Infection, Genetics and Evolution 44 (2016) 459–470

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

# Identification of three cytochrome P450 genes in the Chagas' disease vector *Triatoma infestans*: Expression analysis in deltamethrin susceptible and resistant populations

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ARTICLE INFO

*Article history:*
Received 18 May 2016
Received in revised form 7 July 2016
Accepted 21 July 2016
Available online 25 July 2016

*Keywords:*
*Triatoma infestans*
Chagas' disease vector
P450 genes

ABSTRACT

Cytochrome P450 monooxygenases play a predominant role in the metabolism of insecticides. Many insect P450 genes have frequently been associated with detoxification processes allowing the insect to become tolerant or resistant to insecticides. The increases of expression of P450 genes at transcriptional level are often consider responsible for increasing the metabolism of insecticides and seems to be a common phenomenon in the evolution of resistance development in insects. As pyrethroid resistance has been detected in *Triatoma infestans*, it was of interest to analyze genes associated with resistance to insecticides such as those encoding for cytochromes P450. With this purpose, the cDNA sequences of three cytochrome P450 genes (CYP4EM7, CYP3085B1, and CYP3092A6) were identified in this species. Primers and specific Taqman probes were designed from these sequences to determine their expression by quantitative PCR. The mRNA levels of the cytochrome P450 genes identified were determined from total RNA extracted from pools of fat body collected from individuals of different resistant and susceptible strains of *T. infestans*, and at different interval times after the topical application of the lethal doses 50% ($LD_{50}$) of deltamethrin on the ventral abdomen of insects belonging to the different populations analyzed. It was detected overexpression of the CYP4EM7 gene in the most resistant strain of *T. infestans* and the expression of the three cytochrome P450 genes isolated was induced by deltamethrin in the susceptible and resistant populations included in this study. These results suggest that these genes would be involved in the detoxification of deltamethrin and support the hypothesis that considers to the cytochrome P450 genes of importance in the development of pyrethroid resistance.



## 1. Introduction

Chagas' disease (American trypanosomiasis) is produced by infection with *Trypanosoma cruzi*, which is transmitted by hematophagous insects of the subfamily Triatominae (Hemiptera: Reduviidae). The disease is a serious public health problem in Latin America, where about 8 million people are estimated to be infected with *T. cruzi* and >25 million people are at risk of contracting the infection (Rassi et al., 2010; World Health Organization, 2014). From an epidemiological standpoint, the most important species of triatomines involved in the transmission of Chagas' disease are those that combine a high degree of adaptation to the domestic environments, have a wide geographical distribution, possess a high vectorial capacity, and are anthropophilic (Lent and Wygodzinsky, 1979). Among them, *Triatoma infestans* is the main vector of *T. cruzi* in the Southern Cone of Latin America between latitudes 10° S and 46° S, where it is primarily restricted to domestic and peridomestic environments.

The interruption of transmission of this parasitic and infectious disease consists of vector control by insecticide treatment of infested dwellings. *T. infestans* has been the target of control programs as part of the Southern Cone Initiative (Moncayo and Silveira, 2009). Pyrethroid insecticides remain the first choice of chemical for indoor residual spraying due to their low mammalian toxicity, rapid breakdown in the environment and efficacy. However, vector control has proven to be difficult, in part as a consequence of the variability and extension of endemic areas, and because of the difficulties to implement sustained

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http://dx.doi.org/10.1016/j.meegid.2016.07.027
1567-1348/

entomological vigilance to prevent the recovery of treated bug populations (Tarleton et al., 2014). In this regard, high levels of *T. infestans* reinfestation after spraying were observed in Argentina, Bolivia, and Paraguay (Gürtler et al., 2007). Coincidently, pyrethroid resistance in the vector insect has been reported in some of those countries and emerges as one of the main explanations of the unsatisfactory control observed (Vassena et al., 2000; Picollo et al., 2005; Santo Orihuela et al., 2008; Germano et al., 2010; Lardeux et al., 2010; Gurevitz et al., 2012; Mougabure-Cueto and Picollo, 2015). Analyses of genes involved in insecticide resistance in insect vectors of diseases as *T. infestans* are of considerable importance. Particularly, the importance of studying the expression patterns of genes involved in their resistance lies in its potential for exploitation in novel insect control strategies.

