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Caracterização e distribuição da resistência a deltametrina de populações silvestres e domésticas de *Triatoma infestans* da Bolívia

por

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Tese apresentada ao Programa de Pós-graduação em Ciências da Saúde, como requisito parcial para obtenção do título de doutor em Ciências - área de concentração Doenças Infecciosas e Parasitárias

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"No estalla como las bombas ni suena como los tiros.
Como el hambre, mata callando.
Como el hambre, mata a los callados:
Los que viven condenados al silencio y mueren condenados al olvido.
Tragedia que no suena, enfermos que no pagan, enfermedad que no vende…
El mal de Chagas no es negocio que atraiga a la industria farmacéutica,
ni es tema que interese a los políticos ni a los periodistas.
Elige a sus víctimas en el pobrerío.
Las muerde y lentamente, poquito a poco, va acabando con ellas.
Sus víctimas no tienen derechos, ni dinero para comprar los derechos que no tienen.
Ni siquiera tienen el derecho de saber de qué mueren...". (Eduardo Galeano, 2005).

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RESUMO

A persistência do *Triatoma infestans* e a transmissão contínua de *Trypanosoma cruzi* nos Vales Inter-Andinos e no Grande Chaco da Bolívia são de grande importância. Na última década, focos silvestres desta espécie foram descritos em amplas áreas, na qual o alcance das estratégias de controle do vetor é limitado, sendo frequentes os relatos de resistência do *T. infestans* a inseticidas, que incluem populações silvestres e domésticas.
O presente estudo teve como objetivo caracterizar o perfil de suscetibilidade (1) e hereditariedade (2) de populações silvestres e domésticas de *T. infestans* da Bolívia à deltametrina, bem como descrever a área de distribuição das populações resistentes a diferentes piretróides na América do Sul (3).
1) Foram avaliadas nove populações de *T. infestans* silvestres e domésticas do Grande Chaco e dos Vales Inter-Andinos da Bolívia. Três amostras silvestres de *T. infestans* (dark morph) do Chaco (Santa Cruz) foram suscetíveis a deltametrina (RR_{50} de <2), com 100% de mortalidade em resposta a dose diagnóstica (DD). A população doméstica de Villa Montes do Grande Chaco (Tarija) apresentou altos níveis de resistência (RR_{50} = 129,12 e 0% DD). Do mesmo modo, as populações domésticas dos Vales Inter-Andinos (Cochabamba) apresentaram RR_{50} > 9, sendo as populações silvestres menos suscetíveis, com RR_{50} >5 do que SRL.
2) Cruzamentos experimentais foram realizados entre uma colônia suscetível RR_{50}=0,62 (S), uma resistente RR_{50}=129,12 (R) e com susceptibilidade reduzida RR_{50}=5,04 (SR), em ambas as direções (♀x♂ e ♂x♀). O modo de herança do caráter resistente foi determinado pelo grau de dominância (DO) e dominância efetiva (D_{ML}). A hereditariedade (h_2) foi estimada a partir da colônia R selecionada durante duas gerações, utilizando a dose diagnóstica (10 ng.i.a./ninfa). O resultado para DO e D_{ML} (<1) indica que a resistência é um caráter de dominância incompleta e de herança autossômica. A Dose Letal 50% (DL_{50}) para F1 de ♀Sx♂R e ♂Sx♀R foi de 0,74 e 3,97 respectivamente, revelando efeito de diluição da resistência inicialmente observada. Por outro lado, foi observado um incremento da RR_{50} de 2,25 vezes (F1) e 26,83 vezes (F2) na população selecionada em comparação com a colônia parental.
3) Foi compilado um total de 24 artigos que avaliaram a suscetibilidade a diferentes piretróides em 222 populações de *T. infestans* coletadas no campo dos países de Argentina, Bolívia, Brasil e Paraguai. A relação entre resistência aos inseticidas (avaliada por critérios diferentes) e diferentes variáveis ambientais foi estudada utilizando modelo linear generalizado. A DL_{50} mostrou uma forte relação linear com a RR_{50}. Análise estatística descritiva demonstrou que a distribuição de frequência da Log (DL_{50}) é bimodal, sugerindo a existência de dois grupos estatísticos (um grupo de menor e outro com maior Log (DL_{50}). Finalmente, o modelo significativo incluindo 5 variáveis ambientais referentes a temperatura e precipitação, revelou concentração das populações com altas DL_{50} sobre a região identificada como o centro de dispersão de *T. infestans*. Os dados obtidos neste estudo contribuem com informações sobre a variabilidade do perfil de resistência, ocorrência e distribuição de populações resistentes na Bolívia.

Palavras chaves: *Triatoma infestans*, Triatomíneos, resistência a piretróides, Bolívia.

ABSTRACT

Both the persistence of *Triatoma infestans* and the uninterrupted transmission of *Trypanosoma cruzi* in the inter-Andean valleys and Grande Chaco regions in Bolivia are of high relevance. Wild foci of this species have been described within a wide area this past decade. In places where the reach of the vector control strategies is limited, accounts of wild and domestic populations of insecticide resistant *T infestans* are frequent. The following study aimed to characterize the deltamethrin susceptibility profile (1) and heritability (2) in wild and domestic *T. infestans* Bolivian populations. Additionally, we describe the geographic distribution of pyrethroid resistant South American populations of this species.1) We evaluated 9 populations of wild and domestic *T. infestans* from Grande Chaco and two from the Bolivian inter-Andean valleys. Three wild *T. infestans* (dark morph) from Chaco (Santa Cruz) were susceptible towards deltamethrin (RR_{50} de < 2) presenting a 100% mortality in response to the diagnostic dose (DD). In contrast, the Villa Montes domestic population in Gran Chaco (Tarija) showed high levels of resistance (RR_{50} = 129.12 and 0% DD). Concordantly, the domestic populations from the inter-Andean valleys (Cochabamba) presented a RR_{50} > 9, whereas the wild populations were less susceptible (RR_{50} > 5 than SRL).2) We performed experimental crosses between susceptible (S) [RR_{50}=0.62], resistant (R) [RR_{50}=129.12], and a reduced susceptibility (SRL) [RR_{50}=5.04] colonies, in both directions (♀x♂ e ♂x♀). The heritability mode of the resistance trait was determined by the degree of dominance (DD) and effective dominance (D_{ML}). Heritability (h_2) was estimated considering R colony individuals selected after two generations using the diagnostic dose (10 ng.a.i./nymph). The results regarding the DD and D_{ML} (<1) indicate that resistance is an incomplete dominance trait of autosomal inheritance. The lethal doses 50% (DL_{50}) for the F1 ♀Sx♂R and ♂Sx♀R crosses were 0.74 and 3.97 respectively. This revealed a dilution effect upon the resistance observed initially. Oppositely, we observed a 2.25 fold increase in the RR_{50} for the F1 and 26.83 fold for F2 when comparing between the selected population and the parental colony.3) We reviewed 24 scientific publications that assess pyrethroid susceptibility in 222 *T. infestans* field collected populations from Argentina, Bolivia, Brasil, and Paraguay. The relation between insecticide resistance (assessed with various criteria) and multiple environmental variables was studied using a General Linear Model. The DL_{50} presented a strong linear relation with RR_{50}. Descriptive statistical analyses revealed that the frequency distribution of log (DL_{50}) values is bimodal, which suggests the existence of two statistical groups of lower and higher log (DL_{50}) values. Finally, a significant model considering 5 environmental variables, regarding temperature and precipitation revealed the concentration of the populations with high DL_{50} on the region identified as the *T. infestans* origin of dispersion.The data collected and results obtained in this study bring forth valuable information regarding variability in resistance profiles, as well as the occurrence and distribution of resistant populations in Bolivia.

Key words: *Triatoma infestans*; Triatomines; Pyrethroid resistance; Bolivia.

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LISTA DE SIGLAS E ABREVIATURAS

BHC - Hexaclorobenzeno

BID - Banco Interamericano de Desenvolvimiento

CIPEIN- Centro de Investigaciones en Plagas y Insecticidas

CONICET - Consejo Nacional de Investigaciones Científicas y Técnicas

CITEFA- Instituto de Investigaciones Científicas y Técnicas para la Defensa

D – Doméstico

DC - Doença de Chagas

DD – Dose diagnóstica

DDT - Dicloro-difenil-tricloroetano

DEET - N,N-dietil-m-toluamida

DL_{99} - Dose letal 99%

GABA - Ácido Gama-aminobutírico

HCH - Hexaclorociclohexano

IIBISMED - Instituto de Investigaciones Biomédicas

INCONSUL - Iniciativa do Países do Cone Sul

IRD – Institute de Recherche pour de developemente

KD – Knockdown

Kdr - Resistência Knockdown do inglês “Knockdown resistance”

LATEC - Laboratório de Referência em Triatomíneos e Epidemiologia da Doença de Chagas

FIOCRUZ – Fundação Oswaldo Cruz

ONGs - Organizações Não-Governamentais

OPAS - Organização Panamericana de Saúde

OMS- Organização Mundial da Saúde

PNCCh - Programa Nacional de Controle de Chagas

PNU- Programa das Nações Unidas

POMA- Programa Mundial de Alimentos

RR - Razão de Resistência

UNGECH - Unidade de Gestão para o Controle da Doença de Chagas

UNICEF - Fundo das Nações Unidas para a Infância

SUMÁRIO

1 INTRODUÇÃO

1.1 Aspectos gerais da doença de Chagas

A doença de Chagas (DC) também conhecida como Tripanossomíase Americana é uma enfermidade parasitária endêmica da América Latina causada pelo protozoário flagelado *Trypanosoma cruzi* (Schofield *et al.,* 2006; Yacoud *et al.,* 2008). O parasito é transmitido ao homem através das fezes de insetos hematófagos da subfamília Triatominae (Heteroptera, Reduviidae), sendo a via vetorial responsável por mais de 80% dos casos humanos (Schofield, 1994; WHO, 2002). Os reservatórios silvestres e os vetores do *T. cruzi* apresentam ampla distribuição geográfica, que vai desde os Grandes Lagos da América do Norte até o sul da Argentina e do Chile (Coura & Dias, 2009).

A transmissão do *T. cruzi* pela via vetorial depende de três fatores básicos: a) presença do *T. cruzi*, o agente etiológico da doença, b) triatomíneos domiciliados, c) hospedeiros humanos e outros animais vivendo no ambiente domiciliar. A DC ainda representa um importante problema de saúde pública na América Latina, representando a principal causa de lesões cardíacas em jovens e adultos economicamente produtivos, estima-se entre 8 a 10 milhões de pessoas infectadas (Moncayo & Silveira, 2009; Rassi-Jr *et al.,* 2012, WHO, 2014). Na Bolívia a área de risco corresponde aproximadamente 60% do território, sendo que cerca de 3.700.000 vivem nestas áreas e estima-se que cerca de 1.800.000 estariam infectados (PNCh, 2008).

Nos últimos anos tem sido atribuída atenção especial à transmissão vertical da doença de Chagas em países não endêmicos, nos quais se estima que o número de pessoas infectadas distribuídas entre os quatro continentes (América do Norte, Europa, Ásia e Oceania), seja superior a 390 mil indivíduos (Coura & Viñas, 2010; Rassi-Jr *et al.,* 2010). Neste contexto, os principais mecanismos de transmissão do *T. cruzi* estão ligados à transfusão sanguínea, a transmissão vertical e ao transplante de órgãos por imigrantes latinoamericanos chagásicos para estas áreas (Coura & Vinas, 2010; Rassi-Jr *et al.,*2012).

1.2 Vetores da doença de Chagas

A subfamília Triatominae é um grupo de insetos amplamente distribuído. A maioria das espécies de triatomíneos ocorre exclusivamente no Novo Mundo, entre a latitude 42ºN (nordeste dos USA) e 46ºS (Patagônia- Argentina) (Lent & Wygodzinsky, 1979; Carcavallo, 1999). Apenas *Triatoma rubrofasciata* é comum, tanto no Novo Mundo (principalmente nordeste do Brasil) como também em muitas regiões tropicais da Ásia e da África (Schofield & Galvão, 2009). Sete espécies do gênero *Triatoma* e seis espécies de *Linshcosteus* são conhecidas apenas na Ásia e na Índia, respectivamente (Galvão *et al.,* 2003). Alguns autores sugerem que as espécies do Velho Mundo são derivadas de *T. rubrofasciata* e transportadas da América do Norte, associada aos ratos em navios (Gorla *et al.,* 1997; Patterson *et al.,* 2001; Hypsa *et al.,* 2002; Schofield & Galvão, 2009). Atualmente são reconhecidas 148 espécies pertencentes a 18 gêneros e 6 tribos (Galvão, 2007; Justi *et al*., 2014). Nas Américas, três são os principais gêneros de triatomíneos de maior importância epidemiológica, a saber: *Triatoma*, *Panstrongylus* e *Rhodnius*. Os triatomíneos mais importantes na epidemiologia da doença de Chagas são *Triatoma infestans*, *Triatoma brasiliensis*, *Triatoma pseudomaculata*, *Triatoma sordida*, *Triatoma dimidiata*, *Panstrongylus megistus* e *Rhodnius prolixus* (WHO, 2002) cuja importância varia dependendo da área geográfica.

1.3 Importância epidemiológica de *Triatoma infestans* na saúde pública

T. infestans foi e continua sendo o vetor de maior importância epidemiológica da DC nos países do Cone Sul (Schofield, 1994, Dias, 2007). Em 1964, a máxima distribuição de *T. infestans* foi predita em 6.28 milhões de km^2, incluindo áreas da Argentina, Bolívia, Brasil, Chile, Paraguai, Peru, Uruguai, sendo responsável pela transmissão do *T. cruzi* a mais de 9 milhões de pessoas (WHO, 1991). Após o início do controle vetorial por iniciativas dos países do Cone Sul sua distribuição foi reduzida a menos de um milhão de km^2, resultando em uma forte diminuição na incidência da DC (Gorla, 2002). Na Bolívia atualmente populações domésticas de *T. infestans* persistem nos vales Interandinos e na Região do Grande Chaco de Argentina, Bolívia e Paraguai, representando um grande desafio para os programas de controle (Schofield *et al.,* 2006; Gürtler *et al.,* 2005).

Uma das características que distingue *T. infestans* dos outros triatomíneos é sua alta antropofilia. Estes costumam permanecer em refúgios constituídos por materiais de construção precária como adobe abrigando-se em buracos, fendas das moradias humanas e locais de criação de animais. É comum observar agrupamentos destes insetos nas moradias em torno dos animais criados para consumo local ou familiar como galinheiros, currais de cabras e porcos (Forattini, 1980).

Gorla & Schofield (1989) determinaram uma forte correlação entre a densidade dos insetos e a densidade de hospedeiros, mediante a simulação do ciclo de vida natural deste triatomíneo em galinheiros experimentais de adobe. Desta forma as características macroambientais percebidas pelo inseto podem ser suavizadas pelas condições microambientais do habitat doméstico, definindo as áreas que podem ser colonizadas. Ainda nesse sentido, Gorla (2002) descreveu a influência de diferentes fatores ambientais na colonização das casas e observou que a amplitude térmica e a densidade da vegetação representam preditores diretos e indiretos da presença de *T. infestans,* indicando que a distribuição de *T. infestans* nem sempre está associada com a disponibilidade de alimento, como ser presença de galinheiros nas moradias, se não também com as condições ambientais favoráveis para seu desenvolvimento.

1.3.1 Populações silvestres de *T. infestans*

Acreditava-se que os focos silvestres de *T. infestans* eram bastante restritos. Torrico em 1946 relatou pela primeira vez um foco silvestre de *T. infestans* em uma colina no Vale de Cochabamba na Bolívia, fato confirmado 40 anos depois (Bermúdez *et al.*, 1993). A partir de 1990 até a atualidade mais focos silvestres tem sido reportados nos Vales Interandinos na Bolívia bem como na região do Chaco da Bolívia, Paraguai e Argentina (Noireau *et al.,* 1999; Noireau *et al.,* 2005; Cortéz *et al.,* 2007; Yeo *et al.,* 2005; Ceballos *et al.,* 2009; Buitriago *et al.,* 2010). Estes trabalhos sugerem que as populações de *T. infestans* silvestres não se restringem aos Vales Interandinos da Bolívia e que estariam amplamente distribuídos na região do Chaco. Não entanto outros focos silvestres de *T. infestans* têm sido relatados nas regiões Metropolitana e de Valparaiso de Chile (Bacigalupo *et al.,* 2006).

O fato de *T. infestans* ser encontrado no ambiente silvestre é de suma importância epidemiológica porque pode manter o ciclo da infecção por *T. cruzi* tanto no ciclo

silvestre como doméstico. Existem evidências de que a transmissão de *T. cruzi* foi difundida inicialmente entre marsupiais e pequenos mamíferos antes da transmissão ao homem através da colonização de ambientes domésticos por *T. infestans* durante o desenvolvimento das primeiras culturas agrícolas na América do Sul (Guhl *et al.,* 2000). Neste contexto, a DC pode apresentar dois ciclos epidemiológicos distintos: 1) o ciclo silvestre, no qual *T. cruzi* circula entre os triatomíneos e uma diversidade de mamíferos de pequeno e médio porte que constituem reservatórios naturais; 2) o ciclo doméstico, antropozoonótico, no qual o parasito circula entre o homem e animais de criação doméstica por meio do vetor sinantrópico (Barreto, 1979; Lainson *et al.,* 1979).

Apesar do grande número de focos silvestres de *T. infestans* relatados, bem como da diversidade destes, pouco se sabe sobre a importância da ecologia destes triatomíneos na transmissão do *T. cruzi* (Noireau, 2009). Na região Andina, *T. infestans* silvestres ocorrem principalmente em afloramentos rochosos, que independentemente da sua dimensão, se apresentam como refúgios adequados para a espécie (Cortez *et al.,* 2007; Noireau, 2009). Estas populações Andinas de *T. infestans* apresentam um padrão cromático (Tabela 1) semelhante ao de seus homólogos domésticos em todos os países do Cone Sul. Entretanto vale destacar que dentre estas existe uma população que se diferencia das demais silvestres sendo conhecida como "Mataral morph". Este padrão foi relatado exclusivamente no Sudeste do departamento Cochabamba ($\underset{\sim}{}$ 1.800 m de altitude), sendo estes insetos maiores, com diferenças no conexivo e bandas mais claras e amplas que o padrão (Cortez *et al.,* 2007).

