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Exploring novel pyrethroid resistance mechanisms through RNA-seq in *Triatoma dimidiata* from Colombia

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ABSTRACT

Pyrethroids are the most widely used insecticides for controlling insect vectors carrying medically and economically significant pathogens. In Colombia, studies on triatomine insecticide resistance are limited. Due to the increasing challenge of insecticide resistance, this work focuses on determining resistance to different pyrethroid insecticides in populations of *Triatoma dimidiata* from Colombia. To define the possible causes of resistance, three potential molecular mechanisms were explored: 1) mutations in the coding region of the voltage-gated sodium channel gene (*vgsc*), the insecticide target site; 2) modulation of enzymatic activity associated with metabolic resistance; and 3) changes in the mRNA profiles using RNA-seq. The results showed that the field population of *T. dimidiata* was resistant to lambda-cyhalothrin and deltamethrin insecticides. Insects surviving sublethal doses of insecticides did not exhibit the classical mutations in the *vgsc* gene. Transcriptomic profile analyses of *T. dimidiata* revealed differentially regulated genes in field and laboratory populations under selective pressure with lambda-cyhalothrin. Gene enrichment analysis showed the positive regulation of transcripts related to detoxifying enzymes and mitochondrial proteins, which could play a significant role in insecticide resistance. This comprehensive investigation is crucial for providing insights into resistance mechanisms and generating strategies to manage these critical vector species.

1. Introduction

Pyrethroids are the most broadly used insecticides for controlling insect vectors of pathogens of medical and economic importance. Triatomines, vectors of *Trypanosoma cruzi*—the etiological agent of Chagas disease—are widely distributed across the Americas. Currently, neither vaccines nor effective treatments are available for the chronic phase of Chagas disease; therefore, reducing the population of triatomine bugs remains the most effective method to decrease transmission. Residual house spraying with insecticides has proven efficacious in interrupting parasite transmission. However, excessive use of insecticides has led to the emergence of resistance.

Pyrethroid resistance has been reported across various American countries, including Argentina (Fronza et al., 2016; Germano et al., 2010; Picollo et al., 2005), Bolivia (Lardeux et al., 2010; Santo-Orihuela et al., 2013), Brazil (Pessoa et al., 2014), Peru (Yon et al., 2004), Mexico (Davila-Barboza et al., 2019b, 2019a), and Venezuela (Vassena et al., 2000). These reports confirm the existence of resistance and shed light on crucial resistance mechanisms. These mechanisms include metabolic resistance (Santo-Orihuela et al., 2013), alterations in the site of action associated with mutations in the insecticide target, specifically the voltage-gated sodium channel (Capriotti et al., 2014; Fabro et al., 2012), and reduced penetration (Pedrini et al., 2009). These findings have been extensively described in *Triatoma infestans.* These data are also supported by findings in other species, such as *T. dimidiata, T. pallidipennis, T. picturata, T. brasiliensis, Panstrongylus herreri,* and *Rhodnius prolixus* (Flores-Ferrer et al., 2018).

In Colombia, triatomine insecticide resistance is limited to laboratory populations, specifically in *R. prolixus, T. dimidiata*, and *T. maculata.* Notably, only one study has been conducted on insecticide resistance in field populations of *R. prolixus.* This study revealed the potential emergence of resistance to deltamethrin in *R. prolixus* populations, suggesting an associated rise in the activity of detoxifying enzymes. Despite these results, more information must be given regarding insecticide

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susceptibility in other species of triatomine bugs. Therefore, these species, equally crucial as vectors of *Trypanosoma cruzi* in certain regions, warrant comprehensive investigations to enhance our understanding of their resistance status and possible mechanisms of this resistance.

This work aims to determine the resistance profile of natural populations of *T. dimidiata* from Boyacá, Colombia, towards pyrethroid insecticides—specifically permethrin, lambda-cyhalothrin, and deltamethrin. As the second most crucial epidemiological vector of *Trypanosoma cruzi* in Colombia, *T. dimidiata* assumes a pivotal role and even replaces *R. prolixus* in municipalities recently certified as free of *Trypanosoma cruzi* intra-domiciliary transmission (Cantillo-Barraza et al., 2020). Our approach evaluates metabolic resistance mechanisms and *kdr* mutations and identifies new genes associated with pyrethroid resistance in *T. dimidiata* using RNA-seq. The findings of this study will help direct future strategies for controlling *T. dimidiata* populations in regions where this vector is prevalent in Colombia.

2. Material and methods

2.1. Specimen collection

Triatoma dimidiata insects were collected in the field from Socotá, Boyaca, between October and November 2021. The insects were captured through active searches by trained Department of Health Service agents. The insects were transported to the BCEI laboratory at the University of Antioquia and identified using taxonomic keys (Lent and Wygodzinsky, 1979). The insects were established as a colony with 80 individuals (nymphs and adults) and were fed weekly with chicken blood as a food source. These individuals were called F_0. The colony was maintained in the laboratory under controlled temperature (25 ± 1 °C), humidity (60–70 %), and photoperiod (12:12 h). We used a reference *T. dimidiata* laboratory strain from the same municipality collected and established in 2017. This reference strain consisted of triatomines raised under laboratory conditions without insecticide exposure or the introduction of wild individuals.

2.2. Determination of resistance profiles to pyrethroid insecticides

For these experiments, we used the offspring obtained from field F_0 individuals, referred to as F1. First instar nymphs (5–7 days old) of the first generation (F_1) not fed were topically treated with one of three insecticides at selected doses, following the World Health Organization protocol (WHO, 1994). We tested different concentrations of three pyrethroid insecticides to determine the amount required to kill between 10 and 90 % of the individuals. We used five doses and the diluent (ethanol) as a control in each test. The experiment was repeated three times, and ten insects were used for each replicate.

For the bioassay, we applied 0.2 μL of the insecticide permethrin [dose range 3 - 50 ppm] – 96.1 % active ingredient [a.i.], lambda-cyhalothrin [dose range 0.375 - 6 ppm] – 99.8 % a.i., or deltamethrin [dose range 1.5 - 75 ppm] – 98 % a.i., (Sigma-Aldrich, St Louis, MO, USA) diluted in absolute ethanol (Merck,) on the abdomen using a 1-μL Hamilton syringe (Hamilton Co., Reno NV). We determined the mortality rate after 72 h of exposure according to the criteria described by the WHO (WHO, 1994). Nymphs were considered dead when they did not respond to mechanical stimulation. The laboratory-susceptible reference strain (Lab) was included in each experiment as the control group.

Mortality rates were determined for each population and each insecticide using log-probit regression to estimate the lethal doses 50 and 90 (LD_{50} and LD_{90}, respectively) with 95 % confidence intervals. The analyses were performed using the statistical software Polo Plus v0.03 (LeOra S, 2007). The populations were considered statistically different from the reference strain if the LDR (Lethal Dose Ratio) or RR (resistance ratio) and the 95 % confidence interval did not include the number 1.0 (Robertson et al., 2017, 2007).