In insects, cytochrome P450 monooxygenases (cytochrome P450s) play a predominant role in the metabolism of insecticides, which often results in the development of insecticide resistance in insect populations (Zhou et al., 2010). Most insect cytochrome P450 genes belong to microsomal CYP4, CYP6, CYP9, CYP28, CYP321 and mitochondrial CYP12 families and many insect P450 genes have frequently been associated with detoxification processes allowing the insect to become tolerant or resistant to insecticides (Feyereisen, 2005; Li et al., 2007). An important characteristic of insect P450s that is associated with enhanced metabolic detoxification of insecticides is the constitutively increased levels of P450 proteins and P450 activity that result from constitutively transcriptional overexpression of cytochrome P450 genes in insecticide resistant insects (Carino et al., 1994; Liu and Scott, 1997, 1998; Kasai et al., 2000; Feyereisen, 2005; Daborn et al., 2002, 2007). In addition, the expression of some cytochrome P450 genes can be increased by exogenous and endogenous compounds (Feyereisen, 2005), a phenomenon known as induction. In insects, the induction of P450s and their activities would be involved in the adaptation of insects to their environment and the development of insecticide resistance (Terriere, 1983, 1984).

Resistance to deltamethrin was associated with oxidative metabolism in specimens of *T. infestans* from Brazil and Argentina and *Rhodnius prolixus* from Venezuela. Deltamethrin resistance in both species was decreased by piperonyl butoxide, an inhibitor of cytochrome P450 enzymes (Vassena et al., 2000; Picollo et al., 2005). Gonzáles Audino et al. (2004) and Picollo et al. (2005) measured cytochrome P450 activity on individual *T. infestans* through ethoxycoumarin-deethylase (ECOD) activity. The ECOD activity was significantly higher in a deltamethrin-resistant colony than in a susceptible colony. These results suggested that cytochrome P450s play an important role in the resistance to deltamethrin. On the other hand, other mechanism that has been associated to pyrethroid resistance in insects involves changes in the voltage-gated sodium channel which is the target site of these insecticides. To this respect, Fabro et al. (2012) identified the presence of a resistant-conferring mutation (L1014F) in a pyrethroid-resistant population of *T. infestans* from Argentina. Subsequently, Capriotti et al. (2014) identified a new pyrethroid resistant-conferring mutation (L925I) in *T. infestans* that was associated with inefficiency in the control campaigns.

Because the increases of expression of P450 genes at transcriptional level are often consider responsible for increasing the metabolism of insecticides and seems to be a common phenomenon in the evolution of resistance development in insects (Carino et al., 1992, 1994; Liu and Scott, 1997, 1998; Li et al., 2007), it was of interest to analyze in *T. infestans* genes associated with resistance to insecticides such as those encoding for cytochromes P450. With this purpose, in this study initially the cDNA sequences of three cytochrome P450 genes were identified. Comparative analysis of transcriptional expression of these P450 genes in susceptible and resistant strains of *T. infestans* was performed. Besides, the expression patterns of the P450 genes isolated were determined at different interval times after the application of deltamethrin in insects from the different strains analyzed.