Na região do Vale de Cochabamba, na localidade de Cotapachi, foram coletadas ninfas e adultos de *T. infestans* silvestres em afloramentos rochosos associados a abrigos de marsupiais *Thylamys* e algumas espécies de roedores dos gêneros *Bolomys*, *Philotys* e *Akodon*. Tanto os mamíferos como os triatomíneos capturados apresentaram elevados níveis de infecção pelo *T. cruzi* (> 60%). Apesar da prevalência das linhagens de TCI e TCII no ciclo doméstico no Vale Cochabamba, somente TCI parece ser transmitida entre pequenos mamíferos e *T. infestans* silvestres (Cortez *et al.,* 2006, 2007). Populações de *T. infestans* rupícolas foram relatadas no Norte do departamento de Potosí (área do Parque Nacional Toro Toro), apresentando uma associação interessante com aves tendo sido capturados em fissuras de penhascos onde a arara de peito

vermelho (*Ara rubrogenys*) bem como outras aves de ninho vivem (comunicação pessoal, M Baune em Noireau *et al.,* 2009).

Diferentemente, as populações silvestres de *T. infestans* do Chaco (<400 m de altitude) são arbóreas. Estes apresentam coloração mais escura e são conhecidas como "dark morphs" (DM) distinguindo-se claramente da forma padrão de *T. infestans*, sejam elas domésticas ou silvestres (Noireau *et al.,* 1997, 2005). *T. infestans* DM são encontrados dentro dos troncos de árvores emergentes, em ocos ocupados por Papagaios verdadeiros (*Amazona aestiva*) ou bromélias (Noireau *et al.,* 2009). A baixa taxa de infecção por *T. cruzi* relatada neste morfotipo (~2.5%) está relacionada a sua tendência à ornitofilia (Noireau *et al.,* 2000; Ceballos *et al.,* 2009). A estrutura populacional de dark morph ainda é desconhecida. Enquanto que em *T. infestans* domésticos observam-se duas gerações/ano no clima quente do Chaco (Gorla & Schofield, 1989) acredita-se que as variações na disponibilidade de abrigo em habitats silvestres podem colocar os DM em desvantagem resultando em aumento no tempo de desenvolvimento dos insetos (Noireau *et al.,* 2009).

Destaca-se a plasticidade comportamental e cromática exibida pelos *T. infestans* silvestres. De acordo com a região e as características biogeográficas, estes vetores ocorrem em habitats rupícolas a maior altitude dos 2000 m.s.n.m. (afloramentos rochosos ou penhascos) ou arbóreos em altitude >2000 m.s.n.m. (figura 1) (Noireau *et al.,* 2009).

Tabela 1: Diferentes morfotipos de *Triatoma infestans* silvestres e algumas características de sua bioecologia.

Nome	**Padrão Andino**	**Padrão Andino**	**Mataral morph Andino**	**Dark morph Não andino**
Distinção morfo cromática, com relação ao padrão	-	-	Tamanho (> 30 mm), grandes marcas amarelas no conexivo.	Em geral coloração escura, pequenas marcas amarelas no conexivo.
Área de endemismo	Inter- Andino, Floresta seca (>2000 m.s.n.m.)	Área de Toro Toro Inter – Andino Floresta seca (~ 2000 m.s.n.m.)	Inter – Andino Floresta seca, sudeste do Departamento de Cochabamba (~ 1800 m.s.n.m.)	Terras baixas do Gran Chaco (< 400 m.s.n.m.)
Habitat	Afloramentos rochosos	Penhascos	Afloramentos rochosos	Ocos de árvores
Preferência hospedeiro - alimentação	Pequenos mamíferos	Aves (possivelmente araras)	Provavelmente pequenos mamíferos	Aves (possivelmente papagaios)
Taxa de infecção *Trypanosoma cruzi*	Alta	Baixa	Alta	Baixa

m.s.n.m. = metros sobre o nível do mar. (Noireau, 2009)

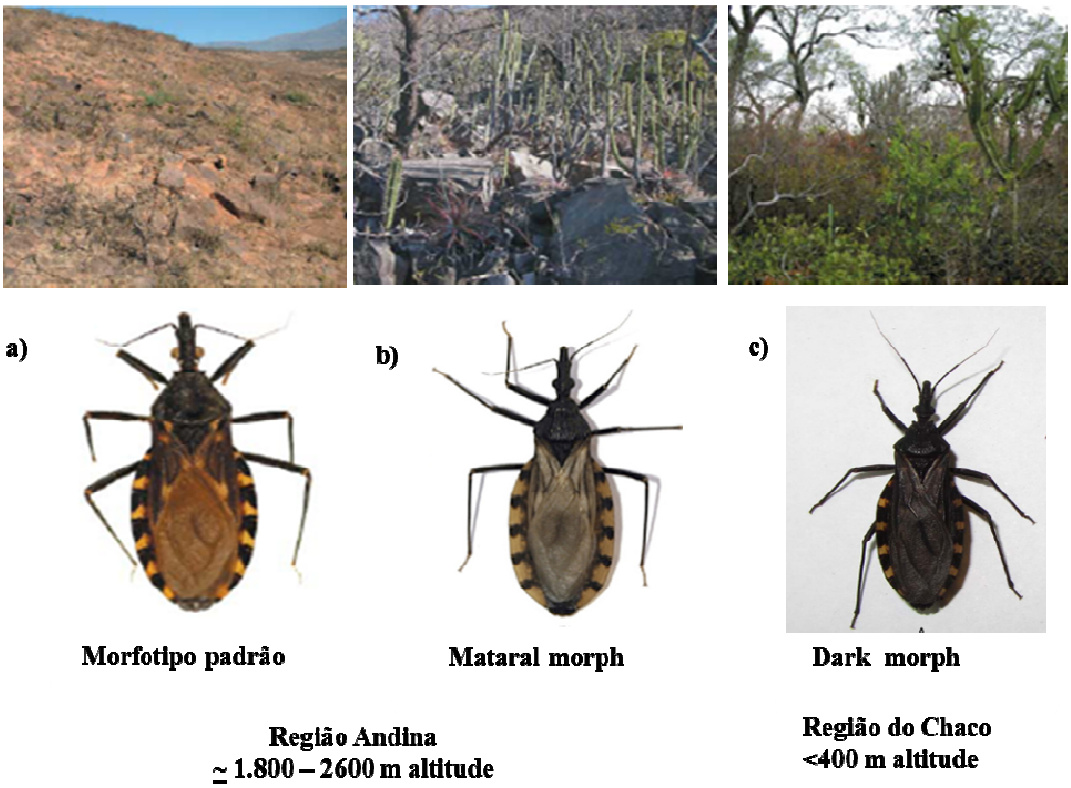

Figura 1. a) *Triatoma infestans* silvestre morfotipo padrão e habitat b) *Triatoma infestans* mataral morph e habitat c) *Triatoma infestans* silvestre dark morph e habitat.

1.4 Controle vetorial da doença de Chagas

A vulnerabilidade de qualquer doença ao controle depende basicamente de duas variáveis ou condições: de um lado, do conhecimento que se tem dela, e de outro lado da potência do instrumental tecnológico disponível. Isso vai determinar o nível de controle que se pretende (Silveira *et al*., 2002; Dias *et al.,* 2002). No caso da DC há limitantes epidemiológicos importantes: como a falta de proteção da população sob risco como ser a falta de imunização e por se tratar de uma enzootia, é caracterizada por apresentar grande número de reservatórios animais; por apresentar uma fase aguda às vezes clinicamente inaparente, impedindo a prevenção secundária; a transmissão transplacentária com a transmissão congênita (Silveira *et al.,* 2002; Silveira & Dias, 2011). Deve-se considerar também que a DC por se uma doença ligada a extrema pobreza é afetada pela dependência das populações pobres e das ações do Estado, a vontade política dos governos, especialmente quanto aos ítens de cobertura e

continuidade e, a fundamental questão da vigilância epidemiológica na etapa de consolidação. Em resumo estes são determinantes primários de natureza econômica observada geralmente em populações em pobreza a extrema pobreza.

Neste sentido, a principal estratégia para controle da DC consiste basicamente na interrupção da transmissão vetorial, pelo combate aos triatomíneos domésticos mediante o controle químico com inseticidas, e maior controle nos bancos de sangue, através do monitoramento da infecção dos doadores por *T. cruzi* (Massad, 2008). A melhoria habitacional também é uma alternativa, mas apresenta algumas limitações pela extensão da área de risco de transmissão e pelo alto custo em relação ao controle químico (Silveira *et al.,* 2002).

Sabe-se que em termos práticos todas as espécies de Triatominae são capazes de transmitir o *T. cruzi*. No entanto, só aqueles triatomíneos que mantêm contato com os seres humanos têm importância epidemiológica, chegando a ser os principais alvos do controle químico. Esta espécie de triatomíneo está frequentemente associada ao peridomicílio e seus anexos, e muito ocasionalmente instalam colônias dentro das casas, sendo, portanto, nestas situações, um transmissor secundário da doença de Chagas. (Silveira *et al*.,1993; Schofield, 1994; Catalá *et al.,* 2007).

No início de 1990, cerca de 80% dos casos de DC foram atribuídos à transmissão vetorial estimando-se uma prevalência de aproximadamente 16 milhões de pessoas infectadas pelo *T. cruzi* (Schofield, 1994). Estes dados justificam a relevância do controle vetorial no contexto da DC como um componente primário nas estratégias para diminuir a transmissão do *T. cruzi*, atrelado a triagem sorológica de doadores de sangue com vistas a reduzir a transmissão.

Países como Brasil, Argentina Chile e Uruguai foram os precursores do controle da transmissão vetorial nas Américas, quando instalada a iniciativa do Cone Sul (INCOSUL) por Argentina, Brasil, Bolívia, Chile, Uruguai, Paraguai, a situação das condições entre os países era bastante desigual tanto como à situação epidemiológica como a capacidade operativa. Entretanto as ações desta iniciativa na maior parte sempre foram limitadas e provisórias já que não obedeciam aos requisitos de continuidade no tempo e contiguidade espacial, condições necessárias para a sustentabilidade ou permanência dos resultados. Esse fato ocorreu basicamente devido ao aporte irregular

de recurso, não suficientes para dar íntegra cobertura a toda a área de risco de transmissão vetorial (Silveira *et al*., 2002)

Este esforço conjunto na luta contra os triatomíneos foi estabelecido em 1991 com o objetivo de interromper a transmissão do *T. cruzi* pela transfusão de sangue bem como a transmissão vetorial pelo *T. infestans* (WHO, 1991). Neste sentido, foram consideradas as características biológicas e ecológicas de *T. infestans*, mas principalmente a sua vulnerabilidade ao controle químico e quase exclusividade como um vetor domiciliar, apesar da existência de algumas populações silvestres na Bolívia (Schmunis, 1996). O controle da infestação doméstica por *T. infestans* foi baseado principalmente por meio do controle químico, que consistiu na pulverização interna e externa das casas com inseticidas de ação residual, na melhoria da habitação rural e do monitoramento contínuo da reinfestação (Dias *et al*., 2002b; Schofield *et al*., 2006).

Vinte anos após a criação do INCOSUL os resultados foram espetaculares, reduzindo significativamente a incidência da DC em aproximadamente 99% da área de interesse. Desta forma Uruguai (1997), Chile (1999) e Brasil (2006) foram certificados como livres da transmissão de *T. cruzi* pelo *T. infestans*, enquanto que nos demais países os resultados mostraram-se limitados (Dias *et al*., 2002b, Schofield *et al.,* 2006). Em sete províncias na Argentina e a área leste do Paraguai a transmissão pelo *T cruzi* foi interrompida (Coura, 2009); na Bolívia os Departamentos de La Paz y Potosí foram recentemente certificados livres de transmissão vetorial (Salvatella *et al.,* 2014)

1.4.1 Histórico do controle vetorial na Bolívia

Com o financiamento do Fundo das Nações Unidas para a Infância (UNICEF) as primeiras medidas para combater a DC, foram dirigidas nas áreas endêmicas fundamentalmente direcionadas aos aspetos da educação em saúde, à promoção das ações de limpeza e organização das casas com a participação comunitária (Guillen *et al.,* 1999). Assim, as atividades voltadas ao combate aos triatomíneos na Bolívia tiveram início em 1980, quando foram organizados pela primeira vez esforços conjuntos para combater a DC, mesmo não sendo este o principal objetivo.

De 1986 a 2000, o Programa Mundial de Alimentos (POMA) vem financiando projetos como “Atenção Primária em Saúde em Áreas Afetadas pela Doença de Chagas”. Esta iniciativa consistia na remuneração do trabalho por uma sesta básica, dentro deste

projeto um dos componentes principais foi dirigido ao fomento da melhoria das moradias com tecnologia própria e uso de materiais locais para combater a DC. Os primeiros resultados foram muito importantes resultando em ações de limpeza em aproximadamente 80.000 casas e melhoria habitacional em aproximadamente 45.000. Este fato e os ótimos resultados despertaram o interesse de outros projetos e de Organizações Não-Governamentais (ONGs) iniciando-se a implantação de diferentes tipos de intervenções nas áreas mais afetadas (Guillen *et al.,* 1999).

A partir de 1984, a unidade Sanitária de Tupiza, no Departamento de Potosí iniciou a luta contra a DC, com base no Projeto de Desenvolvimento Agrícola "Cotagaita San Juan del Oro". Com financiamento do Fundo Internacional de Desenvolvimento Agrícola, da Organização dos Países Exportadores de Petróleo, pelo Programa Mundial de Alimentos e por aporte local, este projeto promoveu o desenvolvimento rural com componentes de assistência técnica, microirrigação, crédito, extensão agrícola, infraestrutura e, dentre outros o componente de saúde mediante o combate da DC. (Guillen *et al.,* 1999; Villena *et al.,* 2007). Tal componente foi baseado principalmente em atividades de educação em saúde, capacitação comunitária, melhoramento das unidades domiciliares, utilização de inseticidas químicos e vigilância epidemiológica participativa nas províncias de Modesto Omiste, Norte e Sud Chichas, ambas do departamento de Potosí. Considerando os elevados índices de infestação encontrados na região, foram trabalhadas aproximadamente 326 comunidades perfazendo um total de 23.417 unidades domiciliares (Guillen *et al.,* 1999; Villena *et al.,* 2007). Em 1992 este projeto ficou sob a responsabilidade do Ministério da Saúde da Bolívia, constituindo-se na primeira experiência de intervenção integral e sistemática de caráter regional no país (Guillen *et al.,* 1999; Villena *et al.,* 2007).

Em 1991, o Ministério de Prevenção Social e Saúde Pública propôs a criação do Programa Nacional de Controle de Chagas (PNCCh) com ação integral e sistemática com vistas ao alcance de toda a área na qual a DC estava presente. Contudo, somente entre 1992 e 1993 o governo da Bolívia assinou um convênio entre o Programa das Nações Unidas (PNUMA), a Organização Pamanericana da Saúde (OPAS) e a Organização Mundial da Saúde (OMS) que permitiu a criação de uma Unidade de Gestão destinada ao Controle da Doença de Chagas (UNGECH), apoiada pelo PNCCh. Neste contexto, em 1993 as atividades de controle da DC nas sete regiões endêmicas da

Bolívia foram iniciadas. Ressalta-se que em 1991, Bolívia integrou a equipe envolvida no INCOSUL (WHO, 1991).

No ano 2000, fomentado pelo crédito outorgado pelo Banco Interamericano de Desenvolvimento (BID), o programa de controle vetorial na Bolívia foi desenvolvido de forma permanente e continuado por 10 anos. Através das campanhas sistemáticas e intensivas de borrifação observou-se uma significativa redução da transmissão vetorial refletindo-se na taxa de incidência da DC, inicialmente presente em 90% para 60% do território nacional (Guillen *et al.,* 1999; PNCh, 2008).

Entretanto, vinte anos após o estabelecimento do INCOSUL na Bolívia, apesar dos esforços realizados, a transmissão do *T. cruzi* pelos triatomíneos continua na região do Grande Chaco e algumas áreas dos vales Interandinos, nas quais a colonização das unidades domiciliares por *T. infestans* tem sido frequentemente relatada.

1.4.2 Uso de inseticidas no controle vetorial da doença de Chagas

Dado que não existe uma vacina suficientemente eficaz, e a expectativa de melhora clínica da doença entre os portadores crônicos é pouco provável, a estratégia básica de controle da transmissão da DC é o combate aos vetores, considerando-se que o controle químico é a ferramenta de maior importância e eficiência (Dias, 2007).

Nas primeiras tentativas para eliminar triatomíneos foram utilizados querosene, soda cáustica ou água fervendo sob as paredes das moradias infestadas (Dias & Schofield, 1999). No entanto, as medidas de controle sistêmico começaram nos princípios da década de 1940 com a utilização dos inseticidas sintéticos. Durante esta década e até a seguinte, o Dicloro-Difenil-Tricloroetano (DDT), que se mostrou sumamente exitoso para o controle de insetos de importância médica (a exemplo de anofelinos, transmissores da malária nas regiões do Pacífico e da Itália) foi testado em laboratório pelo Emanuel Dias. Contudo observou-se nos diferentes testes, sucesso extremamente limitado considerando-se: 1) presença de vias metabólicas de glutation e NADPH dependentes em *T. infestans* (Agosin *et al.,* 1964), 2) lenta velocidade de penetração do ativo através do tegumento do inseto e 3) ausência de ação ovicida (Fotán & Zerba, 1992).

Na atualidade, foi comprovada a resistência cruzada do DDT com os piretróides em vários insetos de importância medica. Já no caso de *T. infestans* a resistência cruzada do

DDT com os piretróides, é uma das razoes para seu fracasso, isto porque debe-se considerar que na Bolívia há a ocorrência de áreas de malária (longamente trabalhadas com DDT) e onde existia grande ocorrência de infestação pelo *T. infestans*. Assim por exemplo, em Bermejo (Departamento de Tarija), a resistência da espécie à deltametrina foi comprovada e esta foi uma área malárica na época.