2.3. Selection of populations under insecticides lethal dose 50 (LD50)

At least 100 first-instar nymphs (5–7 days old) of the F_1 generation were topically treated with a lethal dose (LD_{50}) of lambda-cyhalothrin or permethrin to select the surviving individuals (Table 1). After 72 h of exposure, we offered a blood meal to the surviving nymphs. We established colonies of insects with a minimum of 60 individuals from the surviving nymphs. We maintained these insects until they reached adulthood. With the offspring of these adults (F_2), we determined the resistance ratio using the same thorough methodology. All experiments were conducted similarly with the laboratory-susceptible reference strain (Lab) as controls for the three insecticides mentioned previously.

2.4. Evaluation of metabolic enzyme activity through biochemical assays

To determine whether detoxifying enzymes caused resistance, we measured the activity of metabolic enzymes associated with insecticide resistance, following the parameters reported for *Aedes aegypti* (Ministry of Health of Brazil, 2006) with some modifications (Calderón et al., 2020). After obtaining the necessary specimens, we stored the nymphs at −80 °C until processing. The nymphs were individually homogenized in 300 μL of deionized water on ice. We calculated each population's enzymatic activity from forty first-instar nymphs (3 days old). All experiments compared the laboratory-susceptible reference strain (Lab) without insecticide pressure with the offspring obtained from laboratory insects pressured with the three insecticides mentioned before. We compared the field F_1 population without pressure with the F_2 population obtained after pressure with lambda-cyhalothrin. Briefly, the nymphs were homogenized individually, and with the total homogenate extract, the concentration of proteins was measured in a 10 μL sample after five minutes of incubation using the Bradford method (Kruger, 2009). Acetylcholinesterase (ACE) activity was estimated in 25 μL reactions after four hours of incubation, and multiple function oxidase (MFO) activity was determined by measuring the heme content in 20 μL reactions after 90 min of incubation. Next, the extract was centrifuged at 4000 rpm for 3 min at 4 °C, and the supernatant protein concentration was measured in a 20 μL sample using the Bradford method. Alpha-esterase (α-EST) and Beta-esterase (β-EST) activities were measured using α-naphthyl and β-naphthyl esters as substrates in 10 μL after 15 min of reaction. Glutathione-S-transferases (GST) were measured by conjugating the thiol group of glutathione to the substrate, 1-chloro-2,4-dinitrobenzene, in 15 μL after 10 and 20 min of reaction.

Enzymatic activity was measured in 96-well microplates, following the protocol described for each enzyme (Ministry of Health of Brazil, 2006). Each nymph was counted in duplicate, and absorbance was measured using a Multiskan Spectrum microplate reader (Thermo Fisher Scientific, USA).

2.5. Enzyme activity statistical analysis

The Kolmogorov-Smirnov test was performed to assess normality in the data obtained from the biochemical assays. The Mann-Whitney U test was conducted to determine if there was a significant difference between the two group medians ($p < 0.05$). The Kruskal-Wallis H test was used to determine if there was a statistically significant difference between the medians of the groups ($p < 0.05$). Multiple comparisons to precisely identify the different groups were made using Dunn's test ($p < 0.05$). Enzyme activity was visualized using violin plots.

ACE activity was considered altered if the inhibition of its activity in the presence of propoxur was ≥ 70 % according to Hemingway's functional criterion (Hemingway, 1998). All analyses were conducted using the GraphPad Prism software (version 8.0.1, GraphPad Software, USA, www.graphad.com). To determine if there was a correlation between enzyme activity and LD_{50} and between enzyme activity and RR50, the Pearson correlation ($p < 0.05$) was calculated if the data met the assumptions of normality.

Table 1
Resistance ratio to pyrethroids insecticides of *T. dimidiata* populations from field and laboratory.

Insecticide	Pop	n	LD_{50} [ng/ins] (CI 95 %)	RR_{50} (CI 95 %)	LD_{90} [ng/ins] (CI 95 %)	RR_{90} (CI 95 %)	Slope ± SE
Permethrin	Lab	327	3.5 (2.46–4.43)	**1.0**	9.26 (6.80–18.70)	**1.0**	3.03 ± 0.36
	Field F_1	148	3.80 (1.79–11.20)	**1.09** (0.87–1.35)	14.65 (6.51–497.8)	**1.58*** (1.07–2.34)	2.19 ± 0.20
	Lab-P-P	269	3.99 (2.63–6.67)	**1.20** (0.87–1.44)	18.33 (9.54–89.15)	**1.97*** (1.17–3.36)	1.91 ± 0.23
Lambda-cyhalothrin	Lab	161	0.19 (0.16–0.23)	**1.0**	0.59 (0.46–0.87)	**1.0**	2.68 ± 0.32
	Field F_1	354	0.37 (0.30–0.46)	**1.95*** (1.45–2.61)	2.17 (1.58–3.30)	**3.65*** (2.26–5.89)	1.68 ± 0.16
	Lab-P-L	299	0.29 (0.21–0.40)	**1.53*** (1.05–2.21)	5.47 (2.51–22.25)	**9.22*** (3.27–25.41)	1.08 ± 0.17
	Field F_2	233	1.07 (0.67–2.62)	**2.87*** (1.51–5.44)	15.16 (4.90–200)	**7.00*** (1.36–36.02)	1.11 ± 0.23
Deltamethrin	Lab	430	1.61 (1.04–2.57)	**1.0**	5.48 (3.622–32.46)	**1.0**	2.13 ± 0.213
	Field F_1	369	4.51 (3.01–5.82)	**2.79*** (2.11–3.70)	32.68 (20.66–64.81)	**5.05*** (2.66–9.61)	1.49 ± 0.16
	Lab-P-D	121	3.27 (2.46–4.47)	**2.03*** (1.46–2.80)	13.08 (8.45–28.24)	**2.02*** (1.06–3.86)	2.13 ± 0.35

Pop: Studied populations (Lab: Laboratory-susceptible; Field F_1: individuals obtained from F0; Lab-P: Individuals from Laboratory under insecticide pressure; Field F_2: population obtained from individuals field F_1 under insecticide-pressure); n: number of nymphs tested; LD: Lethal doses ng/insect, CI: Confidence Interval; RR: Resistance ratios calculated based on Lab or Field, RR is considered significant if the CI does not contain the number 1 (Robertson et al., 2007). *: significance statistic.

2.6. Sequencing the coding region of voltage-gated sodium channel gene

To identify *kdr* mutations, we sequenced a fragment of the coding gene for the voltage-gated sodium channel (*vgsc*) corresponding to domain II of the protein, specifically between subunits 4 and 6, encompassing amino acids 906 to 1095. We sequenced lab and field F1 nymphs without insecticide exposure and field F1 nymphs that had survived sublethal doses of insecticides. To select insecticide-pressured nymphs, we exposed first-instar field F1 nymphs to an LD_{50} (permethrin and lambda-cyhalothrin) and, after 72 h of exposure, stored the surviving nymphs at −80 °C until processing.

Following the manufacturer’s instructions, nymph RNA was individually extracted using TRIzol reagent (Invitrogen). The RNA was quantified using Nanodrop (Thermo Fisher Scientific, USA). To determine if the RNA was contaminated with genomic DNA, we amplified the nuclear-encoded elongation 1-alpha factor (EF-1α) gene, as previously reported (Díaz et al., 2016). From 2 μg of the RNA, we synthesized cDNA using M-MLV reverse transcriptase (New England BioLabs) and Oligo (dT)20 (Invitrogen), according to the manufacturer’s instructions. Thermal cycler conditions for reverse transcription were 42 °C for 60 min and 72 °C for 10 min.