## 2. Material and methods

### 2.1. Insects

Four different laboratory strains of *T. infestans* were studied. Two strains were provided by the Centro de Referencia de Vectores of the Servicio Nacional de Chagas de Córdoba (Córdoba province, Argentina). One belonging to a colony susceptible to deltamethrin (CRV-susceptible strain) that was originated in 2006 from insects collected in the locality of Chuña (Department of Ischilín, Córdoba province, Argentina) (30° 28′ S, 64° 40′ W) and other belonging to a 2nd generation of a colony resistant to deltamethrin (CRV-resistant strain) that was originated from individuals collected in the locality of Mataral (Department of Santa Cruz, Bolivia) (18° 06′ S, 64° 12′ W). The other two strains were provided by the Centro de Investigaciones de Plagas e Insecticidas (Buenos Aires province, Argentina). They consisted of the first laboratory generation of a colony susceptible to deltamethrin (CIP-susceptible strain) that was originated from insects collected in the locality of Los Quirquinchos (25° 07′ S, 61° 22′ W) and of a colony resistant to deltamethrin (CIP-resistant strain) that was originated from specimens collected in the locality of La Esperanza (26° 03′ S, 60° 27′ W), both localities belonging to the Department of General Güemes (Chaco province, Argentina). The laboratory colonies were reared at 28 ± 1 °C at a relative humidity of 60–70% with a 6-hour light/18-hour dark cycle and fed once every two weeks on restrained chickens.

### 2.2. Chemicals

The insecticide used in this study was deltamethrin 98.6% (Chemotecnica, Argentina). Dilutions of insecticide were prepared in acetone (Carlo Erba Reagents, Francia).

### 2.3. Bioassays

The determination of lethal doses 50% ($LD_{50}$) was carried out according to the protocol for evaluating insecticidal effect on triatomines (WHO, 1994) with the insects from the CRV-susceptible, CRV-resistant, and CIP-susceptible strains. The $LD_{50}$ of the CIP-resistant strain was provided by the Centro de Investigaciones de Plagas e Insecticidas (Buenos Aires province, Argentina). The CRV-susceptible strain, which has been reared at laboratory since 2006 and never exposed to insecticide treatment, was used as a reference for insecticide susceptibility. Bioassays were conducted on fifth instar nymphs and adults of the laboratory strain of reference and in fifth instar nymphs for the other two samples. Fifth instar nymphs and adults were fed after 7 to 10 days of moulting and 7 days later were used in the assay. The weight of insects was recorded and those with similar weights to an average obtained from numerous individuals fed under the same conditions (200 ± 40 mg for fifth instar nymphs and 300 ± 40 mg for adults) were selected. Then were treated with different doses of deltamethrin by topical application of 1 μL of the insecticide in acetone solutions on the ventral abdomen of insects and controls were treated with acetone alone. Ten insects per dose and control group were used. Subsequently, mortality was recorded after 72 h of topical application. Three independent replicates of each test were performed on different days. $LD_{50}$ were calculated according to the probit method (Litchfield and Wilcoxon, 1949) implemented in PoloPlus 2.0 program (Robertson et al., 2007).

For the experiments, fifth instar male and female nymphs were sexed by the differences described by Espinola (1966) and separated before feeding. Adult females and males were maintained segregated after emergence until they were able to have a blood meal (day 7 post-ecdysis). For the cytochrome P450 cDNA sequences identification, fifth instar nymph fat bodies were extracted after 7 days of the blood feeding. Each sample was a pool of tissue from three specimens. For expression analysis of cytochrome P450 genes, fifth instar nymphs and adults of both sexes from the CRV-susceptible strain and fifth instar

nymphs from the CRV-resistant, CIP-susceptible, and CIP-resistant strains were used. Fifth instar nymphs and adults were fed after 7 days of moulting and 7 days later was performed a topical application of 1 μL of the corresponding $LD_{50}$ of deltamethrin on the ventral abdomen of insects. Subsequently, pools of fat bodies from two adult specimens and three fifth instar nymphs were collected at different time intervals after the application of insecticide. For controls, topical applications with 1 μL of acetone were performed. In all cases, the topical application was carried out at the same time of day and the tissue was dissected under aseptic conditions and stored in liquid nitrogen until used for RNA extraction.