Após o inesperado fracasso do DDT como alternativa no controle dos triatomíneos, justamente no período em que o mercado dos inseticidas estava dominado pelos hidrocarbonetos clorados, surgiu a partir de 1947 a alternativa de utilizar o hexaclorociclohexano (HCH) cujo isômero ativo é o gamma (lindane) e o dieldrin (Dias & Pelledrino 1948).

Na década de 1970, os inseticidas fosforados como o fenitrotion e malation; e carbamatos como o propoxur, foram extensamente usados no controle dos vetores da DC pelo fato de serem menos persistentes e não bioacumuláveis (Zerba, 1999). Apesar da sua efetividade, sua alta estabilidade química e potencial toxicológico estes produtos foram trocados paulatinamente por outros compostos químicos com características mais favoráveis e menos tóxicos os piretróides (Dias & Schofield 1999).

Na década de 1980, os inseticidas piretróides ingressaram no mercado (Casida & Quistad, 1998). O desenvolvimento destes ativos se realizou a partir da modificação da estrutura química de piretrinas naturais, as quais são extraídas do aquênio da flor de piretro (*Chrysanthemum cinerariaefolium*) (Head, 1973). Os piretróides atuam ao nível do canal de sódio dependente da voltagem do sistema nervoso central periférico (McCaffery, 1998). Estas moléculas mantém os canais de sódio abertos por mais tempo que normal, propagando o ingresso de íons durante a polarização e a despolarização da membrana do axônio. Como consequência, diminuem a velocidade de retorno ao estado inativo e não se restabelecem corretamente o potencial de repouso, perto de 70 mV (Stenersen, 2004). O fechamento tardio dos canais de sódio produz uma despolarização após potencial de ação que é suficientemente grande, podendo gerar múltiplas ações de resposta a um só estímulo (Dong, 2007; Lund, 1985). Os piretróides apresentam reduzido risco ecológico e toxicidade para os mamíferos, são inodoros e não deixam manchas na parede das casas (Dias & Schofield, 1999). As baixas doses de piretróides necessárias para o controle dos triatomíneos permitem que sua utilização seja mais econômica. Estas características levaram a utilização deste grupo de inseticidas de

forma massiva como método de controle de pragas, ectoparasitos e vetores transmissores de doenças, dentre eles os triatomíneos (Germano, 2012).

A exposição aos inseticidas piretróides está associada a sintomas de hiperexcitação, incoordenação, tremores e convulsões, seguidas de paralisia e eventualmente a morte. Alguns compostos são muito efetivos por produzir uma rápida queda, precedida pelo estado de incoordenação e instabilidade na locomoção, conhecida como efeito *knockdown* (KD).(Alzogaray *et al.,* 1997; Alzogaray & Zerba 1997). Os piretróides podem ter um efeito repelente e inibir a alimentação do inseto como demonstrado em *T. infestans* (Alzogaray *et al.,* 2000, Sfara *et al.,* 2006).

1.5 Seleção da resistência aos inseticidas

A exposição dos indivíduos ao uso intensivo de inseticidas resulta em uma pressão seleção, na qual, para uma dose determinada de composto químico aplicado sob uma população, uma pequena proporção de indivíduos está pré-adaptada a tolerar, sobreviver e se reproduzir com êxito. O aumento ou seleção de insetos tolerantes ao inseticida resulta no desenvolvimento de uma população resistente. O que significa que tem a capacidade de sobreviver e reproduzir, na presença de doses de inseticidas que são letais para a maioria dos indivíduos normais ou sensíveis da mesma espécie (WHO, 1975; Roush & Daly, 1990).

A partir da definição proposta, surgiu a pergunta sobre o significado da população hipoteticamente normal (aquela que seria suscetível). Existe um padrão generalizado de resposta a um inseticida? A resposta a um inseticida depende da espécie ou da população? Existe influência do meio frente à resposta a um inseticida? Estas questões, entre outras, levaram à postulação de definições de resistência mais modernas, como a "falha de um inseticida para controlar uma população, apesar de sua efetividade no passado" (Robertson *et al*., 2007). Esta definição pode ser ampliada a ocorrência de modificações fisiológicas, genéticas, etc. que determinam a diminuição da efetividade de um composto no controle de pragas (Germano, 2012).

A velocidade em que se desenvolve esta característica depende da frequência dos genes que conferem a resistência na população, da natureza destes genes (recessivos ou dominantes), da intensidade da pressão de seleção e da taxa de reprodução da espécie (Perry *et al.,* 1998). É sabido que a resistência a um inseticida não é exclusiva de um

grupo de inseto, nem de um tipo de molécula. Conhece-se pelo menos 600 espécies de insetos com resistência a um ou mais de um grupo de inseticidas (Brogdon & McAllister, 1998). O desenvolvimento da resistência nos insetos de importância médica como os barbeiros e os mosquitos representam um desafio no controle das doenças emergentes como a malária, DC e dengue.

A resistência aos inseticidas é o resultado de alguma modificação que pode afetar características fisiológicas ou comportamentais dos insetos. Independente do caráter mono ou polifatorial da resistência, os mecanismos responsáveis podem ter origem comportamental, fisiológica e/ou bioquímica além da modificação do sítio alvo (Brogdon & Mcallister, 1998).

1.5.1 Modo de herança da resistência

A resistência aos inseticidas em insetos de importância agrícola e médica é um sério problema para a agricultura e para a saúde pública (Knight & Norton, 1989; Roush & Tabashknik, 1990). Conhecer o modo de herança da resistência, a dominância relativa e o número de genes envolvido é essencial para compreender e manejar tais populações resistentes, elucidando as bases genéticas envolvidas neste fenótipo (Tabashnik, 1991, Abbas *et al*., 2014). O modo de herança na resistência aos inseticidas pode ser investigado através de retrocruzamentos entre uma população resistente e uma sensível podendo fornecer informações do caráter monofatorial ou polifatorial deste fenótipo (Georghiou, 1969). Informações sobre a expressão da resistência podem contribuir para a compreensão do desenvolvimento deste fenótipo. Do mesmo modo, a compreensão do modo de herança da resistência aos inseticidas é de suma importância, principalmente para a sustentabilidade do controle de pragas (Abbas *et al*., 2014; Bouvier *et al*., 2001).

1.5.2 Mecanismos de resistência

1.5.2.1 Redução da penetração do inseticida

Dado que a cutícula do inseto representa a primeira barreira para o ingresso do inseticida, a redução da penetração ou a lentidão para chegar ao sítio alvo implica diminuição do potencial de toxicidade do composto (Germano, 2012). Assim uma baixa taxa de penetração de inseticida piretróides foi observada em *Helicoverpa armigera*

(Lepidoptera: Noctuidae) resistentes a deltametrina e *M. domestica* resistentes a permetrina (DeVries & Georghiou, 1981; Ahmad *et al*., 2006). No caso de *T. infestans,* foi relatado espessamento da cutícula associado à resistência a deltametrina (Juárez, 1994). Pedrini *et al.,* (2009) demonstraram que populações resistentes a deltametrina de *T. infestans* apresentavam maior espessura do exoesqueleto, quando comparadas com populações suscetíveis, sugerindo a redução da penetração do inseticida como mecanismo de resistência.

1.5.2.2 Resistência comportamental

Este mecanismo de resistência consiste na evasão do inseto ao contato a superfície tratada com o inseticida, pelo que se diz que existem mudanças no comportamento. Modificações genéticas nos receptores periféricos dos estímulos e/ou nos sistemas centrais de processamento dos mesmos têm sido incriminadas como responsáveis pelas alterações comportamentais observadas (Lines, 1987; Mbogo *et al.,* 1996; Mathenge *et al.,* 2001). Lokwood *et al.* (1984) e Roberts & Alecrim (1991) relataram também alteração comportamental de *Anopheles darlingi* em resposta ao DDT na Amazônia, tendo sido observado. Hemingway *et al.*, 2004 relatam que compostos do tipo DDT e permetrina podem induzir mudanças comportamentais em mosquitos, reduzindo a proporção de espécimes que entram nas habitações e alterando o período de maior atividade dos mesmos.

No entanto este tipo de resistência não é bem aceita pela comunidade cientifica, pelo fato de que os insetos não possuem um sistema nervoso complexo e isto pode ser uma resposta de ação de repelência mais que comportamental. Neste sentido, a mudança no comportamento para evitar o inseticida estaria relacionada com o aumento da repelência e irritabilidade, tendo como consequência a diminuição do contato do inseto com o inseticida (Vassena *et al.,* 2007).

1.5.2.3 Resistência metabólica ou bioquímica

A resistência metabólica ou bioquímica corresponde a um processo ou mecanismo normal de desintoxicação de xenobióticos, que costuma apresentar mudanças (aumento de enzimas detoxificativas) em insetos resistentes aos inseticidas (Brogdon & McAllister 1998). Assim, o incremento da atividade de diferentes famílias de enzimas pode estar associado com a resistência a distintos inseticidas. As principais famílias

enzimáticas associadas com a resistência aos inseticidas são as oxidases de função mista, glutationa-S-transferase, acetilcolinesterases e esterases (Ranson *et al*., 2002; Zerba, 2002; Hemingway *et al*., 2004). Tratam-se de famílias multifuncionais, muito importantes na desintoxicação de xenobióticos e devido à sua baixa especificidade e ubiquidade são capazes de formar parte do metabolismo e degradação de muitas substâncias. Existem indicativos que apontam que o incremento da família enzimática depende do tipo de inseticida a ser degradado. Assim, Brogdon & Mcallister (1998) sugerem um maior envolvimento de oxidases de função mista na degradação de piretróides e organoclorados; de esterases na degradação de organofosforados e de acetilcolinesterase na degradação de carbamatos. A resistência *T. infestans* à deltametrina tem sido atribuída a um aumento da atividade detoxificativa de oxidases de função mista (Vassena *et al.,* 2000) e de esterases (González-Audino *et al.,* 2004; Santo Orihuela *et al.,* 2008). Contudo, vale destacar que o perfil enzimático de detoxificação varia de uma população a outra, ainda que em resposta a um mesmo inseticida, revelando a inexistência de um padrão de resposta que possa ser utilizado como diagnóstico para todas as populações. Uma possível justificativa para isso pode ser a utilização durante os ensaios bioquímicos tradicionais de um reduzido número de substratos enzimáticos, que podem não necessariamente estar envolvidos na resistência aos inseticidas (Pessoa *et al*. 2015).

1.5.2.4 Modificação do sítio alvo

Este mecanismo de resistência consiste na mutação do sítio alvo no qual o inseticida atua, evitando a união com a molécula do inseticida. Alterações nos sítios alvo, como canal de sódio, acetilcolinesterase e receptores GABA foram relatadas como mecanismo de resistência aos inseticidas (Devonshire & Moores, 1984; Brogdon & Mcallister, 1998). Referindo-se aos inseticidas piretróides, são conhecidas pelo menos dez mutações nos genes codificadores do canal de sódio que conferem insensibilidade no sistema nervoso em diferentes insetos tais como *Musca domestica*, *Anopheles gambiae* (Diptera: Culicidae) e *Blatella germanica* (Dictyoptera: Blatellidae) (Knipple *et al.,* 1994, Dong 1997, Dabiré *et al.,* 2009). Estas mutações conhecidas comumente como *Kdr* (do inglês knockdown resistance), produzem diferentes graus de resistência de acordo com o sítio alterado. Em populações com elevados níveis de resistência de *T. infestans* provenientes da Argentina e Bolívia, Fabro *et al.,* (2012), Capriotti *et al.,*

(2014) e Sierra *et al.,* (2016) relataram a existência de dois pontos de mutação (L1014F and L925I) no canal de sódio.

Os múltiplos mecanismos de resistência descritos podem estar expressos de forma isolada ou associada, como observado em *Sdoptera exigua*, *Helicoverpa armígera,* com aumento da atividade enzimática e penetração reduzida (DeVries & Georghiou, 1981; Delorme *et al.,* 1988); e *Myzus persicae* e *Anopheles* sp, com aumento da atividade enzimática e modificação do sítio alvo (Criniti *et al.,* 2008, Karunaratne *et al.,* 2007).

1.6 Resistência de *T. infestans* aos inseticidas

Há pouco tempo atrás, a resistência dos triatomíneos aos inseticidas era considerada pontual e com pouca probabilidade de ocorrer já que o ciclo destes insetos é bastante longo, apresentando menor oportunidade de selecionar indivíduos resistentes (Brow & Paul, 1971; Champ & Dyte, 1976; Gorla & Schofield, 1989; Schofield, 1989; Pacheco e*t al.,* 1990; Gorla, 1991; Gorla, 1994).

A primeira evidência bem documentada da resistência dos triatomíneos foi a de *Rhodnius prolixus* ao dieldrin e a resistência cruzada ao lindane na Venezuela. A resistência ao dieldrin foi relatada em 1970 no Estado de Trujillo, e diminuição da suscetibilidade ao fention e ao propoxur nos Estados de Yaracuy, Tachira, Cojedes e Portuguesa (Gonzalez-Valdivieso *et al.,* 1971; Cockburn, 1972; Nocerino, 1976). Os estudos realizados durante os anos 1976 – 1978 em 11 Estados da Venezuela demonstraram elevados níveis de resistência de *R. prolixus* ao dieldrin 4% e baixa suscetibilidade ao fention e ao propoxur (Nelson & Colmenares, 1979).

Considerando que a resistência nos triatomíneos era pouco documentada, e a falta de uma metodologia padrão para realização dos testes de suscetibilidade (o que dificultava a análise e comparação dos resultados), a OMS reuniu especialistas no *Centro de Investigaciones en Plagas y Insecticidas,* CIPEIN (CONICET/ CITEFA, Buenos Aires, Argentina) para desenvolver um protocolo para a avaliação da suscetibilidade aos inseticidas de *T. infestans* e *R. prolixus* (WHO 1994; Vassena *et al*., 2007). O protocolo desenvolvido foi utilizado pela primeira vez pelo CIPEIN na década de 1990 (Vassena *et al.,* 2000) investigando-se a suscetibilidade de *R. prolixus* venezuelanos e *T. infestans* brasileiros aos piretróides deltametrina e cipermetrina. Foi encontrada resistência inicial em *R. prolixus* apresentando razão de resistência (RR) igual a 12,4 para cipermetrina e

11,4 para deltametrina e em *T. infestans* (RR 7,0) para deltametrina (Vassena *et al.,* 2000).

Posteriormente o CIPEIN passou a realizar estudos de monitoramento da resistência a deltametrina em *T. infestans* coletados em diferentes províncias da Argentina. Estes estudos demonstraram resistência de algumas amostras de *T. infestans* de San Luis, La Rioja, Mendoza, Catamarca e Salta, mas os níveis de resistência apresentados (2,0-7,9) não comprometeram o controle químico no campo (Vassena & Picollo, 2003; González-Audino *et al.,* 2004; Santo-Orihuela *et al.,* 2008).

Picollo *et al.,* 2005 realizou estudos de suscetibilidade em outras localidades da Província de Salta (El Chorro, La Toma, El Sauzal e Salvador Mazza) e no Departamento de Tarija (Yacuiba) fronteira com Bolívia, as quais apresentaram RR> 55. No entanto estes insetos se mostraram suscetíveis a outras classes de inseticidas como o fenitrotion (organofosforado), o bendiocarb (carbamato) e o fipronil (fenilpirazol). Simultaneamente o Serviço Nacional de Chagas na Argentina vinha advertindo falhas no controle de *T. infestans* (infestação das unidades domiciliares > 80%). Assim, foram realizados vários estudos na região do Chaco e Vale Interandinos da Bolívia, reportando elevados níveis de resistência a deltametrina nas localidades de Entre Rios (RR= 174); Tierras Nuevas (RR= 542), Villa El Carmen (RR= 438), el Palmar (RR= 300), Villamontes (RR= 247) (Departamento de Tarija), Mataral (RR= 17,4, Departamento Cochabamba) e Sucre (RR= 31,3, Departamento Chuquisaca) (Toloza *et al.,* 2008; Germano *et al.,* 2010, 2012).

Estudos realizados em populações domésticas de *T. infestans* por Lardeux *et al.,* (2010) e Depickire *et al.,* (2012) relataram diferentes níveis de resistência a deltametrina em várias localidades de cinco departamentos da Bolívia (La Paz, Cochabamba, Chuquisaca Tarija e Santa Cruz). Foram atribuídos aos departamentos de Tarija, Chuquisaca e Santa Cruz, os maiores valores de resistência (RR>818), nos restantes departamentos a RR vario de 3,7 a 14, evidenciando diferentes níveis de resistência ao nível regional.

É importante destacar que estudos em populações silvestres de *T. infestans* parecem indicar uma baixa suscetibilidade aos inseticidas piretróides e ao fipronil. Embora a maioria das amostras de *T. infestans* silvestres apresentasse suscetibilidade a deltametrina, algumas amostras provenientes das comunidades de Mataral

(Cochabamba), Julo Grande, Kirus Mayu (Potosí) apresentarem diminuição da suscetibilidade a deltametrina (Lardeux *et al.,* 2010, Roca-Acevedo *et al.,* 2011, Depickere et al., 2012).

Os resultados sobre a resistência de *T. infestans* aos piretróides demonstraram a existência de uma área crítica, com elevados valores de RR, que abrange desde o norte da Argentina e o Sul da Bolívia. Estas informações são entretanto, consideradas insuficientes para conhecer a área de resistência para *T. infestans*, sendo necessário avaliar outras áreas de ocorrência desta espécie, já que todos estes trabalhos foram restritos à Argentina e Bolívia, e ampliar este tipo de estudo para outras espécies de triatomíneos de importância epidemiológica na doença de Chagas (Sonoda, 2008).