We performed Touchdown PCR to amplify a fragment of the *vgsc* gene. PCR was carried out using Phusion High-fidelity DNA Polymerase (New England BioLabs) in a total volume of 25 μL, using previously described primers JD5 (Dávila-Barboza et al., 2018) and TiRev2 (Fabro et al., 2012). In brief, a master mix was prepared consisting of 14 μL water, five μL Phusion GC buffer [5 ×] (New England BioLabs), 1.25 μL JD5 primer [10 μM], 1.25 μL TiRev2 primer [10 μM], 0.5 μL dNTP solution [10 mM], 0.75 μL DMSO [100X] and 0.25 μL Phusion High-fidelity DNA Polymerase (New England BioLabs), and two μL cDNA. Thermal cycler conditions for Touchdown PCR were as follows: denaturation at 98 °C for 2 min; 9 cycles of 98 °C for 20 s, touchdown starting at 59 °C for 30 s (decreased by one °C per cycle), and extension at 72 °C for 20 s; 32 cycles of amplification (98 °C for 20 s, 51 °C for 30 s, and 72 °C for 30 s), and final extension at 72 °C for 10 min. Amplified fragments of the expected size PCR product (~500 bp) were verified by gel electrophoresis using a 1.5 % agarose gel.

A total of 30 samples were sent for Sanger sequencing (five from the Lab not pressured with insecticide, five from field F_1 not pressured with insecticide, ten from the field F_1 population that survived exposure to permethrin, and ten from the field F_1 population that survived exposure to lambda-cyhalothrin (Macrogen Inc., Seoul, South Korea).

The nucleotide sequences obtained were edited, aligned, and analyzed using Biological Sequence Alignment Editor software (BioEdit version 7.2.5, USA) (Hall, 1999). The sequences were compared to other available sodium channel sequences from the phylogenetically related species *T. infestans* (GenBank Accession No. JF761319.1), *T. longipennis*, and *T. mazzotti* (Dávila-Barboza, 2017). Multiple alignments were performed using the ClustalW tool with consensus sequences.

2.7. Transcriptome preparation and sequencing

A total of nine individuals were selected for RNA-seq. We selected three individuals from the field F_1 population, three from the reference laboratory population (Lab), and three from the lambda-cyhalothrin-pressured laboratory population (Lab-Press-L). The insects were obtained at three days old, ensuring their color and weight were homogeneous, and stored at −80 °C until processing. RNA extraction was performed using the Quick-RNA Miniprep kit (Zymo Research, USA), and DNA traces were removed by adding DNase I, following the manufacturer’s instructions. The RNA quantity and quality were quantified using a NanoDrop spectrophotometer (Thermo Scientific, USA). The samples were sent to the University of Oklahoma (Oklahoma, USA) for whole-transcriptome sequencing using the platform Illumina NovaSeq 6000 S4- PE Sequencing System Platform (Illumina, USA). Raw data are available at GEO (GSE254270).

2.7.1. Data processing

The raw reads (FASTQ data) were quality-checked for base sequence quality, GC content, base N content, and adapters using FastQC v0.12.0 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). These raw reads were then trimmed using Trimmomatic software v0.39 (Bolger et al., 2014) to remove adapters (TruSeq3-PE.fa:2:30:10), low-quality reads, and reads with a minimum length of 36 bp with the parameters LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 HEADCROP:10 MINLEN:36.

2.7.2. Transcriptome de novo assembly

The nine sample sequences were assembled *de novo* using Trinity software v2.15.0, with default parameters (Grabherr et al., 2011; Haas et al., 2013).

The output files were saved in FASTA format, and the quality of the

transcript assembly was verified using Bowtie2 v2.3.3.1 software (Langmead and Salzberg, 2012).

All the assembled transcripts were functionally annotated using the Trinotate pipeline v3.2.2, with a cutoff E-value of 10−5 (https://trinotate.github.io/).

TransDecoder v5.5.0 (https://github.com/TransDecoder/TransDecoder) was used to predict coding regions within the generated transcripts and translate the most extended open reading frame (ORF).

Trinity transcriptome file was searched for nucleotide sequences homology using BLASTX (NCBI-blast v2.7.1+) against several databases (SwissProt database, Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Ontology (GO))

2.7.3. Quantification and differential gene expression analysis

The counting of transcripts was estimated using Salmon software (Patro et al., 2017). The transcripts with <32 counts were filtered out and normalized using TMM in edgeR (Robinson et al., 2010). Genes were considered up-regulated or down-regulated when |Fold-change| was ≥ 1.5 and the statistical threshold p-value adj < 0.05. For visualization, data were scaled using Z-scores. Principal component analysis (PCA) and heatmap analysis were performed using the R package (R ComplexHeatmap). The differential gene expression analyses were carried out comparing the data obtained from insects pressured with lambda-cyhalothrin (L-P-L) and insects from Field (F1) regarding individuals from the laboratory population (Lab).

2.8. Ethics statement

The Ethical approval (Act No 112 of 2017) reviewed and approved the animal study for analyzing animal specimens (Animal Ethics Committee at the SIU—Universidad de Antioquia).

3. Results

3.1. Triatomine gains insecticide resistance during the F1 generation

When evaluating the lambda-cyhalothrin insecticide susceptibility of the *T. dimidiata* field population, a noticeable increase in resistance to lambda-cyhalothrin was observed under insecticide pressure during only one generation. The RR_{50} changed from 1.95 [1.45–2.61] in the F1 field population to 2.87 [1.51–5.44] in the F2 field population. Similarly, the RR_{50} in the lab population changed from 1.0 to 1.53 [1.05–2.21], after insecticide pressure (Table 1). The susceptibility to deltamethrin also altered in the laboratory population after being pressured with this insecticide, with an RR_{50} of 1.0 to 2.03 [1.46–2.8]. Notably, the susceptibility profile to permethrin remained unchanged after insecticide exposure (RR_{50}: 1.0 vs RR_{50}: 1.20 [0.87–1.44]) (Table 1).

3.2. Enhanced enzymatic activity in insecticide-resistant T. dimidiata populations

To assess the mechanisms involved in resistance, we addressed three experimental approaches: enzymatic detoxification, sequencing the coding region for the *vgsc* gene, and RNA-seq of *T. dimidiata*.

We observed a significant increase in the activity of the detoxification enzymes glutathione S-transferase (GST), Mixed-Function Oxidases (MFO), and Esterase (EST) within populations resistant to permethrin, deltamethrin, and lambda-cyhalothrin (Fig. 1). This pattern held for both laboratory and field populations. Moreover, we found a significant positive correlation between the RR50 of pyrethroid insecticides and the mean EST ($p < 0.05$) and a significant positive correlation between the LD_{50} of permethrin and the mean activity of glutathione S-transferase ($p < 0.05$) (See supplementary materials, Figure S1).

