.g. ventral abdomen). The area and properties of the dorsal versus ventral cuticle in contact with the toxicant are different in each method which may explain the differences in susceptibility (Busvine, 1971; Chapman, 2013). In addition, the bioavailability, and consequently the penetration rate, is different in the topical application where the insecticide is dissolved in acetone compared with impregnated papers where the toxic is partitioned between the paper matrix and the oil. Lardeux *et al.* (2010) used a similar bioassay to that used in the present work and obtained LC_{50}s to deltamethrin for each immature stage (nI = 0.037%, nII = 0.045%, nIII = 0.052%, nIV = 0.074% and nV = 0.050%) comparable to those obtained in the present study. The impregnated paper bioassay has disadvantages compared to the topical bioassay mainly in the quantification of toxicological parameters due to the variation in the amount of insecticide that penetrates in each insect exposed and the ignorance of researcher about of that amount; the topical bioassay minimize such variation by individually administrating a known amount of insecticide (Busvine, 1971; ffrench-Constant & Roush, 1990). However, despite such

Table 10. Percent mortality of the post-embryonic stages of *T. infestans* exposed to papers impregnated with 0.36% w/v of deltamethrin in oil for San Juan, Colonia Castelli and La Rinconada strains.

	San Juan		Colonia Castelli		La Rinconada	
Stage	24 h	72 h	24 h	72 h	24 h	72 h
N I	100%	100%	100%	100%	28.57%	21.43%
N II	100%	100%	73.33%	60%	6.66%	6.66%
N III	100%	100%	33.33%	26.67%	0%	0%
N IV	100%	100%	13.33%	6.66%	0%	0%
N V	100%	100%	20%	0%	6.66%	6.66%
Adult	100%	100%	n/i	n/i	6.66%	6.66%

N, instar nymph; n/i, no insects available.

uncertainty, the impregnated paper bioassay is optimal for use in the field as it is possible to distribute ready-to-use papers to the test sites. In addition, the field test sites do not need infrastructure to breed insects as the protocol proposed here considers testing field insects. With the standardized topical bioassay using nymphs I from the first generation of the laboratory it is not possible to dispense with a laboratory with minimal equipment (stereoscopic microscope, micro-syringes, insectary, etc.). The present manuscript does not propose to replace the topical bioassay by the impregnated paper bioassay, but to expedite large-scale monitoring by using a bioassay suitable for the field and to decentralize monitoring (i.e. insecticide impregnated paper) in phase I and to use a more accurate topical bioassay for confirmation of resistance and quantification of toxicological parameters (e.g. LD_{50} and level of resistance) in phase II by specialized laboratories.

Using the developed methodology, no differences in susceptibility were detected in response to variation in age, nutritional status and sex. Therefore, the methodology is more favourable for the development of a protocol for monitoring insecticide resistance in insects from the field because: (a) the toxicological data could be pooled obtaining a few discriminant concentrations, and (b) the methodology developed will assess the insect regardless of the status of the main variables that affect the toxicological susceptibility.

The theoretical discriminant concentration (DC) is one that kills all susceptible individuals but does not kill any resistant individual which allows differentiation or discrimination unequivocally between susceptible and resistant insects (ffrench-Constant & Roush, 1990). Considering this criterion, the DC was based on the CL_{99} of the reference susceptible population. However, there is a historical debate about whether to use the CL_{99} or twice the CL_{99} (Busvine, 1971; Roush & Tabashnik, 1990, Robertson *et al.*, 2007). In the first case, a few individuals can survive (i.e. 1% of the susceptible insects) and will be erroneously considered resistant. In the second case, individuals with low resistance may die and will be erroneously considered susceptible. The choice of the CL_{99} level and the type of error to tolerate depends on the judgement of the researcher, the objectives of the study, the prior knowledge of the insecticide-insect system and the history of control. In the present study, both criteria were considered (i.e. Cl_{99} and $2xCL_{99}$) in a way that the proposed DC assesses the greater possible number of stages in each post-exposure time. Thus, 0.36% of deltamethrin in oil is proposed as DC as that would evaluate all post-embryonic stages at 24 h after exposure and fifth instar nymphs and adults again at 72 h. In practice, as the field sample is mostly composed of fifth instar nymphs and adults, the DC is optimal because most of the field insects will be tested even considering the possible reversal of the knockdown effect between 24 and 72 h post-exposure. The laboratory evaluation of random samples (i.e. different stages with different internal conditions randomly collected) of two deltamethrin-resistant colonies showed that the methodology developed discriminated optimally resistant insects from susceptible insects. Even more, the DC showed high sensitivity discriminating a very low resistant colony.

In conclusion, according to the methodology and discriminate concentration developed in this study the following steps for the phase one of monitoring of deltamethrin resistance in *T. infestans* are proposed: (a) a random sample of insects collected in field is taken, (b) the insects are exposed for 1 h to filter paper impregnated with deltamethrin 0.36% in silicone oil, (c) the insects are removed from paper and kept under temperate conditions to record mortality of all stages at 24 h post-exposure and fifth instar nymphs and adults at 72 h post-exposure. If the result shows 100% mortality at 24 and 72 h, the population is considered susceptible to deltamethrin and ends the study. If, however, survival is recorded in some stage at 24 h or in fifth instar nymph and adult at 72 h, the population is considered possibly resistant and the phase two is carried out. During phase two, the toxicological status of the sample (i.e. resistant or susceptible) will be confirmed through a dose–response study through topical application performed on standardized descendants from field insects (WHO, 1994). The dose–response study should be carried out in laboratories with the required equipment by trained technicians. Thus, a protocol for determining susceptibility/resistance to deltamethrin in *T. infestans* collected in different regions of the endemic area for Chagas disease has been proposed.

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