Em 2005 o Laboratório de Referência em Triatomíneos e Epidemiologia da Doença de Chagas (LATEC/CPqRR/FIOCRUZ) no Brasil estruturou um grupo para avaliar a suscetibilidade não só de *T. infestans* mas também outros triatomíneos de importância epidemiológica neste país (Sonoda, 2008). Com isso, Pessoa (2008) padronizou metodologia para o desenvolvimento destes estudos a partir de 14 amostras de *T. sordida* de Minas Gerais, através de bioensaios com deltametrina, encontrando populações com RR>5, ou seja, com indicativo de resistência incipiente. Populações de *Triatoma brasiliensis* do estado do Ceará, que corresponde ao principal vetor no Nordeste Brasileiro, mostraram-se suscetíveis a deltametrina (Sonoda *et al.,* 2010). O grupo realizou uma série de trabalhos, adquirindo uma experiência que lhe confere atualmente a responsabilidade de atuar como Centro Colaborador da OMS para o controle de Triatomíneos e na coordenação da Rede de Monitoramento da Resistência de Triatomíneos (Pessoa *et al.,* 2015). Mais recentemente foi demonstrado que as populações residuais de *T. infestans* no Brasil provenientes dos estados de Rio Grande do Sul e da Bahia, são suscetíveis a deltametrina, indicando, portanto, que a persistência destas populações é devido à falhas operacionais (Pessoa *et al.,* 2015b).

2 JUSTIFICATIVA

O programa de controle de triatomíneos nos últimos 20 anos foi bem-sucedido na maioria dos países do Cone Sul (Brasil, Chile, Uruguai, e extensas áreas da Argentina), graças à borrifação das casas infestadas, reordenamento ou melhoria das casas, o que colaborou profundamente com a transformação da realidade das populações residentes nas áreas rurais.

No entanto, o controle químico do vetor apresentou sucesso limitado nos vales Interandinos da Bolívia e principalmente na região do Grande Chaco da Argentina, Bolívia e Paraguai, lugares estes nos quais apesar dos esforços contínuos a transmissão endêmica continua. Nestas regiões o processo de reinfestação das casas tratadas com inseticidas é um fenômeno grave que acontece particularmente rápido, estando possivelmente relacionados aos elevados níveis de resistência relatados nesta área.

Ainda neste contexto, diferentes níveis de resistência a deltametrina e ao fipronil foram detectados em populações silvestres de *T. infestans* da Bolívia. Dessa forma, a preocupação hoje também é direcionada aos focos silvestres de *T. infestans* com variáveis fenotípicas diferentes, as quais apresentam uma ampla distribuição não só na Bolívia, se não também na Argentina, Chile e Paraguai. Acredita-se que estas populações poderiam ter um papel na reinfestação das casas tratadas com inseticidas, por que ao acaso ou não, é justamente nessas regiões onde o sucesso do controle vetorial é limitado, a resistências aos inseticidas e focos silvestres deste vetor foram relatadas. Diante disso, deve-se considerar a possibilidade de ocorrência deste fenômeno em regiões ainda mais amplas.

A presença de *T. infestans* com morfotipos diferentes e amplamente distribuídos na Bolívia são indícios de existe uma alta variabilidade genética desta espécie neste país. Neste sentido, Dias & Schofield (2007) consideram que isso explicaria o porquê do difícil controle vetorial neste país. Desta forma existiria maior chance da ocorrência do fenótipo “resistente” e não seria estranho que a resistência natural e os altos níveis de resistência aos inseticidas se desenvolveram justamente na Bolívia, fato que está sendo observados nos estudos de suscetibilidade os inseticidas de *T. infestans*.

Partindo de tal pressuposto, justificamos a importância deste trabalho que se propôs a caracterizar o perfil de suscetibilidade a deltametrina, modo de herança de populações silvestres e domésticas de *Triatoma infestans* da Bolívia a distribuição geográfica das populações resistentes aos piretróides nos países do Cone Sul.

Acreditamos que é de suma importância a ampliação dos conhecimentos da resistência e/ou suscetibilidade aos inseticidas dos triatomíneos, como uma ferramenta complementar da vigilância entomológica em regiões onde são relatados focos silvestres e domésticos de uma mesma espécie, assim como a identificação de áreas críticas com elevadas razoes de resistência. Os dados aqui obtidos poderão auxiliar de forma prática na eleição de ferramentas e estratégias racionais e factíveis para o controle vetorial na área de interesse.

3 OBJETIVOS

3.1 Objetivo geral

Caracterizar o perfil de suscetibilidade a deltametrina, modo de herança de populações silvestres e domésticas de *Triatoma infestans* da Bolívia e a distribuição geográfica das populações resistentes aos piretróides nos países do Cone Sul, assim como avaliar tais parâmetros e discuti-los no contexto e necessidades das ações e programas em curso.

3.2 Objetivos específicos

- Caracterizar o perfil de suscetibilidade e/ou resistência a deltametrina de populações de *T. infestans* silvestres e domésticas da Bolívia;

- Caracterizar a herança e hereditariedade do caráter de resistência de *T. infestans* com diferentes graus de resistência a deltametrina;

- Avaliar a distribuição geográfica da resistência aos piretróides de *T. infestans nos países do Cone Sul* e correlaciona-la com variáveis ambientais.

4 ARTIGOS

4.1 Artigo 1

Gomez et al. Parasites & Vectors 2014, 7:497
http://www.parasitesandvectors.com/content/7/1/497

RESEARCH Open Access

Susceptibility to deltamethrin of wild and domestic populations of *Triatoma infestans* of the Gran Chaco and the Inter-Andean Valleys of Bolivia

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Abstract

Background: The persistence of *Triatoma infestans* and the continuous transmission of *Trypanosoma cruzi* in the Inter-Andean Valleys and in the Gran Chaco of Bolivia are of great significance. Coincidentally, it is in these regions the reach of the vector control strategies is limited, and reports of *T. infestans* resistance to insecticides, including in wild populations, have been issued. This study aims to characterize the susceptibility to deltamethrin of wild and domestic populations of *T. infestans* from Bolivia, in order to better understand the extent of this relevant problem.

Methods: Susceptibility to deltamethrin was assessed in nine, wild and domestic, populations of *T. infestans* from the Gran Chaco and the Inter-Andean Valleys of Bolivia. Serial dilutions of deltamethrin in acetone (0.2 μL) were topically applied in first instar nymphs (F1, five days old, fasting, weight 1.2 ± 0.2 mg). Dose response results were analyzed with PROBIT version 2, determining the lethal doses, slope and resistance ratios (RR). Qualitative tests were also performed.

Results: Three wild *T. infestans* dark morph samples of Chaco from the Santa Cruz Department were susceptible to deltamethrin with RR_{50} of <2, and 100% mortality to the diagnostic dose (DD); however, two domestic populations from the same region were less susceptible than the susceptibility reference lineage (RR_{50} of 4.21 and 5.04 respectively and 93% DD). The domestic population of Villa Montes from the Chaco of the Tarija Department presented high levels of resistance (RR_{50} of 129.12 and 0% DD). Moreover, the domestic populations from the Valleys of the Cochabamba Department presented resistance (RR_{50} of 8.49 and 62% DD), the wild populations were less susceptible than SRL and *T. infestans* dark morph populations (RR_{50} < 5).

Conclusion: The elimination of *T. infestans* with pyrethroid insecticides in Brazil, Uruguay, Chile, and its drastic reduction in large parts of Paraguay and Argentina, clearly indicates that pyrethroid resistance was very uncommon in non-Andean regions. The pyrethroid susceptibility of non-Andean *T. infestans* dark morph population, and the resistance towards it, of Andean *T. infestans* wild and domestic populations, indicates that the Andean populations from Bolivia are less susceptible.

Keywords: *Triatoma infestans*, Control, Bolivia, Insecticide resistance, Deltamethrin

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Background

Triatoma infestans (Hemiptera, Reduviidae) is the main vector of *Trypanosoma cruzi* (Trypanosomatidae), pathogenic agent of Chagas disease in the countries of the Southern Cone of Latin America [1,2]. Traditionally, the vector control programs focus on the interruption of transmission cycles using insecticides with residual action, especially pyrethroids [2]. Triatominae control strategies were originally based on ecoepidemiological characteristics of the group, such as their slow reproduction and vulnerability to chemical control, and aimed to eliminate *T. infestans*, despite the fact that this species has restricted wild foci [3]. Thereby in the last decades, Uruguay, Chile and Brazil were certified as free of transmission of *T. cruzi* by *T. infestans* [4,5].

However, in the Gran Chaco in Argentina, Bolivia and Paraguay and in some areas of the Inter-Andean Valleys of Bolivia, despite the constant efforts to control *T. infestans* the success of these actions was limited and thus the species still persists [6,7]. In addition to this important problem, in the last few years, wild foci of *T. infestans* have been described, mainly in the Inter-Andean Valleys and in the Gran Chaco [8,9]. This fact was also observed in Argentina, Paraguay and Chile, showing that wild *T. infestans* have dispersed more widely than expected [10-14]. In Bolivia, the epidemiological significance of wild foci of *T. infestans* has been stressed [10].

The process of reinfestation of houses treated with insecticides is a serious phenomenon, and it is occurring quickly in the Gran Chaco [6,15-17]. It is considered that vector control failures, are due to high levels of insecticide resistance in this area [18-21]. In the last few years, more studies demonstrated that the phenomenon of resistance to insecticides in domestic *T. infestans* populations presents an extensive distribution in southern Bolivia and northern Argentina [21-23], with high resistance ratios, and different toxicological profiles [14,18,19,24-26]. Recently, susceptibility and resistance to deltamethrin and fipronil were detected in wild populations of *T. infestans* from Bolivia [14,27].

Coincidence or not, it is in those regions that the reach of the vector control strategies is limited, and insecticide resistance in *T. infestans* populations has been reported, including wild populations [14,27-29]. Thus, this study proposes to characterize the susceptibility to deltamethrin of wild and domestic *T. infestans* from the Gran Chaco and Inter-Andean Valleys in Bolivia, in order to understand better the extent of this relevant problem.

Methods

Populations of *T. infestans*

Nine populations of *T. infestans* five wild (S) and four domestic (D) were collected in the period from 2010 to 2011, in the region of Gran Chaco and Inter-Andean Valleys in Bolivia.

Wild *T. infestans* were captured using traps described by Noireau *et al.* [30,31]. In the Gran Chaco, they were captured in hollow tree trunks, whereas in the Inter-Andean Valleys in rock outcrops.

The collection of the domestic *T. infestans*, both in the Inter-Andean Valleys and in the Gran Chaco, was performed through active searches in intradomicile and peridomicile, with assistance of technicians from the National Chagas Disease Control Program of Bolivia (NCHDCP) (Table 1).

All insects collected were identified using the taxonomic key of Lent and Wygodzinsky [35] and maintained under controlled conditions of temperature and humidity (25°C ±1°C; 60% ±10% RH). They were fed weekly with chicken blood (*Gallus gallus*), ethical approval Comissão de Ética no Uso de Animais (PROTOCOL Nº 41/14-2).

Insecticide

Deltamethrin (pyrethroid) technical grade (S) – cyano-3-pehoxybenzyl (1R) -cis-3- (2,2-dibromovinyl) -2,2-dimethylcyclopropane Carboxylate, (99.6% - Bayer®, Brazil) was used.

Bioassays

The susceptibility reference lineage (SRL) of *T. infestans* came from Centro de Investigaciones de Plagas e Insecticidas (CIPEIN) [36], preserved in the laboratory for more than 30 years, without contact with insecticide and inclusion of external material was used.

Serial dilutions of deltamethrin in acetone were prepared. For each concentration, three repetitions were carried out with ten first instar nymphs of F1 generation (five days old, fasting, weight of 1.2 ± 0.2 mg). The treatment consisted of the application of 0.2 μL of insecticide dilution on the dorsal abdomen, according to the World Health Organization-WHO [37] and Pessoa [38] procedures, with the aid of a Hamilton micro-syringe mounted on a repeating dispenser. For each population, a minimum of eight doses of insecticide active ingredient (a.i.) ranging from 0.42 to 300 ng and killing between >0% to <100% of the individuals, were applied per insect. Acetone was applied to the control group. The mortality was assessed 72 hours after application and it was determined by the inability or lack of coordination of the nymphs to move from the center to the edge of the filter paper (7 cm diameter). Signs of paralysis and lack of response to external stimuli was considered as well. During and after the experiment, the insects were kept under controlled conditions of temperature and humidity (25°C ±1°C; 60% ±10% RH).

Diagnostic dose

The diagnostic dose (DD) applied was twice the minimum concentration of the insecticide that causes 99% of

Table 1 Samples of wild (S) and domestic (D) populations of *Triatoma infestans*, geographical origin, capture site (ecotope), morphs and cytogenetic classification

Site of collection	Department/Province	Latitude/longitude	Altitude meters	Capture site Ecotope	Morphs[a]	Cytotype[b]
CIPEIN [SRL]	Susceptible reference strain	-	-	-	-	-
San Silvestre - S	Santa Cruz de la Sierra/Cordillera	19°21'21"S/62°34'10"W	400	Tree trunk	Dark morph	Non-andean
Terra Plem –S	Santa Cruz de la Sierra/Cordillera	19° 09'27"S/62° 38'8"W	400	Tree trunk	Dark morph	Non-andean
Tita Chaco – S	Santa Cruz de la Sierra/Cordillera	18°55'39"S/62°34'28"W	400	Tree trunk	Dark morph	Non-andean
Mataral - S	Cochabamba/Aquile	18°36'06"S/65°07'20"W	1,700	Rock outcrop	Mataral morph	Andean
Cotapachi – S	Cochabamba/Quillacollo	17°25'29"S/66°15'56"W	2,556	Rock outcrop	Common morph	Andean
Mataral - D	Cochabamba/Aquile	18°35'44"S/65°08'58"W	1,750	Domestic (intra and peridomicile)	Common morph	Andean
Tamachindi - D	Santa Cruz de la Sierra/Cordillera	19°28'41"S/19°28'41"W	410	Domestic (intra and peridomicile)	Common morph	Non-andean
Rancho Nuevo –D	Santa Cruz de la Sierra/Cordillera	19°26'22"S/62°34'05"W	410	Domestic (intra and peridomicile)	Common morph	Non-andean
Villa Montes - D	Tarija/Gran Chaco	21°09'02"S/63°21'56"W	463	Domestic (peridomicile)	Common morph	Intermediate

[a]Morphs classification according to Noireau *et al.* [32], Cortez *et al.* [33].
[b]Cytotypes classification according to Panzera *et al.* [34].

mortality in the susceptible laboratory strain [21,39]. According to the World Health Organization [39], when mortality is <80% the tested population is considered resistant, and if >98% it is considered as susceptible. The LD_{99} to deltamethrin of the SRL was determined (5.50 ng (a.i.) per insect) and with it the DD was estimated.

Data analysis

Data from dose – response tests from each population were analyzed using the PROBIT program version 2 [40]. The slope and the lethal doses required to kill 50% of treated individuals (LD_{50}) were estimated, as well as the Resistance Ratio (RR_{50}), which was calculated by dividing each field population LD_{50} by the SRL LD_{50} value.

Results

Wild populations

From the five wild populations studied, the ones collected at the San Silvestre, Terra Plem and Tita Chaco communities (Chaco region) were identified as *T. infestans* dark morph. These populations had a RR_{50} lower or equal to the SRL, and 100% mortality to DD (Table 2). In agreement with PAHO criteria, they were considered as susceptible to deltamethrin since all had a RR <5.

Regarding the populations from the Inter-Andean Valleys, *T. infestans* from the Cotapachi community were identified as common morph and presented a RR_{50} of 2.90 and 100% mortality to the DD. Furthermore, Mataral community *T. infestans* individuals were identified as Mataral morph and presented a RR_{50} of 4.24 and 96% mortality to the DD (Table 2).

Interestingly, we observed that *T. infestans* dark morph from the Chaco had lower slopes than the SRL (<2.83), whereas Mataral and Cotapachi populations had higher slopes (4.36 and 4.69, respectively).

Domestic populations

Out of the four domestic populations studied, all bugs were identified as *T. infestans* common morph. Domestic populations from Mataral had a RR_{50} of 8.49 and 62% mortality to the DD. In the Rancho Nuevo and Tamachindi populations the RR_{50} was 4.21 and 5.04 respectively with 93% mortality to the DD for both communities. The level of resistance estimated for the Villa Montes population (RR_{50} = 129.12) and the mortality % to the DD (0%) drew our attention (Table 2).

Regarding the estimated slopes, the Tamachindi and Rancho Nuevo populations presented higher values than the SRL (5.46 and 4.43 respectively). On the other hand, resistant individuals from Mataral and Villa Montes presented slopes similar to the SRL (2.92 and 2.25 respectively).

Discussion

This study shows the high susceptibility to deltamethrin determined for three wild populations of *T. infestans* dark morph from the Gran Chaco region of Bolivia, which in turn corresponds to the non-Andean region according to the cytogenetic classification of Panzera *et al.* [34]. These populations presented RR_{50} values equal to or less than the SRL. According to the criteria established by PAHO [41] they were considered susceptible to the tested insecticide (RR_{50} < 5). Notwithstanding, wild Andean populations

Table 2 Toxicological profile to deltamethrin in wild and domestic *Triatoma infestans* from Gran Chaco and the Inter-Andean Valleys of Bolivia

Population	N°	Slope +/− SD	X^2 (df) P	LD_{50} (95% CI)	RR_{50} (95%)	DD% (N°)
CIPEIN (SRL)	240	2.83 +/−0.04	3.43 (4) 0.51	0.42 (0.35 - 0.49)	-	-
San Silvestre – S	300	1.97+/−0.05	0.51 (6) 0.00	0.26 (0.21 - 0.32)	0.62	100 (60)
Tera Plem – S	300	2.61 +/− 0.03	2.72 (6) 0.15	0.39 (0.33 - 0.46)	0.93	100 (60)
Tita Chaco – S	270	2.72+/−0.04	0.54 (5) 0.01	0.48 (0.41 - 0.58)	1.16	100 (60)
Matoral - S	390	4.36 +/− 0.02	1.75 (9) 5.16	1.78 (1.63 - 1.93)	4.24	96 (60)
Cotapachi – S	240	4.69+/−0.02	1.72 (6) 0.05	1.22 (1.11 -1.34)	2.90	100 (66)
Tamachindi - D	390	5.46 +/− 0.02	1.43 (9) 2.35	1.75 (1.62 - 1.87)	4.21	93(60)
Rancho Nuevo –D	390	4.43+/− 0.02	2.41 (10) 7.94	2.09 (1.93 - 2.27)	5.04	93 (46)
Matoral - D	480	2.92+/− 0.30	1.84 (2) 0.00	3.52 (3.15 - 4.02)	8.49	62 (60)
Villa Montes - D	360	2.25 +/− 0.04	3.05 (9) 0.04	54.23 (45.54 - 63.32)	129.12	0 (50)

SD: standard deviation; X^2: chi-squared; df: degrees of freedom; P: probability value; LD_{50} insecticide dose that killed 50% of the population (ng/insect); CI: confidence intervals; RR: resistance ratio; DD: % mortality of the discriminating dose; SRL: susceptible reference lineage; S: Sylvatic (wild); D: Domestic; N°: number of individuals used.

from Matoral and Cotapachi were less susceptible than SRL and *T. infestans* dark morph populations.