3.3. Sequencing reveals that resistant T. dimidiata populations did not exhibit the classical mutations in the vgsc gene

Mutations in critical genes are crucial for acquiring resistance to pyrethroids in triatomines. We thoroughly examined mutations in a fragment of the *vgsc* coding region, a gene known for its pivotal role in pyrethroid resistance. This scrutiny aimed to unravel the genetic basis of resistance in this vital vector species. The sequence of the *vgsc* coding region revealed a 95 % identity with the *T. infestans* orthologous gene, shedding light on the evolutionary context. Sequence alignment indicated that the *vgsc* gene from nymphs surviving sublethal insecticide doses lacked mutations previously linked to triatomine insecticide resistance (Figure S2). No differences existed between populations.

3.4. RNA-seq analysis reveals the regulation of key processes in resistant triatomines, such as the cuticle, respiratory chain, and muscle tissue

Transcriptomic analysis showed 723,166,336 reads obtained across all replicates. After filtering for quality, size, and adapter removal, 658,518,872 reads (91.1 %) were used for *de novo* transcriptome assembly (See supplementary materials Table S1).

Differentially regulated genes were used to conduct an initial visual analysis of the data using Principal Component Analysis (PCA). The two principal components explained 85.7 % of the total variance and showed apparent clustering among replicates (Fig. 2A). A total of 179 genes were differentially regulated in the field F_1 population (32 up-regulated and 147 down-regulated) and 367 in the laboratory population under Lab-Pres-L pressure (90 up-regulated and 277 down-regulated). Fourteen common up-regulated and 112 down-regulated genes were found between the two populations (Fig. 2B, C).

Unsupervised hierarchical clustering analysis showed that differentially expressed genes were organized into five distinct clusters (I–V) (Fig. 2D): Cluster I ($n = 51$) included up-regulated genes only in the Lab-Press-L population (yellow bar), cluster II ($n = 66$) comprised down-regulated genes only in the Lab-Press-L population (red bar), cluster III ($n = 39$) (green bar) featured up-regulated genes only in the field F_1 population, and clusters IV ($n = 113$) (light blue bar) and V ($n = 129$) (gray bar) contained down-regulated genes in both populations.

3.4.1. Electron transport chain complex

When we explored the identity of the differentially expressed genes, we found that the field F_1 population showed increased transcription of genes participating in the electron transport chain. Notably, there was a substantial increase in the expression of cytochrome C oxidase subunit 1 [Log_2 FC = 10.6] (COX1 or COI), cytochrome B complex [Log_2 FC = 11.7] (CYB), NADH-ubiquinone oxidoreductase complex chain 4 [Log_2 FC = 15.2], and chain 5 [Log_2 FC = 10.8] (NU4 M or ND4, NU5 M or ND5) (Fig. 3). Likewise, the laboratory population under lambda-cyhalothrin pressure (Lab-P-L) showed an increase in the transcription of electron transport chain genes, such as cytochrome c oxidase subunit 5A (COX5A) [Log_2 FC = 3.7], NADH-ubiquinone oxidoreductase complex chain 4 (NU4 M or ND4), [Log_2 FC = 10], and chain 5 (NU5 M, or ND5) [Log2 FC = 9.2].

3.4.2. Cuticle genes

Upon further examination of the field F_1 population in the differentially expressed genes, a down-regulation of genes primarily associated with cuticular processes was observed. Specifically, there was a decrease in the expression of OVGP1 [Log_2 FC = −3.8], genes related to cuticle structure (CUP7, CU15, CU198) (Log_2 FC = −2.7, −2.7, −2,2), cuticle development (CUO6) [Log_2 FC = −2.5], endocuticle (CUD9) [Log_2 FC= −1.8] and pigmentation (YELL, TY3H) [Log_2 FC = −3.2]. Simultaneously, in the laboratory population under lambda-cyhalothrin selection pressure, a decrease in the regulation of genes related to cuticle structure (MBN, CU15, CU198) [Log_2 FC = −3.8, −2.6, −4.2] and cuticle development (CUO6) [Log_2 FC= −3.9] and endocuticle (CUD9)