### 2.4. Isolation of total RNA and cDNA synthesis

Total RNA was extracted from pools of insect tissues using TRizol reagent according to the manufacturer's specifications (Invitrogen, Carlsbad, CA) and eluted in 20 μL of nuclease–free water. For quantitative PCR (qPCR) total RNA was isolated from pools of fat bodies from two adult specimens and three fifth instar nymphs using MasterPure RNA Purification Kit (Epicentre, Madison, WI) following the manufacturer's protocol and eluted in 33 μL of nuclease-free water. This kit includes a DNase treatment in order to eliminate potential genomic DNA contamination. The RNA concentration was determined by absorption at 260 nm.

First-strand cDNA synthesis was performed with 1 μL of Oligo-$dT_{20}$ (50 μM) (Invitrogen), 1 μg of total RNA, and 400 U of SuperScript III RT (reverse transcriptase, Invitrogen) in a 20 μL reaction volume incubated at 55 °C for 1 h.

### 2.5. Amplification and sequencing of cytochrome P450 genes

Rapid amplification of 3′ cDNA end (3′-RACE) was performed using the GeneRacer commercial kit (Invitrogen). 1 μg of total RNA isolated from fat bodies of fifth instar nymphs from the CRV-susceptible strain was reverse transcribed with GeneRacer Oligo-dT and Superscript III RT. Forward primers designed on conserved amino acid sequences of P450 from other insects and a GeneRacer 3′ primer (homologous to GeneRacer Oligo-dT Primer) (Table 1) were used to amplify the first-strand cDNA and to obtain the 3′ cDNA encoding for P450. Only mRNA with a polyA tail was reverse transcribed and amplified using the polymerase chain reaction (PCR). After the PCR products were electrophoresed, bands corresponding to the expected sizes were excised from the agarose gel and purified using QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). The PCR products were then sequenced in an ABI 3130XL automated DNA sequencer (Applied Biosystems, Foster City, CA). The comparative analysis of the cDNA fragments of *T. infestans* with cDNA sequences of cytochrome P450 genes of other insect species revealed that they were the 3′ end of P450 genes. Following initial identification of the *T. infestans* cytochrome P450 genes, conventional PCRs were performed using specific primers designed from the 5′ end of the 3′ cDNA partial sequences of *T. infestans* P450 genes and degenerate or unspecific primers (Table 1) to amplify longer fragments of cytochrome P450 cDNA. To obtain sequences closest to the 5′ end of the genes, subsequent PCRs were carried out using unspecific or degenerate primers and specific primers designed from the new sequences obtained (Table 1). All PCR amplifications were carried out in a Thermocycler (MyCycler; Bio-Rad, Hercules, CA) in 15 μL of a solution containing 1 μL of first-strand cDNA (template), 1–4 μM of each unspecific or degenerated primer, 0.7 μM of each specific primer, 10 mM Tris-HCl (pH 9), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 0.6 U of Amplitaq DNA polymerase (Amersham Biosciences, Piscataway, NJ). The degenerate and unspecific primers were designed from the conserved regions of sequence of cytochrome P450 from other insects. Thermal profiles consisted of an initial denaturation step at 94 °C for 5 min, followed by 40 cycles at 94 °C for 60 s, 48–65 °C for 1 min, and 72 °C for 60 s, with a final extension step of 10 min at 72 °C. Reaction products were visualized after electrophoresis on 1.5% agarose gels (Tris-acetate EDTA buffer, pH 8) containing 0.5 μg/mL of ethidium bromide. The bands corresponding to the expected size were purified and sequenced as explained above. Some of the resulting PCR products were cloned into the pCR4-TOPO TA cloning vector (Invitrogen) before being sequenced. The comparative analysis of the cDNA fragments obtained with cDNA sequences of other insects' cytochrome P450 genes showed that they were part of a cytochrome P450 gene.