Interestingly, wild *T. infestans* Matoral morph had a RR_{50} = 4.24 and thus they are less susceptible than the SRL, but less than RR_{50} = 5 (PAHO criteria) and thus would be considered as susceptible. However, previous studies performed by Roca-Acevedo *et al.* [27] on the same region had reported individuals resistant to deltamethrin and fipronil (RR_{50} = 11.9 e 23.4 respectively). Additionally, in our study of domestic *T. infestans* in the same area we observed a deltamethrin RR_{50} of 8.49 whereas Roca-Acevedo *et al.* [27] reported a RR_{50} of 17.4. The differences between the values may be due to the fact that Roca-Acevedo *et al.* [27] used a different SRL to estimate the RR_{50}.

Depickère *et al.* [14] reported 96% mortality to the deltamethrin DD in individuals from a wild Matoral population. Our results for individuals from the same population agree with this report. The same authors reported the susceptibility of eight wild populations of *T. infestans*, corresponding to the Andean region [34] from Bolivia. Among the populations they tested, the one from Cotapachi, presented a 100% mortality to the DD. Our study also evaluated a wild population from the same region, and similar qualitative test results were obtained (100% mortality to the DD). Nevertheless, the individuals we tested were less susceptible to deltamethirn than the SRL (RR_{50} = 2.90).

The DD is a qualitative method for rapid detection of resistant populations. Several *T. infestans* insecticide resistance studies have evaluated toxicological profiles using RR and DD criteria [14,18-21,27]. Nevertheless, the results obtained by both criteria are not always congruent. Thus, Picollo [42] proposed that the single dose killing 99% of the SRL (1X LD_{99}) would be a more appropriate DD value. This dose permits detecting high mortality among susceptible individuals and low mortality among resistant. Increasing the DD value to twice the LD_{99}, as proposed by Lardeux *et al.* [21], carries the risk of identifying resistant individuals as susceptible due to the high mortality % that would be estimated. This could occur mainly in populations that are in the process of selection for the resistance character, populations in which the toxicological profile would be masked. In contrast, populations with an established resistance character would not present this problem.

We consider that the sample number is an important limiting factor when assessing insecticide resistance. In addition, stochastic variability sources within the studied population must be taken into account. The study developed by Amelotti *et al.* [43] showed that females within an age range can produce individuals with different susceptibility profiles. Due to that, they recommended increasing the sample number to at least 60 individuals with no less than 10 females and with different ages represented per population. This approach would increase the reliability of the obtained results, avoiding false negatives and reducing incorrect interpretation.

During our study domestic populations of *T. infestans* from the Tamachindi and Rancho Nuevo communities, both from the Gran Chaco of the Santa Cruz Department (non-Andean region), were also evaluated. These populations were less susceptible than the SRL with a RR_{50} of 4.21 and 5.04 respectively. They both had 93% mortality to the DD. In several communities of the same region Depickère *et al.* [14] reported reduced susceptibility to deltamethrin in domestic *T. infestans*. It is possible that lower levels of insecticide susceptibility play an important role in the reinfestation process of domestic dwellings.

Population genetics studies performed by Quisberth *et al.* [44] have confirmed that the reinfestation of houses in these communities happens due to residual populations and not due to invasion of wild bugs.

However, the resistance levels registered for populations collected in communities from the Gran Chaco of the Santa Cruz Department (non-Andean region) both in our study and by Depickère *et al.* [14], are not as high as the levels we registered for the Villa Montes population (RR >129.12 and 0% mortality to the DD). The latter, is considered as an Intermediate group, a result of the cross between Andean and non-Andean individuals [45]. Lardeux *et al.* [21] and Depickère *et al.* [14] emphasized that *T. infestans* populations from the Santa Cruz Department, where non-Andean populations occur, are more susceptible to deltamethrin. So Tarija populations (Intermediate), where high levels of resistance have been observed, also show low levels of mortality to the DD (<20%) [14,19-21]. These observations are very important since they correspond to a border area between Argentina and Bolivia, where high resistance levels have been reported [18,20].

Our study has also evaluated different *T. infestans* domestic and wild morph populations from different regions in Bolivia, both from the Inter-Andean Valleys (Andean region) and Gran Chaco (non-Andean region). For each population we obtained different susceptibility profiles. Recent studies indicate that most wild populations of *T. infestans* of the Andean regions in Bolivia are susceptible to deltamethrin [14,21,27]. However, resistance to deltamethrin and fipronil has also been reported in some wild populations from the Julo Grande and Kirus Mayu (Potosí Department) and Mataral (Cochabamba Department) in Bolivia [14,27]. Thus, our data and the aforementioned reports, support the idea that populations of *T. infestans* from different geographic areas and morphs have different toxicological profiles [15,21,22,27].

Population genetics studies consider Bolivia as a center of origin and dispersion of *T. infestans* [8,45-48]. The existence of wild foci and different morphs of this species, added to its wide distribution in that country [8-10,49], suggest a high genetic variability of this species in Bolivia. Therefore, Dias & Schofield [50] consider that the high genetic variability of *T. infestans* would explain why natural resistance and high levels of resistance to insecticides have developed in Bolivia.

Slope values have been used as indicators of population heterogeneity [27]. High slope values are related to low genetic variation, whereas populations in process of selection and thus showing genetic variation relate to less steep slopes (when compared to SRL slope) [51]. In this study 4 out of the 9 tested populations had values that suggest phenotypic variation. The three wild dark morph susceptible populations, and the domestic Villa Montes resistant population (Table 1).

The different toxicological profiles determined for domestic and wild populations of *T. infestans* could be the result of selective pressure from insecticide application plus the genetic variability of the highly structured populations present in this country [52-56]. Moreover, genetic studies through chromosomal markers performed by Panzera *et al.* [45] suggest that pyrethroid resistant populations from the Argentinean-Bolivian border are most likely the result of recent secondary contact between both chromosomal groups (Andean and non-Andean) suggesting a correlation between genomic variability and insecticide resistant populations.

The origin of resistance is unknown in wild Bolivian populations, because they have never had contact with insecticides and is probably due genetic variability. However, more genetic studies should be performed to characterize the resistance phenotype.

Conclusion

Wild and domestic *T. infestans* populations from the Inter-Andean Valleys (Andean region) and Gran Chaco (non-Andean region) from Bolivia, have different susceptibility profiles towards deltamethrin. Although most wild populations are susceptible, insecticide resistance was observed in one. The existence of wild foci and different morphs of *T. infestans* plus its wide distribution in Bolivia are indicative of genetic variability. This could explain the occurrence of resistance in wild populations and thus we suggest that more genetic studies are performed on these populations and the resistance phenotypes are tested under field conditions.

Abbreviations

Ng: Nano grams; mg: Milligrams; ai: Active Ingredient; °C: Celsius degree; µL: Microliters; RH: Humidity relative.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All the authors have contributed substantially to this study. Conceived and designed the experiments: MBG, GCDP, LGD. Performed the experiments: MBG, ACLR. Analyzed the data: MBG, GCDP. Contributed material biologic: ALGO, MRC, FN. Wrote the paper: MBG, GCDP, LGD. All authors read and approved the final manuscript.

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4.2 Artigo 2

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Parasites & Vectors

RESEARCH Open Access

Inheritance and heritability of deltamethrin resistance under laboratory conditions of *Triatoma infestans* from Bolivia

Marinely Bustamante Gomez*, Grasielle D'Avila Caldas Pessoa, Aline Cristine Luiz Rosa, Jorge Espinoza Echeverria and Liléia Gonçalves Diotaiuti

Abstract

Background: Over the last few decades, pyrethroid-resistant in *Triatoma infestans* populations have been reported, mainly on the border between Argentina and Bolivia. Understanding the genetic basis of inheritance mode and heritability of resistance to insecticides under laboratory conditions is crucial for vector management and monitoring of insecticide resistance. Currently, few studies have been performed to characterize the inheritance mode of resistance to pyrethroids in *T. infestans*; for this reason, the present study aims to characterize the inheritance and heritability of deltamethrin resistance in *T. infestans* populations from Bolivia with different toxicological profiles.

Methods: Experimental crosses were performed between a susceptible (S) colony and resistant (R) and reduced susceptibility (RS) colonies in both directions (♀ x ♂ and ♂ x ♀), and inheritance mode was determined based on degree of dominance (DO) and effective dominance (D_{ML}). In addition, realized heritability (h^2) was estimated based on a resistant colony, and select pressure was performed for two generations based on the diagnostic dose (10 ng. i. a. /nymph). The F1 progeny of the experimental crosses and the selection were tested by a standard insecticide resistance bioassay.

Results: The result for DO and D_{ML} (< 1) indicates that resistance is an incompletely dominant character, and inheritance is autosomal, not sex-linked. The LD_{50} for F1 of ♀S x ♂R and ♂S x ♀R was 0.74 and 3.97, respectively, which is indicative of dilution effect. In the resistant colony, after selection pressure, the value of h^2 was 0.37; thus, the LD_{50} value increased 2.25-fold (F2) and 26.83-fold (F3) compared with the parental colony.

Conclusion: The inheritance mode of resistance of *T. infestans* to deltamethrin, is autosomal and an incompletely dominant character; this is a previously known process, confirmed in the present study on *T. infestans* populations from Bolivia. The lethal doses (LD_{50}) increase from one generation to another rapidly after selection pressure with deltamethrin. This suggests that resistance is an additive and cumulative factor, mainly in highly structured populations with limited dispersal capacity, such as *T. infestans*. This phenomenon was demonstrated for the first time for *T. infestans* in the present study. These results are very important for vector control strategies in problematic areas where high resistance ratios of *T. infestans* have been reported.

Keywords: *Triatoma infestans*, Inheritance, Heritability, Insecticide resistance, Deltamethrin, Control

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Background

The prevention of Chagas disease is primarily based on vector control, using mainly pyrethroid insecticides [1]. Successful control of *Triatoma infestans* in Brazil, Chile, Uruguay, and drastic reduction of it in large parts of Paraguay and Argentina, are both clearly indicative of its susceptibility to pyrethroid insecticides in its largest area of distribution [2]. By contrast, resistance to pyrethroids in *T. infestans* has been detected since the year 2000 [3] in several areas of Argentina, Bolivia and Paraguay [4–11]. Failure in vector control is considered to be due to low insecticide efficacy when applied to peridomestic structures, unsustainability of vector control interventions, and high levels of insecticide resistance, reported mainly on the border between Argentina and Bolivia, in the biogeographic region of the Gran Chaco [6, 9, 10, 12].

Genetic factors and intensive insecticide application are responsible for the rapid selection of resistance in many insects and mites [13]. Several factors influence the development of insecticide resistance in insect vectors: volume and frequency of insecticide applications (selective pressures), operational failures, and inherent bionomical characteristics of the insect species involved, such as life cycle, abundance, rapid reproduction, and insect migration [14].

Laboratory models are key to understanding the underlying genetics of inheritance and heritability of insecticide resistance. Conclusions drawn from models are fundamental to improve vector management and resistance monitoring [15]. The inheritance mode of pyrethroid resistance was studied in *T. infestans*, and it showed that resistance is autosomal and shows incomplete dominance, involving at least three genes [16, 17]. Therefore, this study aims to characterize inheritance and heritability of insecticide resistance of *T. infestans* populations from Bolivia to deltamethrin with different toxicological profiles.

Methods

Insects

We used the F2 generation of three colonies of *T. infestans* from Bolivia, with different toxicological profiles to deltamethrin, previously characterized by Gomez et al. [18]: a susceptible colony (S) from San Silvestre (19°21'21"S/62° 34'10"W) Santa Cruz Department; a reduced susceptibility colony (RS) from Rancho Nuevo (19°26'22"S/62°34'05"W) Santa Cruz Department, and a resistant colony (R) from Villa Montes (21°09'02"S/63°21'56"W) Tarija Department. Details of lethal dose 50 (LD_{50}) and resistance ratio 50 (RR_{50}) are shown in Table 1.

The calculation of the RR_{50} of the experimental cross was performed with the susceptible colony (S), because during the study carried by Gomez et al. [18], this colony was significantly more susceptible than the reference lineage (SRL) of *T. infestans* from Centro de Investigaciones de Plagas e Insecticidas (CIPEIN) (X^2 $p < 0.05$). However, the calculation of the RR_{50} for estimation of heritability was performed with the SRL of *T. infestans*.

Experimental matings

Resistance inheritance (cross)

To study inheritance, male and female fifth-instar nymphs were first identified and maintained individually, until the imaginal moult. Then, triatomine couples were formed, and reciprocal crosses were carried out as follows: ♀S x RS♂; S♂ x ♀RS; ♀S x R♂ and S♂ x ♀R. All couples were maintained in plastic cages (5 cm diameter, 10 cm height), feeding was carried weekly with chicken blood (*Gallus gallus*) (ethical approval of FIOCRUZ No 41/14-2). The F1 progeny was tested through a standard bioassay.

Estimation of realized heritability

The evaluation of heritability of resistance was performed with the F1 generation of the resistant colony from Villa Montes Tarija Department (RR_{50} = 129.12), and the individuals selected were those that survived the

Table 1 Toxicological profiles to deltamethrin for colonies of *T. infestans*: *susceptible (S), *reduced susceptibility (RS), *resistant (R) and reciprocally cross progenies: ♀S x ♂RS; ♂S x ♀RS; ♀S x ♂R; ♂S x ♀R

Population	N°	Slope +/− SD	X^2 (df)	P	LD_{50} (95 % CI)	RR_{50} (95 % CI)	DO	D_M
*S	300	1.97+/− 0.05	0.51 (6)	0.00	0.26 (0.21–0.32)	-	-	-
*RS	390	4.43+/− 0.02	2.41 (10)	0.01	2.09 (1.93–2.27)	5.04		
*R	360	2.25 +/− 0.04	3.05 (9)	0.04	54.23 (45.54–63.32)	129.12		
♀S x ♂RS	210	5.44 +/− 0.76	5.34 (4)	0.23	0.65 (0.55–0.76)	2.64 (2.12–3.29)	−0.08	−1.37
♂S x ♀RS	300	4.33 +/− 0.43	4.30 (7)	0.04	1.30 (1.18–1.43)	5.27 (4.22–6.52)	0.54	−8.07
♀S x ♂R	360	4.44 +/− 0.46	3.18 (7)	0.21	0.74 (0.67–0.81)	2.99 (2.39–3.73)	−0.61	0.00
♂S x ♀R	210	5.86 +/− 0.78	3.17 (6)	0.40	3.97 (3.62–4.30)	16.06 (12.93–19.94)	0.02	0.76

*: Colony characterized by Gomez et al. [18]; N°: number of individuals used; SD: standard deviation; X^2: chi-square test; df: degrees of freedom; P: probability value; LD_{50}: insecticide dose that killed 50 % of the population (ng/insect); CI: confidence intervals; RR: resistance ratio; ♀: female; ♂: male; DO: Degree of dominance; D_M: effective dominance

application of the diagnostic dose of deltamethrin (10 ng a.i./nymph) for successive generations (F2 and F3 survivors); they were then tested by standard bioassays.

Bioassays

Serial dilutions of technical grade deltamethrin were prepared in acetone. For each concentration, three repetitions were carried out with ten first-instar nymphs, descendant from each of the experimental crosses (the corresponding F generation for each experiment) (five days old, fasting, weight = 1.2 ± 0.2 mg). Diluted insecticide (0.2 μL) was applied on the dorsal abdomen, according to the procedures recommended by the World Health Organization - WHO [19] and Pessoa [20]. For each experimental cross, at least eight doses of insecticide were applied in order to kill between >0 % to <100 % of the individuals (0.35 to 1500.00 ng a. i./nymph). Acetone was applied to the control group. Mortality was assessed 72 h after application, and it was determined by the inability or lack of coordination of the nymphs to move from the center to the edge of the filter paper (7 cm in diameter). Signs of paralysis and lack of response to external stimuli were also taken into consideration. During and after the experiment, the insects were kept under controlled temperature and humidity conditions (25 °C ±1 °C; 60 % ±10 % RH).

Data analysis

Toxicological data

Data from dose response tests from each experiment were analyzed using the PoloPlus software, version 2.0 [21]. Estimations were made of the slope, the lethal doses required to kill 50 % of treated individuals (LD_{50}) and confidence intervals (CIs.). The resistance Ratio (RR_{50}) was calculated by dividing the LD_{50} value of each experimental cross by the LD_{50} value of the susceptible colony. Parallelism tests were also conducted according to Robertson et al. [22].

Degree of dominance

Degree of dominance (DO) for resistance was calculated according to Stone [23] and Preisler et al. [24] using the following formula:

$$DO = \frac{2X_3 - X_2 - X_1}{X_2 - X_1}$$

Where: X_1 = log (LD_{50}) of the susceptible colony (S); X_2 = log (LD_{50}) of the RS or R colony and X_3 = log (LD_{50}) in the reciprocal progeny (F1).