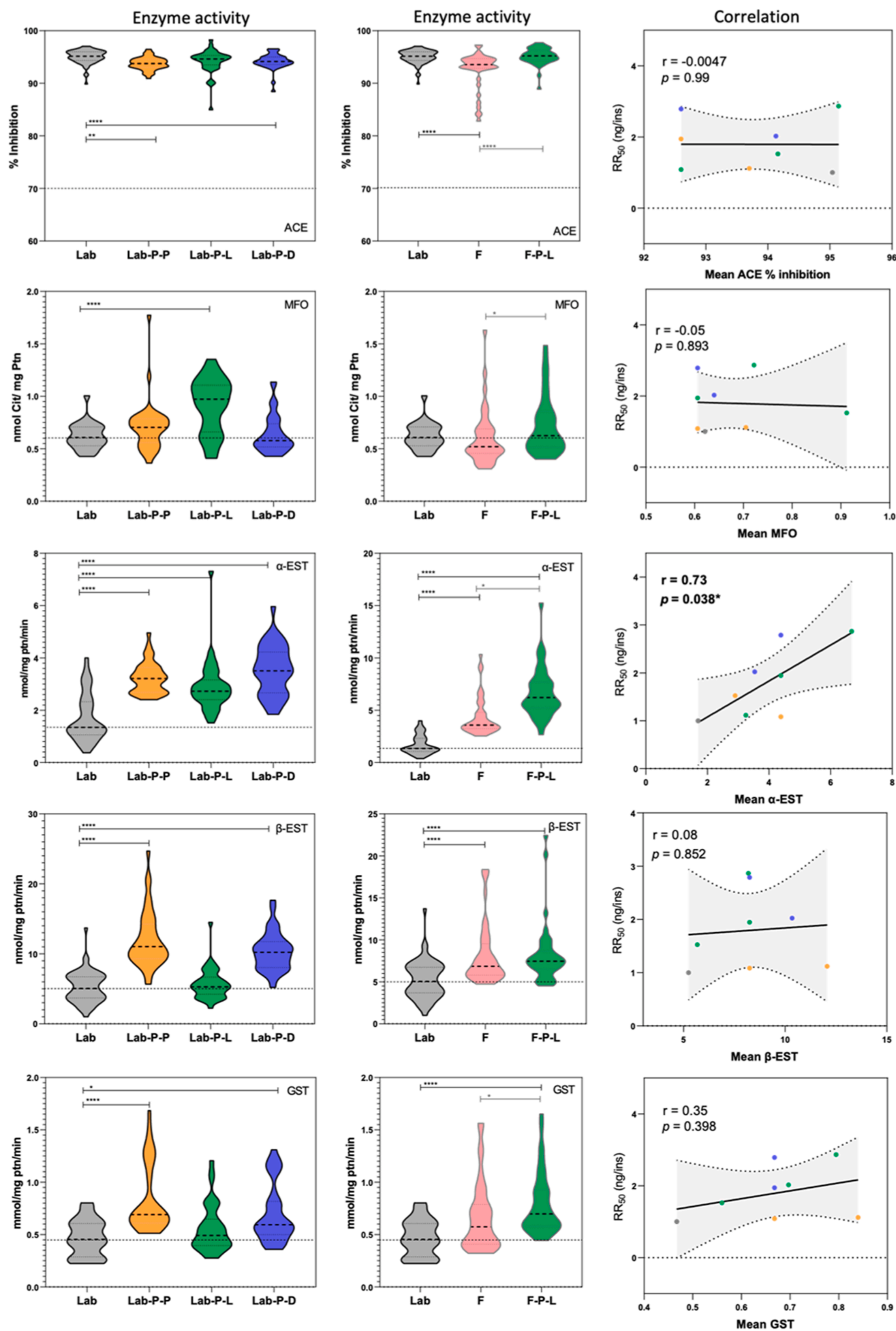

Fig. 1. Enzymatic activity levels and correlation with insecticide resistance in *T. dimidiata.* Violin Plot of enzymatic activity levels (left and center) and correlation plot (right) between resistance ratio (RR_{50}) of insecticide and mean of enzymatic activity. *T. dimidiata* populations with elevated enzymatic activity compared to the Lab or field are marked * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. The Pearson correlation r and p values are shown for each correlation (* $p < 0.05$). ACE: insensitive acetylcholinesterase (iAChE); MFO: mixed-function oxidases; α-EST: α-esterases; β-EST: β-esterases; GST: glutathione-S-transferases. Lab: Laboratory-susceptible; Lab-P-Per: Lab under pressure with permethrin; Lab-P-L: Lab under pressure with lambda-cyhalothrin; Lab-P-D: Lab under pressure of deltamethrin; F: Field F1; F-P-L: Field F2 under pressure with lambda-cyhalothrin. The colors of the points in the correlation plot correspond to each population described in the violin plot.

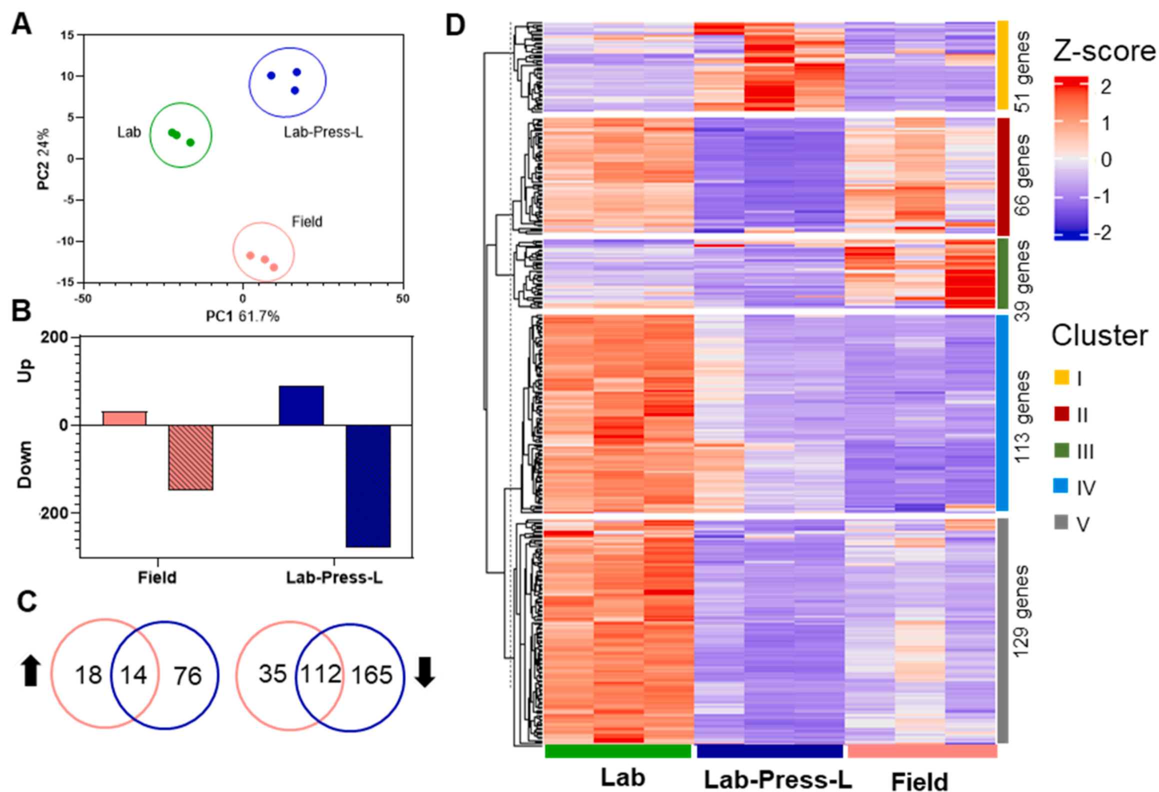

Fig. 2. Overview of the RNA-Seq. (A) Principal Component Analysis (PCA) of the transcriptome of the three populations of *T. dimidiata*. (B) The number of up-regulated and down-regulated genes in Field F1 and Lab-Press-L populations. (C) Venn diagram showing the number of differentially regulated genes. (D) A heatmap of 398 Z-score normalized differentially regulated genes in *T. dimidiata* populations identified the clusters by unsupervised hierarchical clustering analysis. Threshold for up- and down-regulation (|fold change| ≥ 1.5).

[Log_2 FC= −2.9] was also noted (Fig. 3).

3.4.3. Muscle genes

In both the field F_1 and laboratory populations under lambda-cyhalothrin pressure, genes associated with the assembly and function of striated muscles and tendons of adult and embryonic muscles were upregulated (TITIN) [Log2 FC = 8.5 and 2.05, respectively].

3.4.4. ABC transporter

Both groups of triatomines (field F_1 population and lambda-cyhalothrin pressured laboratory population) showed downregulation in an ABC transporter belonging to the G subfamily (ABCGN) [Log_2 FC = −3.2] and [Log_2 FC = −4.2], respectively.

3.4.5. Metabolic resistance-related gene families

In the laboratory population under lambda-cyhalothrin pressure, there was an increase in the regulation of genes related to monooxygenase and oxidoreductase activity of cytochrome P450 (CP307) [Log2 FC= 2.5] and (CP3A2) [Log2 FC= 2.6]. The field F_1 population also showed upregulation of MFO genes (CP307) [Log2 FC= 2.3]. On the other hand, downregulation in genes of carboxylesterase activity (EST6) in the pressured population [Log2 FC= −3] and the field population [Log2 FC= −1.8] was also observed. Other esterase enzymes, such as ACES, were downregulated in the pressure population [Log2 FC= −6.9] and the field population [Log2 FC= −5.6]. We did not find differentially regulated genes in the GST family; however, further analysis revealed that the glutathione S-transferase gene (GSTT4) exhibited up-regulation in both populations, although it did not surpass the selected threshold in the pressure [Log2 FC= 1.1] and field population [Log2 FC= 0.8].

4. Discussion

T. dimidiata, a vector of epidemiological importance for *Trypanosoma cruzi* in Colombia and other countries in South and Central America, is controlled through pyrethroid insecticide spraying. However, limited information exists regarding the expression of resistance to these insecticides in Colombia. This study addresses this gap by investigating whether *T. dimidiata* from a Chagas disease endemic region in Colombia resisted pyrethroid insecticides. Furthermore, the study aimed to identify the mechanisms underlying this phenotype.