**Table 1**
Sequences of all PCR primers used in this study.

| Name | Sequences | Function |
|---|---|---|
| *PCR degenerate primers (for conserved cDNA isolation)* | | |
| CYP4BF | 5′-TTYATGTTYGARGGIYAYGAY-3′[a] (Karunker et al., 2008) | Sense |
| CYP4BR | 5′-AAYTTYTGICCDATRCARTT-3′[a] (Karunker et al., 2008) | Antisense |
| *Unspecific primers (for RT-PCR)* | | |
| MixFe | 5′-TATTAGTAAACTGCTATGGG-3′ | Sense |
| AF | 5′-TGCTGCACAATTTTTCGTCG-3′ | Sense |
| AR | 5′-GTCAATAGTTAACCCATCTGG-3′ | Antisense |
| BF | 5′-GACCCTCTATCGTATCAACC-3′ | Sense |
| BR | 5′-GGTACAGTAAATTATGGAACC-3′ | Antisense |
| CF | 5′-GATATGTGGAATATGATGCTG-3′ | Sense |
| CR | 5′-GGTCCGTATTAATAATCTTGC-3′ | Antisense |
| GenA5′F | 5′-CGATCCTGACGACTTGGAGG-3′ | Sense |
| GenC5′F | 5′-GCAAATGTGGAAGACATTGCG-3′ | Sense |
| *Specific primes (for RT-PCR)* | | |
| TinR | 5′-GCCTTTTCCTGTGCTTGAGG-3′ | Antisense |
| F1–3 | 5′-GATGTTCACTCTCTACTTACTTGG-3′ | Sense |
| R3 | 5′-TGATCTGAATAGGAGTTTCCAATG-3′ | Antisense |
| RACEA3 | 5′-CCTGTGTGTTTAATGAATGTCCC-3′ | Antisense |
| GenCR | 5′-CCACTTCTCTAGCTTCATCAGCATC-3′ | Antisense |
| *Cloning* | | |
| M13F | 5′-GTAAAACGACGGCCAG-3′ | Sense |
| M13R | 5′-CAGGAAACAGCTATGAC-3′ | Antisense |
| *RACE primers (for 3′ cDNA isolation)* | | |
| AF | 5′-TGCTGCACAATTTTTCGTCG-3′ | Sense |
| BF | 5′-GACCCTCTATCGTATCAACC-3′ | Sense |
| GenC3′ | 5′-GATGCTGATGAAGCTAGTGAAGTGG-3′ | Sense |
| GeneRacer3′ | 5′-GCTGTCAACGATACGCTACGTAACG-3′ | Antisense |
| *qPCR primers (for real time PCR)* | | |
| RTFA | 5′-GGACAGTGATGGCAACAATGAT-3′ | Sense |
| RTRA | 5′-GCGGCTATATACAAGTTTTACACATCTT-3′ | Antisense |
| TaqBF | 5′-GCACAATCTAAAGCCCGTGAA-3′ | Sense |
| TaqBR | 5′-ACGTCCAATCGCCATGCT-3′ | Antisense |
| RTCF1 | 5′-CCAACTATTTATATTCTTTATCGCTGGAT-3′ | Sense |
| RTCR1 | 5′-CGATTGCACTTCAAGTTCCTTTG-3′ | Antisense |
| QβactinaF | 5′-CCCCTTTCAGTGAGGATCTTCA-3′ | Sense |
| QβactinaR | 5′-CGCCATCCTTCGATTGGA-3′ | Antisense |

[a] Y = C/T, R = A/G, D = A/G/T, I = inosine.

### 2.6. cDNA sequence analysis

The cytochrome P450 cDNA sequences of *T. infestans* were compared with those of other insects deposited in GenBank using the "BLAST-N" tool available on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nhi.gov) and the program Clustal W2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The *T. infestans* amino acid P450 sequences were deduced from the corresponding cDNA using the translation tool from the ExPASy Proteomics website (http://www.expasy.org/tools/dna.html).