The level of dominance ranges from 0 to 1.0 (i.e. values below 1.0 indicate complete recessiveness and values equal to 1.0 indicate complete dominance).

Effective dominance

Effective dominance (D_{ML}) was estimated according to Bourguet et al. [25] and Abbas et al. [26] using the following formula:

$$D_{ML} = \frac{MX_3 - MX_1}{MX_2 - MX_1}$$

Where: MX_1, MX_2 and MX_3 represent the mortality percentages for the susceptible, the RS or the R colony and the reciprocal cross progeny (F1), for doses that cause 100 % mortality of the SRL (2.76 ng a. i./nymph treated). D_{ML} expresses effective dominance at a given dose of use, and ranges between 0.0 (survival is recessive) and 1.0 (survival is dominant).

Estimation of realized heritability

Following the method of Falconer et al. [27] and Tabashnik [28], realized heritability (h2) of deltamethrin resistance was estimated as follows:

$$h^2 = \frac{R}{S}$$

In the above equation, R (selection response) was estimated as follows:

$$R = \frac{Log_{final}\, LD_{50} - Log_{initial}\, LD_{50}}{N}$$

Final LD_{50} is the LD_{50} of the population after 2 selected generations; initial LD_{50} is the LD_{50} of the field population before selection and N is number of generations selected with deltamethrin.

Whereas, S (selection differential) was calculated as follows:

$$S = i x \sigma p$$

Where i means intensity of selection calculated according to Falconer et al. [27],

σp means phenotypic standard deviation calculated as follows:

$$\sigma p = [(\text{initial Slope} + \text{final Slope})\, 0.5]^{-1}$$

Results

Inheritance of resistance (cross)

The LD_{50} values to deltamethrin of all reciprocal crosses carried out of *T. infestans*, (♀S x ♂RS; ♂S x ♀RS; ♀S x ♂R and ♂S x ♀R) were significantly different and inferior than their parental RS and R colonies ($p < 0.05$). This is indicative that deltamethrin resistance in *T. infestans* is inherited autosomal, not sex-linked (Table 1).

The LD_{50} progeny values of ♀S x ♂R and ♂S x ♀R were 0.74 and 3.97, respectively (Table 1), showing that they are 46.11-fold and 8.34-fold less tolerant to deltamethrin than

their parental insects of the resistant colony. These results indicate a dilution effect among the resistant and susceptible colonies.

Degree of dominance (DO) values for reciprocal crosses were < 1. This result indicated that the deltamethrin resistance character in *T. infestans* is incompletely dominant. The results of effective dominance (D_{ML}) (<1) suggested that resistance is a recessive character at the discriminant doses used for deltamethrin (Table 1).

Estimation of realized heritability

After 2 generations of continuous selection of *T. infestans* with deltamethrin, it was observed that the LD_{50} value increased from 54.23 to 121.93 (F2) and 1455.32 (F3); it is 2.25-fold and 26.83-fold more resistant compared with the parental resistant colony (Table 2). The value calculated for estimation of realized heritability (h^2) was 0.37 (Table 3). These results indicate increased resistance to deltamethrin from one generation to another, under pressure selection, under laboratory conditions.

Discussion

For *T. infestans*, few studies have been conducted about resistance inheritance. Cardozo et al. [16] and Germano et al. [17], suggested that deltamethrin resistance of *T. infestans* is autosomal, incompletely dominant, and the maternal effects are null. This fact was confirmed during our study; regardless of the direction where crossing was performed (female or male; susceptible colony with resistant or with reduced susceptibility colony), degree of dominance and effective dominance values were < 1, even lower to 0, indicating incomplete dominance. The LD_{50} values to deltamethrin for reciprocal crosses were significantly different and lower than their parental colonies ($p < 0.05$).

Khan et al. [29] stressed that intense selection pressure leads to an increase in resistant genotypes in the population due to the removal of susceptible individuals, increasing the frequency of resistant individuals in a population. However, epistatic interactions can occur, mainly in populations with inbreeding, when each allele contributes to resistance, and the introduction of a susceptible allele dilutes the effect under laboratory conditions [30]. Thus, during our study, the LD_{50} values of experimental crosses among resistant and susceptible colonies were 0.73 (R♀ x S♂) and 3.98 (S♀ x R♂) (Table 1), showing a 46.11-fold and an 8.34-fold decrease in the LD_{50} values when compared with resistant parental insects (LD_{50} = 50.23). This suggests that there is a dilution effect of the resistant character to deltamethrin under laboratory conditions.

Several studies demonstrated high levels of structuring of *T. infestans* populations [31–35, 36] and indicated limited capacity of active dispersal and restricted interbreeding. Germano et al. [37] suggested that geographical structure is present at the microgeographic level, and demonstrated that *T. infestans* populations in different dwellings in the same area have different toxicological profiles to deltamethrin. Selection pressure for insecticide application has a greater effect in populations with limited capacity of dispersal, as is the case of *T. infestans*, than on populations with high dispersal ability. The persistent bug populations that survived insecticide application at local spatial scales support two distinct, but equally worrying scenarios. In the first scenario, operational failures may hinder the control of Triatomine populations, as a result of low quality of the spraying technique and/or low efficacy of pyrethroids, especially in peridomestic structures. In the second scenario, difficulty in control may be due to the intrinsic features of Triatomine bugs, which make them resistant to chemical agents. [32, 38–40].

Different toxicological profiles and mechanisms of resistance were reported for *T. infestans* [7, 8, 11, 41]. Mutation of two different alleles of knockdown resistance (*kdr*) were reported from different populations in the Gran Chaco region in Argentina [7, 41], spanning a different geographical distribution. Coincidently, the resistant population used for the experiment of heritability was collected in the Gran Chaco region, where it can be seen that the LD_{50} value increased from 54.23 (parental) to 121.93 (F2) and 1455.32 (F3) (Table 2). These results suggest that resistance to deltamethrin in *T. infestans* could increase rapidly. Despite their long life cycle, only two generations of continuous laboratory selection were required to increase the level of resistance 26.83-fold. The rapid selection and high levels of resistance in this population are likely to be caused by *kdr* mutation.

Table 2 Toxicological profiles to deltamethrin in survivors of *T. infestans* resistant colony (F1 and F2), after application of deltamethrin (10 ng a.i./nymph)

Population cross	N°	Slope +/− SD	X^2(df)	P	LD_{50} (95 % CI)	RR_{50} (95 % CI)
CIPEIN (SRL)*	240	2.83+/−0.04	3.43 (4)	0.51	0.42 (0.35–0.49)	-
Resistant (Parental) Colony*	360	2.25 +/− 0.04	3.05 (9)	0.04	54.23 (45.54–63.32)	129.12 (104.32–166.38)
Surviving F1	210	1.68 +/− 0.42	0.59 (5)	0.01	121.93 (98.65–161.66)	299.23 (222.48–402.47)
Surviving F2	180	1.60 +/− 0.45	0.56 (4)	0.00	1455.32 (1022.71–3914.21)	3571.45 (2099.09–6076.53)

SRL: susceptible reference lineage*: Strain characterized by Gomez et al. [18]; N°: number of individuals used; SD: standard deviation; X^2: chi-square test; df: degrees of freedom; P: probability value; LD_{50}: insecticide dose that killed 50 % of the population (ng/nymph); CI: confidence intervals; RR: resistance ratio

Table 3 Estimate of heritability (h^2) to deltamethrin resistance in survivors of *T. infestans* after insecticide application under laboratory conditions

N° of generations selected	Estimation of mean selection response per generation			Estimation of mean selection differential per generation					
	Initial log LD_{50}	Final log LD_{50}	Response to selection (R)	*i*	Initial slope	Final slope	σp	Selection differential (S)	h^2
2	1.73	3.16	0.72	3.79	2.25	1.60	0.52	1.97	0.37

i = Intensity of selection [44]; σp = phenotypic variation; h^2 = Estimation of heritability

Estimation of heritability (h^2) is a proportion of phenotypic variation accounted by additive genetic variation, which may decrease either due to a decrease in additive genetic variance or to an increase in environmental variance [27]. During our study, the value for estimation of heritability (h^2) to deltamethrin was 0.37 (Table 3). This lower value was most likely caused by lower phenotypic variation, mainly environmental variation, which indicates the presence of resistant alleles in the study colony [42]. Similarly, the selection was done under laboratory conditions; it does not completely reflect the development of resistance in the field population because of homogeneous environmental conditions [28]. In addition, polygenic and monogenic resistance may occur naturally in insect populations [29]. However, monogenic resistance is less likely to occur under laboratory selection, given the absence of rare variants that may be present in natural insect populations [43, 44].

The laboratory experiments and the use of only one insecticide (deltamethrin) impose limitations compared to field conditions. However, most studies evaluating the susceptibility of insects in the laboratory used deltamethrin (technical grade) as a standard insecticide, because this molecule is stable and there is a great amount of knowledge available about it. As the results in this study only used deltamethrin, it could be reasonably extrapolated to other pyrethroids, but not to other active ingredients (i.e. carbamates).

Moreover, the estimated heritability values provide evidence for the potential of resistance development in the future [15, 29, 30, 43]. Thus, the results of estimation of realized heritability allow the prediction of the number of generations required for the population to increase the level of resistance [28]. It should be noted that this is the first study on estimation of realized heritability in *T. infestans* to deltamethrin, and despite the long life cycle (around 6 months for adults), the values of LD_{50} could be increased considerably (26.83-fold) within two generations. Characterizing the mode of inheritance and determining the estimation of the h^2 value is an essential tool for assessing sustainability of insecticides on the pest population and vectors of public health importance for resistance management [45]. Therefore, more long-term studies should be conducted to clarify the selection process in bugs, because it is very important for control vector management.

Conclusion

The results obtained in our study indicate that (1) the inheritance mode of deltamethrin resistance of *Triatoma infestans*, under laboratory conditions, is autosomal and an incompletely dominant character. This is a previously known process, and it was confirmed in other *T. infestans* populations from Bolivia in this study; (2) the lethal doses (LD_{50}) and resistance ratio increase from one generation to another rapidly, after selection pressure with deltamethrin. This suggests that resistance is an additive and cumulative factor, mainly in highly structured populations with limited dispersal capacity, such as *T. infestans*. This phenomenon was demonstrated for the first time for *T. infestans* under laboratory conditions in the present study. These results are very important for vector control programs in problematic areas where high resistance ratios of *T. infestans* have been reported.

Abbreviations

°C: Celsius degree; ♀: Female; ♂: Male; ai: Active ingredient; i.e.: For example; mg: Milligrams; ng: Nano grams; RH: Humidity relative; µL: Microliters.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All the authors have contributed substantially to this study.
Conceived and designed the experiments: MBG, LGD. Performed the experiments: MBG, ACLR, JEE. Analyzed the data: MBG, GCDP. Wrote the paper: MBG, GCDP, LGD. All authors read and approved the final manuscript.

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4.3 Artigo 3

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Distribution of Pyrethroid Resistant Populations of *Triatoma infestans* in the Southern Cone of South America

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Abstract

Background

A number of studies published during the last 15 years showed the occurrence of insecticide resistance in *Triatoma infestans* populations. The different toxicological profiles and mechanisms of resistance to insecticides is due to a genetic base and environmental factors, being the insecticide selective pressure the best studied among the last factors. The studies on insecticide resistance on *T. infestans* did not consider the effect of environmental factors that may influence the distribution of resistance to pyrethroid insecticides. To fill this knowledge gap, the present study aims at studying the association between the spatial distribution of pyrethroid resistant populations of *T. infestans* and environmental variables.

Methodology/Principal Findings

A total of 24 articles reporting on studies that evaluated the susceptibility to pyrethroids of 222 field-collected *T. infestans* populations were compiled. The relationship between resistance occurrence (according to different criteria) with environmental variables was studied using a generalized linear model. The lethal dose that kills 50% of the evaluated population (LD_{50}) showed a strong linear relationship with the corresponding resistance ratio (RR_{50}). The statistical descriptive analysis of showed that the frequency distribution of the Log (LD_{50}) is bimodal, suggesting the existence of two statistical groups. A significant model including 5 environmental variables shows the geographic distribution of high and low LD_{50} groups with a particular concentration of the highest LD_{50} populations over the region identified as the putative center of dispersion of *T. infestans*.

Conclusions/Significance

The occurrence of these two groups concentrated over a particular region that coincides with the area where populations of the intermediate cytogenetic group were found might reflect the spatial heterogeneity of the genetic variability of *T. infestans*, that seems to be the cause of the insecticide resistance in the area, even on sylvatic populations of *T. infestans*, never before exposed to pyrethroid insecticides, representing natural and wild toxicological

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phenotypes. The strong linear relationship found between LD_{50} and RR_{50} suggest RR_{50} might not be the best indicator of insecticide resistance in triatomines.

Author Summary

The elimination of *T. infestans* in wide areas of the Southern Cone countries of South America and good results in other vector control initiatives showed the high susceptibility of triatomines to pyrethroid insecticides. Despite the constant efforts of vector control, the success was not complete in several areas of the Gran Chaco region of Argentina, Bolivia and Paraguay and parts of the Inter-Andean Valleys of Bolivia, and southern Peru, where persistent populations of domestic and wild *T. infestans* still persist. Additionally, high levels of insecticide resistance leading to control failures were described in the biogeographic region of the Gran Chaco, within the area of persistent house reinfestation after insecticide application. The influence of environmental variables on the geographic distribution of triatomine was previously studied for several species, showing significant correlations between Triatominae species occurrence and a number of environmental variables. We investigated the association between the spatial distribution of pyrethroid resistant populations of *T. infestans* and environmental variables. This study shows that pyrethroid resistance in *T. infestans* causing control failures is a highly localized event, spatially associated with the putative dispersion origin of the species, the location of the intermediate cytogenetic group, and with a particular combination of environmental variables, near the border between Argentina and Bolivia. The strong linear relationship found between LD_{50} and RR_{50} suggest RR_{50} might not be the best indicator of insecticide resistance in triatomines.

Introduction

Chagas disease is the most important vector-borne infection in Latin America, affecting approximately 5–6 million individuals [1]. The disease is caused by the protozoa *Trypanosoma cruzi* (Trypanosomatidae) and the most frequent transmission mechanism is through the feces of infected blood-sucking insects belonging to the subfamily Triatominae (Heteroptera: Reduviidae). The main vector of *T. cruzi* in the countries of the southern cone of South America is *Triatoma infestans* (Klug). This species lives mainly in warm and dry rural areas and in close association with human dwellings, including domiciles and peridomiciliary structures [2, 3]. During the last years, a number of wild foci of *T. infestans* have been described, mainly in the Inter-Andean Valleys of Bolivia, in the Gran Chaco of Argentina, Bolivia and Paraguay [4–8] and in a Metropolitan region from Chile [9].

By 1960, the maximum geographic distribution of *T. infestans* occupied an estimated area of 6.28 million km^2 [10], including parts of Argentina, Bolivia, Brazil, Chile, Paraguay, Peru and Uruguay. This species was responsible for well over half of the 18 million people infected by *T. cruzi*, as estimated by WHO [11] for the 1980 decade. After the establishment of the Southern Cone Initiative (INCOSUR) in 1991, wich had the main goal of interrupting the *T. cruzi* transmission using chemical insecticides to eliminate *T. infestans* populations and through blood transfusion, the vectorial transmission of *T. cruzi* was interrupted in Uruguay (1997), Chile (1999) and Brazil (2006), according to the certification of the Pan-American Health Organization (PAHO) [12]. In Argentina (seven provinces) and Paraguay (eastern) the

transmission of *T. cruzi* was interrupted in several areas where the disease had been historically endemic [13]. The Departments of La Paz and Potosi in Bolivia recently certified the interruption of the vectorial transmission of *T. cruzi* [14]. As a consequence of the vector control interventions in the region, there was a significant reduction of the distribution area of *T. infestans* to less than 1 million km^2, leading to a strong reduction of the new infections by *T. infestans* [10, 14–18].

Despite the constant efforts of vector control the success was not complete, *T. infestans* persists as domestic populations in several areas of the Gran Chaco region from Argentina, Bolivia and Paraguay and parts of the Inter-Andean Valleys of Bolivia, and southern Peru [19–21]. Persistent bug populations that survived the insecticide application at local spatial scales, were related with sources of peridomestic populations, operational failures, reduced residual effect of insecticide or development of resistance to pyrethroid insecticides that decrease the vector control efficacy [22–26]. Resistance to insecticides is a microevolutionary process, over which the dynamics, the structure of the population and the gene flow between groups of individuals would determine the maximum geographical spread of each process of resistance evolution [27, 28]. In different geographic areas of Argentina and Bolivia, resistance of *T. infestans* to pyrethroids was detected by 2000, [29–38]. The occurrence of insecticide resistance was relatively unexpected [39, 40]for a long life cycled-insect duration (compared with other pest species with insecticide resistance records), relatively low frequency of insecticide applications, unsustained-over-time, and low insecticide efficacy in the peridomestic structures that would leave many residual populations (not necessarily resistant, but susceptible individuals that were not affected by the insecticide that degraded before contacting the insects). Studies showed different toxicological profiles and mechanisms of resistance [31, 35, 36, 41]. High levels of insecticide resistance (populations that need 1000 times the amount of active ingredient to kill the same fraction of a susceptible population) leading to control failures were described in the biogeographic region of the Gran Chaco, coinciding with the area of persistent house reinfestation even after insecticide application.

The accumulation of evidence over the last years suggests that the occurrence of insecticide resistance in *T. infestans* populations is associated with the high genetic variability detected in the historical dispersion site of the species towards the southern cone of South America [21, 42, 43] and the strong spatial structure of the populations derived by low population dispersal rates [24, 44–45].

Why is it that control failure associated with insecticide resistance has only been recorded in this particular area and not anywhere else over the historical geographic distribution of *T. infestans*? The cause of the appearance of pyrethroid resistance is still under discussion. The repeated application of pyrethroid insecticide does not seem to be the only cause of pyrethroid resistance appearance, as resistant populations occurred in areas that received less insecticide pressure than others where resistance did not occur, and because multiple independent resistance mechanisms were detected in several populations. The diversity of resistance mechanisms and the genetic variability around the putative dispersion center of the *T. infestans* encouraged the consideration of the influence of environmental variables as another potential cause of pyrethroid resistance occurrence [28].