In pursuit of the first aim, the results show that the *T. dimidiata* field population from Socotá-Boyacá exhibited resistance to lambda-cyhalothrin and deltamethrin pyrethroids. The resistance ratios were slightly above 1 (RR_{50}: 1.95 lambda-cyhalothrin and 2.79 deltamethrin), suggesting an incipient resistance profile. However, it is essential to highlight that the resistance rates to deltamethrin were notably higher (> 10 times) than those reported previously (Reyes et al., 2007). Our result is unsurprising due to the constant use of deltamethrin for vector control in Boyacá from 2010 to 2019 (Data provided by the Department of Health Secretary). Our study unveils a significant finding as it represents the first report of resistance to permethrin and lambda-cyhalothrin in Colombia for this vector species.

Developing resistance in triatomine populations has been considered challenging to acquire due to low genetic variability and long life cycles (Monteiro et al., 2001; Noireau and Dujardin, 2010; Pessoa et al., 2015). To study this characteristic in triatomines, artificial selection pressure was applied through the direct application of insecticides to both laboratory and field populations through a single topical application to first-instar nymphs under controlled conditions. The populations exposed to insecticide exhibited significantly higher resistance levels than the baseline population (laboratory populations RR50:1.5 lambda-cyhalothrin and 2.0 deltamethrin; field population RR50:2.87

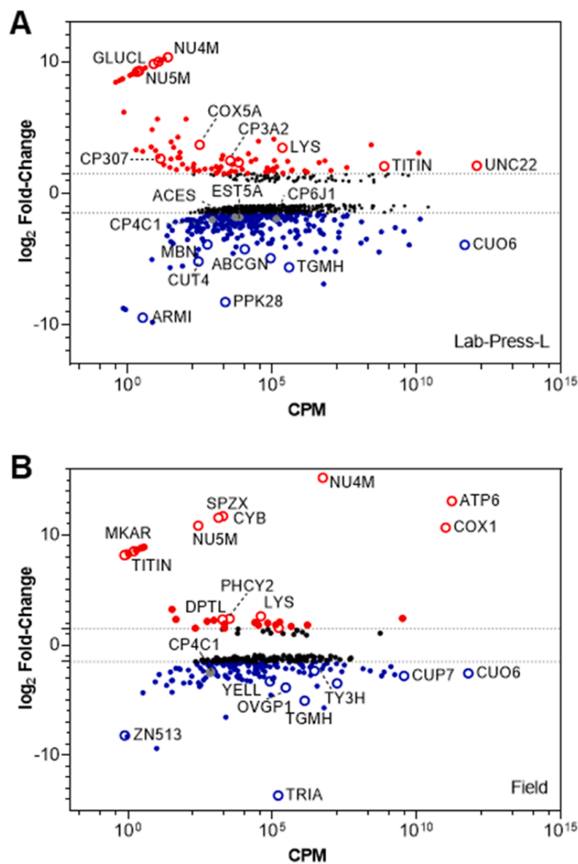

Fig. 3. MA plot from RNA-seq data displaying differentially regulated genes in (A) Lab-Press-L and (B) Field F1 populations. Genes that pass a threshold of *p*-value adj < 0.05 and $|\log2foldChange| > 1.5$ in differential expression analysis are colored in blue when down-regulated in control (Lab) and red when up-regulated in control.

lambda-cyhalothrin). These observations highlight the potential for a rapid development of resistance in these insects, which has relevant epidemiological implications for vector control strategies.

4.1. Enhanced enzymatic activity in T. dimidiata populations

The second aim of this research was to unravel the mechanisms contributing to resistance. We identified increased enzymatic activity, novel mutations in the *vgsc* gene, and differentially expressed genes implicated in resistance. First, we found a significant increase in the enzymatic activity of GST, MFO, and EST through biochemical assays in both field F_1 and laboratory populations under pyrethroid pressure. The increase in the LD_{50} of permethrin exhibited a significant correlation with mean GST enzymatic activity. Similar patterns of increased GST enzyme activity in response to pyrethroids have been found in other species. For instance, the topical application of sublethal doses to *T. infestans* in wild populations in Bolivia and Argentina resulted in a significant (187 %) increase in GST activity (Casabé et al., 1988; Sívori et al., 1997). Comparable trends were noted in *T. mazzotti* and *T. picturata* populations, where 68 % and 49 % of individuals exhibited a significant increase in GST activity, surpassing their reference strain (Dávila-Barboza, 2017). A substantial increase in GST activity was observed in the populations of deltamethrin-resistant *R. prolixus* associated with palm trees (200 %), although not following a linear relationship with the resistance ratio (Calderón et al., 2020).

We found an increase in the RR_{50} to pyrethroid and a significant correlation with mean α-EST. Esterases have been seen in other pyrethroid-resistant triatomines such as *T. infestans* (Picollo et al., 2005; Santo-Orihuela et al., 2017); *T. pallidipennis, T. mazzotti, T. longipennis, T. picturata* (Dávila-Barboza, 2017), and *R. prolixus* (Calderón et al., 2020). The results of the present study are consistent with other studies conducted in triatomines, specifically *T. infestans*, where the MFO enzyme has been associated with pyrethroid resistance. However, this association does not consistently exhibit a linear correlation (Santo-Orihuela et al., 2017, 2008).

As expected, we did not find inhibited ACE (Data not shown); therefore, we confirmed that this enzyme was not correlated with resistance to pyrethroids. ACE enzyme activity is less sensitive to insecticide inhibition in populations resistant to organophosphate and carbamate insecticides. This phenomenon arises from mutations that lead to conformational changes in the active sites of the protein (Guo et al., 2017; Hemingway et al., 2002). These findings suggest that, at least at the tested doses, the evaluated insects are unlikely to resist carbamate and organophosphate insecticides that target acetylcholinesterase. However, this hypothesis merits validation in future studies. These are promising results, and we suggest using insecticides with specific inhibitors for the identified enzymes, primarily for GST and EST, that may play a role in detoxification.

4.2. Sequencing reveals that resistant T. dimidiata populations did not show classical point mutations in the vgsc gene

We analyzed the sequencing data for point mutations at the target sites of pyrethroid insecticides, known as knockdown resistance (*kdr*). To examine this mechanism, we evaluated the presence of point mutations in the *vgsc* gene, the primary target site of pyrethroid insecticides (Soderlund and Bloomquist, 1990; Soderlund and Knipple, 2003). This phenomenon is an example of the rapid evolution of insecticide resistance in insect populations and has been documented in various species worldwide (Rinkevich et al., 2013).

Protein modeling predicted that within the linker region between subunits 4 and 5 of domain II, IIS5, and IIS6 form a binding site for pyrethroids. It has been demonstrated in triatomines that two point mutations, L925I and L1014F, located in these domains, confer pyrethroid resistance. The L925I mutation has been reported in *T. infestans* (Capriotti et al., 2014) and other arthropods, such as *Trialeurodes vaporariorum, Bemisia tabaci, Cimex luctularis, Trialeurodes vaporariorum*, and *Rhipicephalus microplus*. In the present study, we analyzed twenty insects from the field population pressured with lambda-cyhalothrin and permethrin insecticides (individuals F_1) and five from the laboratory. No amino acid changes were identified (Figure S1).