### 2.7. Phylogenetic relationship

Phylogenetic analysis was conducted in order to investigate evolutionary relationships among the putative P450 proteins identified in *T. infestans* and other selected sequences reported in the literature (from *Anopheles gambie*, *Anopheles minimus*, *Drosophila melanogaster*, *Nilaparvata lugens*, *Lygus lineolaris*, *Bemisia tabaci*, *Apis mellifera*, and *Anopheles darlingi*). GenBank accession numbers of *A. gambie* P450s:

CYP12F1 (AAO19582.1), CYP4C25 (AAL58564.1), CYP4D17 (AAL58557.1), CYP4G16 (AHB59619.1), CYP4H19 (AAL58560.1), CYP4J5 (AAL58559.1), CYP6N2 (AAK32956.1), CYP6P3 (AAL93295.1), CYP6R1 (AAK32957.1), CYP6Z3 (AAO24698.1), CYP9L1 (AAL96668.1); *A. minimus*: CYP6P7 (AAR88141.1); *D. melanogaster*: CYP18A1 (CAL69954.1), CYP12C1 (AAF49240.2), CYP4AA1 (AAF58091.2), CYP4C3 (AAF57098.1), CYP4D2 (NP_001284806.1), CYP4G15 (NP_727531.2), CYP4P2 (AAF58963.1), CYP4S3 (AAF48426.1), CYP6A13 (AAF59076.1), CYP6A14 (NP_610389.3), CYP6D2 (AAF46877.1), CYP6G1 (ADJ57460.1), CYP6V1 (AAF50889.1), CYP9B (AAF59291.1), CYP9C1 (AAF47247.1), CYP9H1 (AAF58453.1), CYP6A2 (NP_523628.1); *N. lugens*: CYP4CE1 (CAX94852.1), CYP6AY1 (CAH65682.2); *L. lineolaris*: CYP6X1V3 (AAM94461.1), CYP6X1V2 (AAL15174.1); *B. tabaci*: CYP6CM1 (ACS92724.1); *A. mellifera*: CYP18A1 (CAL69954.1), CYP4G11 (NP_001035323.1), CYP9E2 (XP_006562363.1); *A. darlingi*: CYP4AA1 (ETN62442.1). Multiple alignment of sequences was performed using the multiple alignment program Clustal W in MEGA version 5.1 (Tamura et al., 2011). Tree construction was performed by the neighbor-joining method, using MEGA version 5.1 software (Tamura et al., 2011). The reliability of the trees was tested by the bootstrap procedure with 1000 replications.

### 2.8. Quantitative PCR (qPCR)

The transcript levels of the cytochrome P450 genes identified in *T. infestans* were measured by qPCR, using β-actin to normalize the expression levels. Gene specific primers (Table 1) and Taqman probes were designed according to the corresponding cDNAs using Primer Express program (Applied Biosystems). Reverse transcription polymerase chain reaction (RT-PCR) analysis was used to verify that the PCR products showed a single band and the expected sizes. The PCR products corresponding to the expected size were cloned into the pCR4-TOPO TA cloning vector (Invitrogen) and sequenced to confirm the identity of the amplified products. Relative qPCR was performed to investigate changes in cytochrome P450 expression. Quantitative PCR was carried out using Mx3005P qPCR System with Brilliant qPCR Core Reagent Kit (STRATAGENE, La Jolla, CA). The reaction conditions were 40 cycles of 10 min at 95 °C, 15 s at 95 °C, and 60 s at 55 °C for one of the cytochrome P450 genes isolated and 60 °C for the other two. The relative copy number of cytochrome P450 mRNA was calculated according to $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001). The threshold cycle value difference $^{\Delta}$CT between cytochrome P450 mRNA and β-actin RNA of each reaction was used to normalize the level of total RNA.

### 2.9. Statistical analyses

For the studies involving gene expression analysis by qPCR, two independent experiments were performed and data for each point were registered by triplicate to account for intra-experimental variation. Graphs and statistical tests were performed using GraphPad Prism version 5.00 for Windows (GraphPad software, San Diego, CA, USA). One and two-way ANOVA with Bonferroni posttest were used for comparisons. The results were presented as mean ± Standard Deviation (SD) and a $P$ value < 0.05 was considered statistically significant.