As far as we know, there is no demonstration of a causal relation between the effect of environmental variables and insecticide resistance, partly because of the difficulty of identifying the individual contribution of the genetic background and the abiotic factors. However, a number of studies have shown direct or indirect effects of environmental variables over the appearance of insecticide resistance in several insect pest species. For example, according to Foster et al [46] the selection for resistance to insecticides in *Myzus persicae* is subject to counteracting selection by cold, wet and windy conditions; whereas [47] showed that adaptive responses and

DNA regions that control their expression have been linked to evolutionary responses to pollution, global warming and other changes. Interestingly, a significantly higher diapause propensities in carriers of the resistance alleles (37.0–76.2%) than in susceptible homozygotes (6.7%) was shown [48]. Although no diapause was ever shown to exist in Triatominae, it was shown that the developmental delays in fifth instar nymphs of Triatominae could be due to an adaptive risk-spreading diapause strategy [49], if survival throughout the diapause period and the probability of random occurrence of "bad" environmental conditions are sufficiently high.

The influence of environmental variables on the geographic distribution of triatomine was studied for several species, showing significant correlations between a number of environmental variables (particularly temperature) and species occurrence e.g. [10, 50]. As other phenotypic characters, the different toxicological profiles and mechanisms of resistance to insecticides is due to a combination of a genetic base and environmental factors [28], with the selective insecticide pressure being the best studied among the last factors. So far, studies on insecticide resistance on *T. infestans* did not consider the effect of environmental factors, that may influence the distribution of resistance to pyrethroid insecticides in *T. infestans* populations.

Guided by the question about the particular occurrence of vector control failures caused by pyrethroid resistance in this particular area, we explored in this study for the first time the geographic distribution of pyrethroid resistance of *T. infestans* populations and its association with environmental variables.

Methods

Data collection

An exhaustive compilation of all available data on studies about susceptibility *of T. infestans* to pyrethroid insecticides was carried out. Repeated data were discarded.

A database containing information on the field-collected specimens and methods used in the susceptibility studies based on topical application of insecticide was created. The database includes collection location coordinates, collection ecotope (intradomestic/peridomestic/sylvatic), value of the lethal dose that kills 50% of the evaluated population (LD_{50}), resistance ratio 50 (RR_{50}) (calculated as LD_{50} of the evaluated population/ LD_{50} of the susceptible population) and diagnostic dose (DD) (defined as percent mortality produced by twice the minimum concentration of the insecticide that causes 99% of mortality in the susceptible laboratory strain). All tests were carried out using first instar nymphs between 3–5 days, topicated with a 0.2 uL droplet applied with a Hamilton microsyringe.

Identifying a *T. infestans* population as resistant to pyrethroids is not easy, because no objective definition of resistance for triatomines exists. At least three criteria have been proposed to operationally define triatomines' resistance. Pan American Health Organization [51] defined as resistant all populations with $RR_{50} > 5$ (PAHO criteria from now on). Zerba and Picollo [52] suggested that a population should be considered resistant when $RR_{50} > 2$ (Z&P criteria from now on). WHO [53] proposed the use the DD and considered a population as resistant if mortality is < 80%, and susceptible if mortality > 98% (WHO criteria from now on), although the latter criteria is used mainly to evaluate resistance in mosquitos.

Analysis of resistance occurrence

Using the three criteria mentioned above, *T. infestans* populations studied for pyrethroid resistance were classified as susceptible or resistant according to 7 different estimates of resistance-occurrence categories as follows. The first three categories derived directly from the three criteria mentioned above (namely, PAHO, Z&P, WHO). A fourth category (RR1) recorded as

resistant any *T. infestans* sample that was classified as resistant by any of the three criteria. A fifth category (RR2) recorded as resistant any *T. infestans* sample that was classified as resistant by at least two of the three criteria. A sixth category (RR3) recorded as resistant a *T. infestans* sample that was classified as resistant by the three criteria. A seventh category (LD_{50}) (strictly not a resistance category) considered the value of the LD_{50} (i.e. the amount of the active ingredient that produced 50% of mortality within the sample under study). It is worth remarking that RR1, RR2 and RR3 are derived variables from PAHO, Z&P and WHO variables, not independent of each other, as they are defined as "both" or "either" of the other criteria.

Environmental data

The analysis of the association between resistance occurrence and environmental variables was carried out using the WorldClim dataset (http://www.worldclim.org) [54], that characterizes climatic conditions over the Earth surface between 1950–2000 in a grid format, with a pixel resolution of 1km. Variables included 19 bioclimatic statistics derived from monthly total precipitation, and monthly mean, minimum and maximum temperature (Bio1 to Bio19 described in full at http://worldclim.org/bioclim). Altitude above sea level was added to the climatic variables.

Modelling resistance occurrence

The distribution of *T. infestans* resistance to pyrethroid insecticides occurrence was carried out using the species distribution modelling approach [55], with the geographic coordinates of resistance occurrence recorded as "presences". We explored two different approaches on the consideration of "absences". On one approach, we considered the coordinates of *T. infestans* populations defined as susceptible, and on the other approach (usual within the context of species distribution modelling [56]), we considered a random selection of 1000 background (pseudo-absence) points taken over the Gran Chaco region and Inter-Andean valleys, the area where *T. infestans* populations still persists after the successful interventions of the Southern Cone Initiative [18].

For the study of the association between environmental variables and resistance occurrence we used a binary response variable, assigning 1 to cases recorded as a site with a *T. infestans* resistant population, according to each of the seven resistance criteria mentioned above, and 0 to the susceptible populations or the randomly selected background points. The case of the LD_{50} data was analyzed similarly as a binary variable based on a threshold value that divided the dataset in high LD_{50} (assigned the value 1) and low LD_{50} (assigned 0) (see the appropriate section in Results for additional details). The analysis was based on a generalized linear model (GLM) with a logit link. Colinearity between bioclimatic variables was estimated using the variance inflation factor (*vif*), of the R *car* package. Only variables with $vif < 10$ were considered for the construction of the model. The evaluation of the model was estimated using the partial area under the receiver operation curve, calculated with the pAUC package of R. Cross-validation (through the *cv.glm* function) was used to measure the robustness of model estimation. Data analysis was carried out with R (version 3.2.0).

In order to qualitatively explore the association between population genetics characteristics of *T. infestans* and the LD_{50} measured on the populations compiled in this study we used the geographic coordinates of the populations categorized by cytogenetics groups (andean, non andean and intermediate), as reported by [21].

Results

The bibliographic compilation produced a total of 24 studies published since 2000, that evaluated 222 field-collected *T. infestans* populations for susceptibility to pyrethroid insecticides,

from Argentina (101), Bolivia (106), Brasil (14) and Paraguay (1) (Fig 1). Not all studies reported RR_{50}, or LD_{50} and DD, and as a consequence, the number of populations considered for the analysis was smaller than 222. Table 1 summarizes the occurrence of pyrethroid resistance, according to each criterium category. The highest recorded RR_{50} and LD_{50} were 1108 and 228, respectively from Campo Largo (Salta, Argentina). The complete dataset is included in S1 Table.

Reported values of LD_{50} show a significant and strong linear relationship with their RR_{50}. However, detailed consideration of compiled data shows that all the reported values by Germano and colleagues [20, 32, 36, 23], together with one reported value by [30] and one by [23] (n = 33, all tests used deltamethrin, except [30], that used B-cyfluthrin) (*T. infestans* populations widely distributed around Argentina, Bolivia and Paraguay) lie over different line functions (with very low residual variability), compared with the rest of the n = 112 reported values by the other 15 authors (slope values of 7.5 and 4.4 for the first and second group, respectively; significantly different, $P < 0.0001$) (S1 Table and S1 Fig).

Pyrethroid susceptibility and environmental variables

A first set of analysis for the first six categories using the recorded resistant and susceptible populations to fit the generalized linear model (GLM) with the environmental variables as predictors showed a low ability to explain the variability of the resistant populations (of any considered category) occurrence. Among the fitted models, the best one explained 43% of the resistance occurrence distribution, based on the PAHO criteria. This model was fitted using 100 points of resistance occurrence and 41 of susceptibility occurrence and included the highest number of environmental variables (Table 2). These 41 susceptibility points is a probably biased sample of the susceptible populations occurrence, driven by the special interest in the region of the border between Argentina and Bolivia; the actual distribution of susceptible populations is probably more widely distributed. A similar result was found when the observed susceptible populations were replaced by the set of 1000 randomly selected points taken from the entire Gran Chaco region and Inter-Andean valleys, where *T. infestans* populations are still patchily present. The analysis showed that none of the environmental variables (either considering the location of the susceptible population or taking background points) were able to account for more than 50% of the resistance occurrence, defined by each of the 6 mentioned criteria.

The geographic distribution of LD_{50}

The descriptive analysis of LD_{50} values, showed that the frequency distribution of the Log (LD_{50}) is bimodal, suggesting the existence of two statistical subpopulations (groups).

The value 2.6 is the threshold value that best separates the two groups. Calculating the descriptive statistics separately for the two groups, the group with lower Log (LD_{50}) has an average = 0.17 and standard deviation = 1.47, whereas the group with higher Log (LD_{50}) has values 3.82 and 0.74, respectively (Fig 2).

Driven by this identified pattern, we plotted the distribution of *T. infestans* populations classifying them in two groups, with low (≤ 2.6) and high (> 2.6) Log (LD_{50}). The geographic distribution of these groups show a particular concentration of populations with highest LD_{50} over the region identified as the putative center of dispersion of *T. infestans*. Thus, we pursued the analysis classifying the two LD_{50} groups assigning 0 to those showing Log (LD_{50}) ≤ 2.6 and 1 to those showing Log (LD_{50}) > 2.6.

The analysis of the geographic distribution of these two *T. infestans* populations based on the Log (LD_{50}), with 2.6 as the threshold value, showed a significant fit of the GLM model with

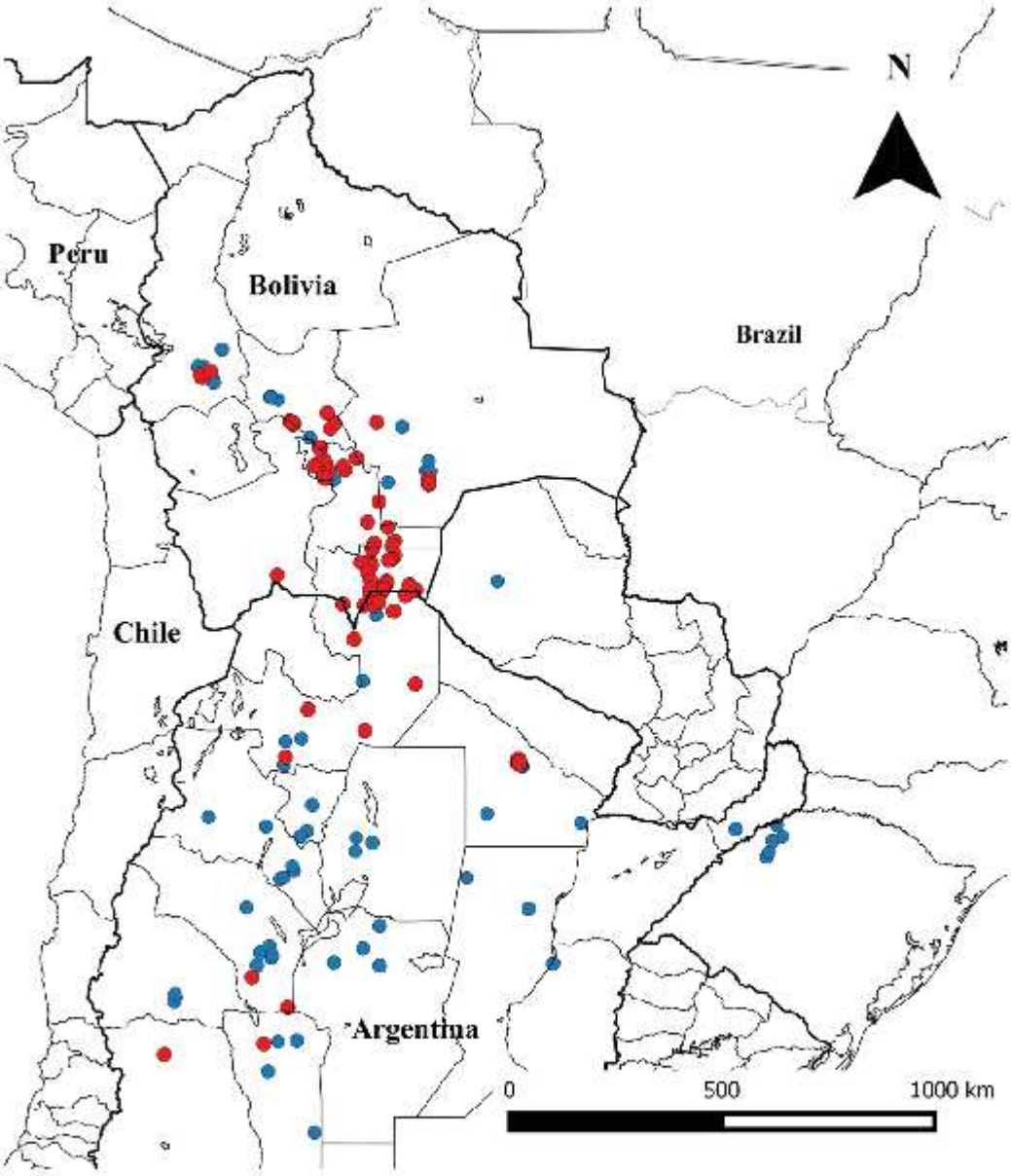
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Fig 1. Geographic distribution of the 222 *T. infestans* populations evaluated for susceptibility to pyrethroids. Red circles: populations identified as resistant by PAHO and WHO criteria (RR_{50} > 5 or mortality DD <80%); blue circles: populations identified as susceptible by PAHO and WHO criteria (RR_{50} < 5 or mortality DD > 80%).

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the environmental variables as predictors. The model was based on 48 population samples where the Log (LD_{50}) > 2.6 and 92 population samples where Log (DL_{50}) was < 2.6. After variable selection to eliminate colinearity (*vif*<10), a model including 5 significant environmental variables was able to explain 55% of the variation in the distribution of the Log (LD_{50}) groups (Table 2). The environmental variables Mean Diurnal Range (Mean of monthly (max temp—min temp)) (Bio2), Mean Temperature of the Driest Quarter (Bio9), Precipitation Seasonality (Coefficient of Variation) (Bio15); Precipitation of the Warmest Quarter (Bio18) are positively correlated, whereas isothermality (Bio2/Bio7) (* 100) (Bio3) is negatively correlated with the occurrence of high LD_{50} populations.

Using the model describing the distribution of populations with low and high Log (LD_{50}), a map with the potential distribution of populations with highest LD_{50} values was created (Fig 3). The area identified as the one where *T. infestans* populations could show highest LD_{50} includes the border between Bolivia and Argentina (see S2 Fig), and southward to the east of Salta and north central Santiago del Estero provinces (Argentina). The model predicts a disjunction area towards the border of La Rioja and San Juan provinces (Argentina) and towards the north of the Cochabamba Department (Bolivia) (see S3 Fig). The model fails at describing the occurrence of one highly resistant population (Log(LD_{50}) >2.6) in Chuquisaca (-65.25, -19.05, a population studied by [28]) and 5 populations (out of 13) concentrated at the south of the Guemes Department (Chaco Province, Argentina, see S4 Fig). The location of the other 40 populations is correct. The model showed a high goodness of fit, with an, AUC = 0.95, pROC = 77.8 (61.9–90.9) and highly robust, with only 3.6% error estimated by the leave-one-out cross validation method.

After identifying the significant model that described the distribution of high and low Log (LD_{50}) groups, separated by the 2.6 threshold, we calculated 3 additional models, using 2, 2.2 and 2.4 as threshold values to separate low and high Log (LD_{50}) groups. All models were significant, included the same environmental variables and explained over 50% of the variation of the geographic distribution of the newly defined groups. From each model, a map showing the prediction of high Log (LD_{50}) occurrence was produced. Fig 3 shows the geographic

Table 1. Number of *T. infestans* populations studied for pyrethroid resistance according to each criterion (response variable) for resistance evaluation in triatomines.

Criteria (resp. variable)	Number of pyrethroid resistant populations	Number of pyrethroid susceptible populations	Total
PAHO	100	41	141
Z&P	119	22	141
WHO	68	83	151
RR1	162	55	217
RR2	24	117	141
RR3	6	74	80
LD_{50}	48	93	141

Response variables represent different ways of identifying susceptible/resistant populations. PAHO: considers resistant populations with RR_{50} > 5. Z&P: considers resistant populations with RR_{50} > 2. WHO: considers resistant a population with mortality <80%. RR1: considers resistant a population that was classified as resistant by any of the three criteria mentioned above. RR2: considers resistant any population that was classified as resistant by two of the three criteria. RR3: considers resistant any population that was classified as resistant by the three criteria.

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Table 2. Best GLM models for each response variable as a function of bioclimatic variables (Bioxx). Response variables are of binary type (0,1).