Position 1014 is a hotspot for divergent substitutions. The well-documented mutation L1014F has been detected in *T. infestans* (Fabro et al., 2012), as well as in other triatomines, such as *T. dimidiata* and *T. pallidipennis* (Davila-Barboza et al., 2019a), along with other insects including *Frankliniella occidentalis, Musca domestica, Myzus persicae, Thrips tabaco, Tuta absoluta, Plutella xylostella*, and *Blattella germanica* (Forcioli et al., 2002; Haddi et al., 2012; Tan et al., 2002). The L1014S mutation has also been reported in *T. picturata, Anopheles gambiae* and *Culex pipiens*, among other species, and the L1014H mutation in *Liriomyza trifolii, M. domestica*, and *Stomoxys calcitrans* (Davies et al., 2007; Rinkevich et al., 2013). We did not detect any of these mutations in our study.

We searched for other amino acid changes within the connector segment IIS5–6. The formation of the outer pore is attributed to the reentrant loops between the transmembrane segments S5 and S6 of each domain, and amino acids at his specific site serve as receptor sites for pore blockers and selectivity filters. One of these alterations was the substitution of phenylalanine with a serine at position 979 (F979S). Phenylalanine is again involved in this alteration; however, it is replaced by a polar amino acid. The F979S mutation has been associated with the L1014F mutation, and these are found together. This was observed in *Myzus persicae*, resistant to the carbamate insecticide pirimicarb (Cassanelli et al., 2005). One possible implication of this result is that

this protein fragment is prone to mutations, and the loss of the amino acid phenylalanine reduces the effectiveness of binding to the insecticide. Notably, no changes were identified at this specific amino acid position in our triatomines.

Additionally, we looked for the point mutation L1024 V, associated with resistance to the fenpropathrin pyrethroid insecticide in *Tetranychus urticae* (Kwon et al., 2010). This mutation is in the transmembrane segment IIS6, a terminal region close to the IIS4–S5 link, forming a pyrethroid-binding cavity, particularly in the open state. This valine substitution (an aliphatic, nonpolar amino acid) is recurrent at several positions in the sodium channel involved in resistance, including M918 V, G933 V, T929 V, I936 V, I1011 V, A1410 V, A1494 V, and D1549 V (Rinkevich et al., 2013). Therefore, substituting Leu with Val in the terminal region of the transmembrane segment IIS6 may alter the binding properties of the sodium channel in the open state, which can lead to reduced sensitivity. Once again, this mutation was not found in any of our insects. Although the sequenced region spans a relevant portion of the gene, it is essential to consider that other regions may also be crucial for interacting with pyrethroids.

4.3. RNA-seq analysis reveals the regulation of key processes in resistant triatomines, such as the cuticle, respiratory chain, and muscle tissue

RNA-seq data analyses compare gene expression profiles due to different treatments. They may not explain why differences are observed; they may be secondary responses to variations in the expression of other genes. The RNA-seq approach examines specific genes of interest and provides an overview of overall changes in gene expression from exposure to insecticides. It may also identify new genes for further study.

Regarding regulated genes associated with resistance in *T. dimidiata*, RNA from three populations was sequenced: laboratory susceptible (Lab), laboratory under pressure with the insecticide lambda-cyhalothrin (Lab-Press-L), and field F1. The susceptible laboratory population was collected from Socotá, Boyacá, in 2017. The insecticide K-othrine (deltamethrin) was used as a control in Boyacá from 2010 to 2019 (Data provided by the Department of Health Secretary). Laboratory populations under pressure with lambda-cyhalothrin (Lab-Pres-L) were derived from nymphs obtained from this colony. The field population was collected in the same municipality at the end 2021. However, since 2020, the insecticide used to control this area has been plaguisan (alphacypermethrin). Therefore, collecting study populations at different times provides valuable information regarding susceptibility. It is worth noting that this municipality was selected to evaluate the interruption of domestic vector transmission of *Trypanosoma cruzi* by *R. prolixus* in 2019, which is why it has undergone continuous monitoring and chemical control (Organización Mundial de la Salud, OMS, 2019).

Transcriptome data showed differentially expressed genes in each population, which were represented and grouped into five clusters. Genes from clusters I and II were hypothesized to be differentially regulated in response to insecticide pressure. These genes might be associated with the parental population's response to recent insecticide exposure or might be the first to exhibit activity or insecticide early response genes. Cluster III suggests that these genes may be responsive to variable conditions in natural and uncontrolled environments. In two significant clusters, IV and V, these genes are particularly intriguing because they are convergent; therefore, they may partially explain the development of insecticide resistance.

4.3.1. Electron transport chain complex

In the present study, up-regulated mitochondrial genes related to the electron transport chain were identified, and some of these genes were shared between the field F_1 and Lab-Pres-L populations, suggesting that they may be involved in resistance or the response to oxidative stress caused by insecticide exposure. The significance of this finding is supported by other studies that have also found similar results. For instance, in *Anopheles sinensis*, upregulation of ND5, ND2, and COII genes was reported in pyrethroids-resistant populations (Ding et al., 2020). In both studies, differentially regulated genes from Complex I of the electron transport chain, NADH dehydrogenase (ND4 and ND5), Complex III Cytochrome c reductase (CytB), and Complex IV cytochrome c oxidase (COX1) were identified.

Other studies have suggested that Complex I inhibitors of the electron transport chain may not be effective for controlling pyrethroid-resistant mosquitoes because of their metabolic cross-resistance, mainly due to an increase in cytochrome P450 (Lees et al., 2020). This finding underscores the complexity of resistance mechanisms and highlights the need to explore alternative strategies, such as inhibitors targeting metabolic pathways and energy production.

These studies suggest that exposure to an insecticide of this type could trigger energy production, thus aiding our better understanding of insecticide resistance mechanisms. Furthermore, these findings support the idea that combining Complex I inhibitors with P450 inhibitors, such as piperonyl butoxide (PBO), could be an effective strategy to overcome pyrethroid resistance, potentially leading to more effective solutions for controlling populations of resistant insects. This implies that understanding the intricacies of energy dynamics associated with insecticide exposure emerges as a pivotal aspect in developing novel insecticide control strategies.

4.3.2. Cuticle genes

In this study, we identified down-regulated genes related to the tyrosine and melanin pathways involved in pigmentation in the laboratory pressure and field populations. Among these genes, the down-regulation of *y* (YELL) is noteworthy. Previous studies have shown that the down-regulation of the *y* gene in *R. prolixus* produces a lighter phenotype without discernible effects on fertility, hatching, viability, or resistance (Berni et al., 2022). This result and the downregulation of other genes related to fatty acid biosynthesis pathways, structural molecules of elasticity, and the chitin matrix seem contradictory to the cuticular resistance mechanism. At the very least, this indicates structural changes in chitin in response to insecticide exposure. In *T. infestans-resistant* populations, insects have 50 % more cuticular hydrocarbons than pyrethroid-susceptible populations (Pedrini et al., 2009). However, it is important to interpret these findings with caution, as in the present study, incipient resistance was found in *T. dimidiata*, in contrast to *T. infestans* populations, which can be up to 1000 times more resistant than susceptible populations.

In mosquitoes, changes in the size of the epicuticle, exocuticle, or endocuticle as well as alterations in cuticular composition and restructuring, affect other biomechanical properties, including a reduction in modulus and hardness, which can lead to resistance (Balabanidou et al., 2018). Therefore, in cases of low resistance, a remodeling of the cuticle, where many genes decrease, alters the formation of this structure.