Response variable	Environmental variables (equation GLM BIOS)	Approx. R^2	AUC	cv.glm
1. PAHO	-7.85 + 0.04 Bio2 + 0.01 Bio9–0.02 Bio13–0.25 Bio14 + 0.01 Bio18	0.43	0.83	0.08
2. Z&P	-2.52 + 0.08 Bio2–0.26 Bio3–0.001 Bio4 + 0.03 Bio9–0.02 Bio13 + 0.02 Bio14 + 0.08 Bio15 + 0.009 Bio18	0.39	0.81	0.09
3. WHO	-2.63 + 009 Bio2–0.26 Bio3–0.001 Bio4 + 0.02 Bio9–0.01 Bio13–0.03 Bio15 + 0.01 Bio18	0.24	0.69	0.08
4. RR1	-7.6 + 0.04 Bio2–0.10 Bio3 + 0.02 Bio9–0.01 Bio13 + 0.05 Bio15 + 0.006 Bio18	0.27	0.73	0.13
5. RR2	-13.48 + 0.08 Bio2–0.13 Bio3 + 0.01 Bio8 + 0.02 Bio9 + 0.01 Bio19	0.18	0.50	0.04
6. RR3	-6.43 + 0.02 Bio9–0.004 Bio16	0.08	0.31	0.01
7. Log (LD_{50})	-25.93 +0.15Bio2–0.34 Bio3 + 0.07 Bio9 + 0.09 Bio15 + 0.006 Bio18	0.55	0.95	0.04

Approx. R^2 = residual deviance/null deviances; AUC = Area Under the Receiver Operator Curve; cv.glm = Estimated error Cross-validation from generalized linear model.

Bio2 = Mean Diurnal Range (Mean of monthly (max temp—min temp)); Bio3 = Isothermality (Bio2/Bio7) (* 100); Bio4 = Temperature Seasonality; Bio8 = Mean Temperature of Wettest Quarter; Bio9 = Mean Temperature of Driest Quarter; Bio13 = Precipitation of Wettest Month; Bio14 = Precipitation of Driest Month; Bio14 = Precipitation of Driest Month; Bio15 = Precipitation Seasonality (Coefficient of Variation); Bio16 = Precipitation of Wettest Quarter; Bio18 = Precipitation of Warmest Quarter; Bio19 = Precipitation of Coldest Quarter.

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distribution of the highest values of Log (LD_{50}) in the four models defined by different threshold values (Log (LD_{50})>2.0 to Log (LD_{50})>2.6 with a step of 0.2).

A map of the distribution of the three *T. infestans* cytogenetic groups (andean, non-andean and intermediate reported by [21]) and the distribution of Log (LD_{50}) measured on *T. infestans* populations shows an almost perfect match between the highly resistant *T. infestans* populations (Log (LD_{50})> 2.6) and the intermediate cytogenetic group (Fig 3).

Discussion

Pyrethroid insecticides were introduced into the pest control market by the end of 1970, and were rapidly identified as a major tool for the control of agricultural pests and vectors of human diseases [56]. At present, pyrethroid insecticides have a 25% share of the insecticide market, and are used in different formulations in the public health sector because of their efficacy, toxicity profile, persistence and low impact over the environment [57–59].

Pyrethroids were incorporated as a tool for the control of domestic triatomines by mid 1980s. The elimination of *T. infestans* in wide areas of the Southern Cone Countries of South America and good results in other vector control initiatives showed the high susceptibility of triatomines to pyrethroids [21, 39]. The reduction of house infestation by *T. infestans* is a

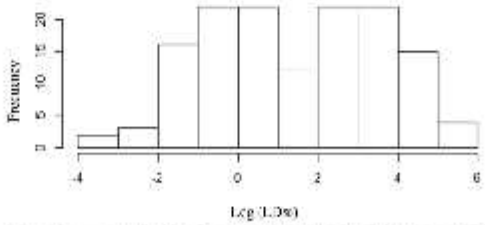

Fig 2. Frequency distribution of Log (LD_{50}) in the *T. infestans* studied populations (n = 141). The value Log (LD_{50}) = 2.6 (equivalent to 13.6 ng a.i./insect) is the threshold value that best separates the two subpopulations.

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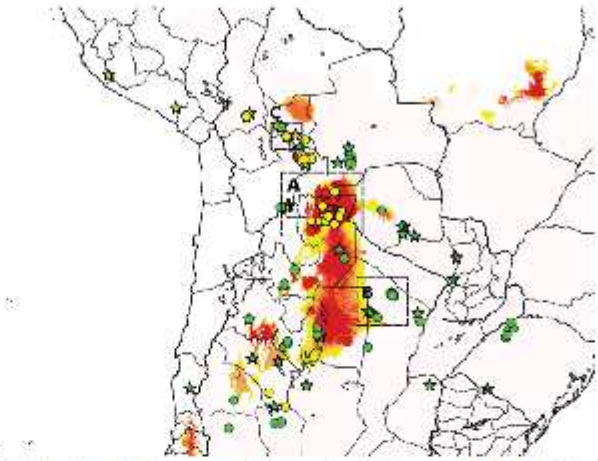

Fig 3. Location of *T. infestans* populations analyzed in the present study (circles) and those studied by [21] (stars). Green, yellow and red stars: Non-andean, Andean and Intermediate cyto-genotypes, respectively (sensu [21]. Green (susceptible populations), yellow and red (resistant populations) circles indicate ranges of Log (LD_{50}): -4.0–-0.15, -0.15–2.6, 2.6–5.4, respectively. Coloured background indicates the gradient of log(DL_{50}) predicted by the GLM (see text): yellow: Log(LD_{50}) >2, to red: Log (LD_{50})> 2.6. A, B and C rectangular areas are enlarged in S2, S3 and S4 Figs.

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success story over about 90% of its maximum geographic distribution area. This success is backed by a long history of vector control programs effort that started in the mid 1950s and made the strongest advances through the INCOSUR, coordinated by PAHO. The main tool for the elimination of house infestation by *T. infestans* was the application of residual insecticides (particularly pyrethroids). However, other socio-demographic factors, such as rural-urban migration, improvement of house quality in rural areas, community education and/or land use changes had contributed to this trend [60].

Although high impact was obtained in the elimination of intradomestic populations of *T. infestans* in most parts of the southern cone of South America, houses of several rural communities in the Gran Chaco are still infested by *T. infestans*. A number of reasons have been mentioned to explain the persistence of *T. infestans* populations in the area, including low insecticide efficacy when applied to peridomestic structures, unsustainability of vector control interventions, and insecticide resistance. Low pyrethroid efficacy is caused by rapid degradation, as has been shown by field measurements of the residual activity of the insecticide sprayed over wood and adobe [61] and by a number of field studies that repeatedly recorded the persistence of frequent residual populations shortly after the insecticide spraying [62]. In addition to the low efficacy of pyrethroid insecticides, the unsustainability of vector control interventions allows the recovery of the even small residual populations of *T. infestans* [58, 63]. Nevertheless, the majority of the sustained vector control failures in the area still occupied by this vector can be attributed to the factors mentioned (low efficacy of pyrethroid insecticides and unsustainability of vector control interventions).

Resistance in the identified hot spot is higher than in other places and it is apparently independent of the frequency of insecticide application in the area, that is not different to the frequency of insecticide application elsewhere. Therefore, we propose that the occurrence of pyrethroid resistant populations in the border between Argentina and Bolivia is not a primary

result of the insecticide selection pressure, but a consequence of the existence of naturally tolerant populations of *T. infestans*, shown by the occurrence of resistant *T. infestans* population of sylvatic origin. The resistance remains high not because of an insecticide-based selection process, but as a natural selection process acting over a population having a naturally high frequency of resistant individuals. A similar interpretation was produced in the review of Mougabure-Cueto and Picollo [28]. The compilation of studies on pyrethroid resistance in *T. infestans* analyzed in this study, shows that the frequency distribution of the $Log(LD_{50})$ for pyrethroids is bimodal, with two well spatially separated statistical groups. This is the first time this resistance feature is shown. The significance of these two groups is not clear. It might reflect the spatial heterogeneity of the high genetic variability of *T. infestans*, that seems to be one possible cause of the insecticide resistance in the area, even on sylvatic populations of *T. infestans*, never before exposed to the pyrethroids, representing natural and wild toxicological phenotypes. The spatial heterogeneity of the LD_{50} is associated with a combination of 3 temperature- and 2 rainfall-derived environmental variables, as shown by the significant fit of the generalized linear model developed in this study. This is the first time the spatial heterogeneity of resistance is shown significantly associated with environmental variables. Panzera et al. [21] speculated that the intermediate cytogenetic group might have appeared recently, as before 1998 house infestation was very low (ranging between 0.5 and 0.8%). These authors suggest that since 1998, and despite continued vector control activities, there has been a gradual increase of insects in houses, reaching house infestation levels of 50 to 80% in 2004. An alternative explanation is that this intermediate cytogenetic group was already in the area and was revealed only after continued vector control activities over a population with high frequency of resistant individuals selected the most resistant ones.

If *T. infestans* showed widespread resistant populations, why is it that control failures have only been reported in a limited area of the *T. infestans* distribution, even though pyrethroid insecticides for the control of the species are in use for more than 30 years now? The vector control failure within a limited area might suggest that the resistance in areas outside of the problematic area is not increasing, even though pyrethroid insecticides are in use, at the same frequency during the last 20 years, or even at higher frequency as it occurred during the last decade in some provinces in Argentina [64].

The occurrence of independent resistance mechanisms suggests that the process is widespread, but that it is not evolving rapidly, as expected by the demographic features of the species. Resistance to pyrethroids is widespread over the arid chaco and Andean valleys of Bolivia, although the high level of pyrethroid resistance (and other active ingredients, such as fipronil) occurs around the putative center of dispersion of the species, where the genetic variability is very high, and a particular combination of environmental variables exists.

We do not have enough information about the process that lead to the occurrence of the highly resistant *T. infestans* populations in the hotspot, to produce a meaningful mechanistic model able to analyze the relation between the occurrence of resistant populations of *T. infestans* and environmental variables. This is a limitation of the study, that can not demonstrate a causal relation between pyrethroid resistance and environmental variables, because the model we based our study on is a statistic one. To be able to demonstrate a causal relationship, we would need a mechanistic model integrating population dynamics, population genetics and environmental variables. Regrettably, we were not able to find publications compiling a geographic database on population genetics characteristics, equivalent to our compilation on pyrethroid resistance, to carry out an equivalent study on the relationship between environmental variables and population genetics.

The compiled published data shows that the highly resistant *T. infestans* populations are geographically limited (except one location in central Chaco province (Argentina) and one north of Potosi (Bolivia)) within an environmental variable space that does not occur towards

the north of Bolivia, but does occur south, down to Santiago del Estero in central Argentina. As we can not claim a causal relation between insecticide resistance and environmental variables, we can not use the resulting model to predict the occurrence of highly resistant *T. infestans* populations. However, we can identify the area highlighted by the model as the one that possesses a similar combination of environmental variable values to the one where the highly resistant *T. infestans* populations occurs. If there is a causal relation between environment and pyrethroid resistance, then the area identified by the model should be carefully considered as an area of potential occurrence of highly resistant *T. infestans* populations. An important consideration should be given to the fact that if insecticide resistance existed in the area, without the need of selection by insecticide pressure, even if the use of pyrethroids is stopped, the frequency of resistant individuals will remain high.

The analysis of the relation between RR_{50} and LD_{50} revealed the existence of two groups of populations in the compiled database. It is difficult to discern the cause of this discrepancy, as it could appear as a consequence of the use of different susceptible populations, or that the studied populations really have a different relation between RR_{50} and LD_{50}. Additional studies on this relation could determine wether these two population groups are artifacts or not. More importantly, if shown that there is only one linear relationship between RR_{50} and LD_{50}, the use of RR_{50} for resistance detection should be revised, as it would mean that LD_{50} multiplied by a constant (the slope) would give the RR_{50} value.

Supporting Information

S1 Table. Compiled database of studies on *Triatoma infestans* susceptibility to synthetic pyrethroids. All studies used topical application of 0,2 μL of the active ingredient on age and weight standardized first instar nymphs produced by adults collected on domestic, peridomestic or sylvatic ecotopes. PAHO, Z&P and WHO columns contains the binary codes (0, 1) identifying susceptible (0) or resistant (1) populations, according to the corresponding three criteria currently used to identify insecticide resistance in Triatominae. LD_{50}: lethal dose 50, RR_{50}: resistance ratio 50, DD: mortality (%).
(XLS)

S1 Fig. Relation between RR_{50} and LD_{50} of 145 *T. infestans* populations. Red circles: data reported by Germano et al [20, 32, 36], Carvajal et al [23] and Picollo et al [30], linear function is $RR_{50} = -2.02 + 7.53\ LD_{50}$, $R^2 = 0.99$, n = 33; black circles: data reported by the rest of the authors reported in the compiled database (S1 Table), linear function is $RR_{50} = -2.76 + 4.41\ LD_{50}$, $R^2 = 0.98$, n = 112.
(TIFF)

S2 Fig. Fig 1 zoomed on rectangle A, over the area where most of highly resistant *T. infestans* populations (Log (LD_{50}) > 2.6) (red circles) and the intermediate cytogenetic group (red stars) are found.
(TIF)

S3 Fig. Fig 1 zoomed on rectangle B, over the Potosi Department (Bolivia) where two highly resistant *T. infestans* populations (Log (LD_{50}) > 2.6) (red circles) are correctly identified by the GLM based on environmental variables.
(TIF)

S4 Fig. Fig 1 zoomed on rectangle C, over the Chaco province (Argentina) where a concentrated group of 5 highly resistant *T. infestans* populations (Log (LD_{50}) > 2.6) (red circles) were not identified by the GLM based on environmental variables.
(TIF)

Author Contributions

Conceived and designed the experiments: MBG DEG LGD. Analyzed the data: MBG DEG. Wrote the paper: MBG LGD DEG. Compilation of data: MBG.

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5 CONSIDERAÇÕES FINAIS

De forma geral os dados apresentados neste trabalho, são um indicativo que não se deve descuidar esforços nos programas de controle vetorial da Doença de Chagas. Consideramos que a continuidade e qualidade do controle realizado pelos programas são de suma importância para evitar o estabelecimento de populações resistentes de *T. infestans*, principalmente em áreas de continua reinfestação.

Além disso devemos sugerir que é de suma importância o fato de reforçar ainda mais a necessidade de monitoramento das populações de triatomíneos no campo, principalmente onde ainda se relatam a ocorrência de populações residuais ou onde existe uma continua reinfestação. Como é o caso da região do Grande Chaco e nos Vales Interandinos na Bolívia onde ainda são relatados focos de infestação pelo *T. infestans*.

Consideramos que a avaliação do perfil toxicológico das populações triatomíneos (quer dizer da avaliação da suscetibilidade aos principais inseticidas usados nos programas de controle), deve ser uma medida adotada por todos os programas como parte de suas atividades. Isto, permitirá contar com a informação necessária par adotar as medidas adequadas, como ser a recomendação da realização de um rodízio de inseticidas, sempre na perspectiva cuidadosa de que fenômenos importantes e ainda desconhecidos estão envolvidos no processo de seleção de populações resistentes de triatomíneos.

Destacamos que um dos principais resultados obtidos em nosso trabalho foi demostrar que os níveis de resistência podem aumentar rapidamente de uma geração a outra, através de pressão de seleção com inseticida no laboratório. Isto chama muito a atenção devido ao aumento em grande magnitude da razão de resistência. Mas, estamos cientes de que não queda esclarecido que mecanismo fisiológico, de fato, estamos observando? Será que os genes podem ser gradativamente ativados sob a pressão do inseticida? Ou selecionamos gradativamente os mais resistentes? Pelo que mais estudos devem ser realizados para determinar que parâmetros ou fatores contribuem para que este

fenômeno ocorra. Acreditamos que dar continuidade a este tipo de estudos será de grande contribuição.

Finalmente devemos salientar que este trabalho foi concluído e possível através de esforços grandes e compartidos entre centros colaboradores da Bolívia (Programa Nacional de Chagas, IIBISMED – UMMS) e Brasil (Laboratório de Referência em Triatomíneos e Epidemiologia da Doença de Chagas, CPqRR- FIOCRUZ Minas e a Secretaria de Vigilância em Saúde - Ministério da Saúde). Acreditamos que esta parceria é um exemplo de colaboração interinstitucional e servirá como referência e indução de propostas similares em outras regiões de importância onde ocorrem, por exemplo, problemas de controle de *R. prolixus, T. brasiliensis e T. dimidiata.*

Por fim é importante salientar que uma das finalidades deste projeto foi o formar uma expertise boliviana na temática de avaliação de resistência em triatomíneos, isto para contribuir com o enfrentamento do problema da resistência na Bolívia.

6 CONCLUSÕES

- As populações silvestres e domésticas de *T. infestans* dos Vales Interandinos e do Grande Chaco da Bolívia, apresentam diferentes perfis de suscetibilidade ao inseticida deltametrina. Das populações estudadas neste trabalho dois das quatro populações silvestres foram suscetíveis. No entanto, a população doméstica de *T. infestans* proveniente de Villa Montes da região do Chaco apresento altos valores de resistência, em comparação com as populações silvestres de *T. infestans* dark morph do Chaco que se mostraram altamente susceptíveis.
- A herança da resistência a deltametrina de *T. infestans* é autossômica e apresenta caráter de dominância incompleta. A pressão continua de inseticida incrementa a razão de resistência de uma geração a outra. Nesse sentido, sugere-se que a resistência em *T. infestans* é um fator aditivo e acumulativo, principalmente em populações altamente estruturadas como es é o caso de *T. infestans*. Este fenômeno demostrado pela primeira vez para *T. infestans* neste estudo. Sendo assim que estes resultados são de suma importância para os programas de controle vetorial e as possíveis estratégias a ser assumidas nas áreas onde foram identificadas altas razões de resistência de esta espécie.
- No modelo de distribuição de resistência foram identificados dois grupos com altos e baixos valores de DL_{50}, distribuídos na região fronteiriça entre Bolívia e Argentina, que coincide com a área das populações do grupo citogenético intermédio de *T. infestans.*

  A ocorrência estos dois grupos concentrados nessa região particular, que coincide com a área onde foram encontradas populações do grupo citogenético intermedio, pode ser um indicativo da heterogeneidade espacial da variabilidade genética de *T. infestans*. Isto parece ser uma dos fatores para a ocorrença da resistência aos inseticidas piretoriides nessa área. Incluindo as populações silvestres de *T. infestans*, que nunca antes foram expostas aos inseticidas piretróides, o que representa fenotipos toxicológicos naturais e silvestres. A forte relação linear encontrada entre DL_{50} e RR_{50} sugere que RR_{50} pode não ser o melhor indicador de resistência a inseticidas para os triatomíneos.

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