4.3.3. Muscle genes

Upregulation of the *sls* or *titin* (TITIN) gene was found in the Lab-Pres-L and field populations. This gene has been reported to be up-regulated in deltamethrin-resistant *Ae. aegypti* mosquitoes and their potential involvement in the neuromuscular junction have been suggested (Lertkiatmongkol et al., 2010). It has also been reported to be up-regulated in *Spodoptera exigua, which* is resistant to chlorantraniliprole. This insecticide targets ryanodine receptors (Wang et al., 2018) and in *Apis mellifera* exposed to cypermethrin, implying that the insecticide induces the upregulation of genes encoding developmental, structural, and muscular function processes (Fent et al., 2020). These findings align with the data obtained, suggesting that titin may also be involved in insecticide resistance in triatomines by counteracting the knockdown effect of paralysis generated by pyrethroids. However, further experiments must be performed to validate whether this gene has a crucial role in insecticide resistance.

4.3.4. ABC transporter

The ABC transporter gene from the G family was downregulated in the two populations analyzed in this study. This finding is consistent with the results obtained by Traverso et al. (2022), who found the downregulation of two genes belonging to the same transporter family in *T. infestans* after nymphs were exposed to deltamethrin (Traverso et al., 2022). Previous studies on *Ae. aegypti* larvae also reported the downregulation of some ABC transporters after exposure to xenobiotics (Sierra et al., 2021). These findings support the hypothesis that transporters are crucial in responding to xenobiotics (Dermauw et al., 2013). In investigations into the detoxification of xenobiotics and *Bacillus thuringiensis (Bt), Ostrinia furnacalis* has been shown to downregulate the ABCG subfamily, which has been linked to resistance (Wu et al., 2019; Zhang et al., 2017). Further research is needed to determine whether the transporter identified in our study is involved in the entry of the insecticide or one of its metabolic byproducts to understand these transporters' role in insecticide resistance fully.

4.3.5. Metabolic-related gene families

This study identified several cytochrome P450 (CYP450) genes as up-regulated and down-regulated in the Lab-Pres-L and Field populations. These results suggest that some of these genes may be involved in insecticide resistance in triatomines. Specifically, higher upregulation of these genes was observed in the Lab-Pres-L group. When comparing these results with the biochemical assays conducted, it was found that the field population did not show significant differences in MFO activity, whereas individuals from the Lab-Pres-L population did. Therefore, these consistent results indicate an essential role for specific CYP450 genes in resistance.

Microsomal mixed function oxidases - MFO enzymes are predominant in insecticide metabolism. The gene CYP307A1 (CP307), which was found to be up-regulated more than two times in this study, has also been associated with metabolic resistance to insecticides in *P. xylostella* (Etebari et al., 2018) and pyrethroids such as deltamethrin in *Cydia pomonella* (Dai et al., 2021). However, the gene CYP307A1 also plays a relevant role in ecdysteroid biosynthesis. For instance, in *Spodoptera litura*, the upregulation of this gene led to an increase in the synthesis of ecdysone, which also resulted in resistance to bistrifluron insecticide (Gong et al., 2022). In *T. infestans*, up-regulated MFO genes were also found in resistant populations (CYP4EM7) and were induced by deltamethrin in both susceptible and resistant populations (CYP3085B1 and CYP3092A6) (Grosso et al., 2016). CYP450 genes are highly regulated in the fat bodies of certain insects; thus, they are strongly associated with the cuticle. Therefore, it is possible to assess CYPs related to the integument and to confirm their role in detoxification (Dulbecco et al., 2018).

The identified genes belonging to carboxylesterases were down-regulated in the analyzed populations. As mentioned earlier, esterase families are broad with multiple functions; therefore, the genes found down-regulated by transcriptomics may not be related to resistance. We hypothesized that the biochemical effect is a cumulative effect of many carboxylesterase genes.

An exciting gene to highlight is the Glutathione S-Transferase Theta-4 gene (GSTT4), which showed a fold change of 1.14 for the Lab-Pres-L population and 0.82 for the field population. Although it did not surpass the chosen threshold (≥ 1.5), it may be of interest concerning resistance. This gene belongs to the theta class, which has been proposed as a group of compounds that contribute to the detoxification of xenobiotics. Traverso et al. (2017) detected two transcripts of *T. dimidiata* from this family; therefore, it would be interesting to induce or inhibit this gene experimentally and confirm whether it contributes to resistant phenotypes in triatomines (Traverso et al., 2017).

In the transcript modulation analysis related to the CYP, GST, and CCE gene families in a population of *T. infestans* with low resistance levels, no differential expression was observed after treatment with deltamethrin. The authors suggest that the genetic modulation of detoxifying enzymes triggered by pyrethroids may depend on the dosage, exposure time, and the species and populations under analysis (Traverso et al., 2022). These findings are consistent with the results of the present study.

This study has some limitations. Analyzing specific tissues or employing techniques for single-cell analysis can increase the probability of identifying resistance-related genes. This approach would enable the targeted analysis of cells responsible for detoxification. Functional validation can be accomplished through knockdown techniques (RNAi) or knockout genetic editing (CRISPR/Cas9) to confirm the roles of different genes.

Overall, the results show that *T. dimidiata* is naturally resistant to lambda-cyhalothrin and deltamethrin; it quickly developed resistance when exposed to these insecticides. Continuous exposure to permethrin increased GST enzymatic activity, contributing to insecticide tolerance. We confirmed a widespread increase in α-EST levels in response to all three pyrethroids. The upregulation of genes related to enzymatic detoxification mechanisms, such as the CYP detoxification enzyme family, and genes connected to muscle and mitochondrial tissues, particularly those associated with the electron transport chain, may play a role in the resistance phenotype. On the other hand, there was evidence of the downregulation of genes related to ABC transporters, which are also involved in the movement of compounds for excretion, and additional genes related to pigmentation, structural molecules of elasticity, chitin matrix, and cuticle formation, which may account for structural changes in response to insecticide exposure.

5. Conclusion

This study is one of the first comprehensive assessments of pyrethroid resistance in *T. dimidiata* in Colombia. This thorough approach helps understand the development of insecticide resistance in triatomines. Recognizing the resistance mechanisms reported in other organisms and understanding the selection response to insecticide exposure through other technologies provides information on potential new genetic and synergistic targets to explore. The findings of this study support the importance of assessing the resistance profiles in populations of domiciled insects in policymaking. Prioritizing action planning before implementing public health measures is essential, as it can significantly reduce the associated economic and environmental costs. By doing so, a sustainable reduction of *Trypanosoma cruzi* transmission could be achieved.

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CRedit authorship contribution statement

Sara Zuluaga Aguirre: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review and editing. **Geysson Fernández:** Writing – review and editing, Data curation, Investigation, Methodology. **Carl Lowenberger**: Funding acquisition, Investigation, review, and editing. **Ana Maria Mejia-Jaramillo**: Methodology, Investigation, Writing – review and editing. **Omar Triana-Chávez**: Conceptualization, Supervision, Investigation, Funding acquisition, Writing – review, and editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data presented in this article are available in three Excel files (see Supplementary Files 1, 2, and 3). Moreover, analyses of transcriptome data are provided. The RNAseq datasets generated for this study are in the Gene Expression Omnibus (GEO) DataSets (https://www.ncbi.nlm.nih.gov/gds) under the accession number GSE254270.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cris.2024.100103.

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