## 3. Results

### 3.1. Sequence and phylogenetic analyses of three cytochrome P450 genes in Triatoma infestans

In this study, three cytochrome P450 cDNAs were isolated using the 3′-RACE methodology. Subsequent reverse transcription PCR (RT-PCR)

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CYP3085B1       ------------------------------------------------------------
CYP3092A6       --------------------QMWKTLRSKLSPTFTSGKLKWMFSQISSCTDILIEYLNEK 40
CYP4EM7         GLEYYLLIPWLQEGLLLSNGEKWHHRRKLLTPAFHFKILEDSLQSLNKHARLLLRNMLKK 60

CYP3085B1       ------------------------------------------------------------
CYP3092A6       ANGEDFLIRDIISCYGFDIIASCAFGLDSQAQKNPDNEFRKKATKIFTPSIKTMMIQTLA 100
CYP4EM7         -NGEPFNVEKMIVPCTLDIICETAMGHSLNTQDSDGNND-------YLRAVRRTCHLIFQ 112

CYP3085B1       ------------------------------------------------------------
CYP3092A6       FMFPNLIRKLGINIIDPEISEFFCTLTKTTLKQRKESGIKRNDFLQLLLQLKDKGYVEYD 160
CYP4EM7         RCVKLVYSREWLYALTLDGRDFFRNLNYLHKFTENIIRNRKMDYLPENKSEEEENFGKKK 172

CYP3085B1       --------------------------AAQFFVAGLDTTSNSMTWLFYELASHPEAQSKAR 34
CYP3092A6       ADEAREVEKIDNIEKLEFTDEILAAQLFIFFIAGFEPVTSLIVFTLYALSKELEVQSKIR 220
CYP4EM7         KAFLDILIETDRKGESKFTDKDIREEVDTFMFEGYDTTSSCLMFTLYLLGRNPQAQEKAY 232
                                          *   *           * *      * *

CYP3085B1       EEIRACLKKHG-DWTYDAIHDMKYVAGCINESLRLHSPIPFIFRTCTRKYVTPDGLTIDK 93
CYP3092A6       DEVMEVKEKFG-LISYDSLKDMTYLESVMAESLRLYPPARLLIRTCTKTYTLKDGNQIEK 279
CYP4EM7         EELYAIFGDSDRAVINKDLHEMHYLEMIIKESLRLYPSVPFISRMLTQDLVLKDNVVIPE 292
                 *                    * *      *****        *  *      *   *

CYP3085B1       GTKVVIPALTLQMNPAVYPDPLSYQPERFIGEE----KNTNLKWLPFGEGPRKCVGIRFS 149
CYP3092A6       DTIIMIPNYSLQRDPKYFPDPEAFKPERFADGEQLSIRQGRGIYLPFGDGPRVCIARRFA 339
CYP4EM7         GSNIGILIFLIHRDPRYYPNPEVFDPERFSIEN--CKNRHPYAYLPFSAGPRNCIGQKFA 350
                     *        *   * *    ****               ***  *** *    *

CYP3085B1       QMEMRTVVARLLENHELIFTENTKFPVVNNDRTFFSRPLNKLFVKLQKVP---- 199
CYP3092A6       LLEAKMALAKIVENFQINASSKNIEPLQRDPKSFIINPIGGLWLKLEKIGQNTN 393
CYP4EM7         MMELKVVLSTILR--FVKIESVNEINCTNLVPSTILRTKDPIQITLMPRS---- 398
                  *                                        *
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**Fig. 1.** Alignment of partial amino acid sequences of *Triatoma infestans* cytochrome P450s. Identical amino acids are denoted with an asterisk. The underlined residues indicate the highly conserved sequences around the heme-binding and meander-binding sites. The sequence of CYP4EM7 that differs by one amino acid from CYP4 family is boxed. Gaps were introduced to maximize sequence identity and are shown by a horizontal dash.

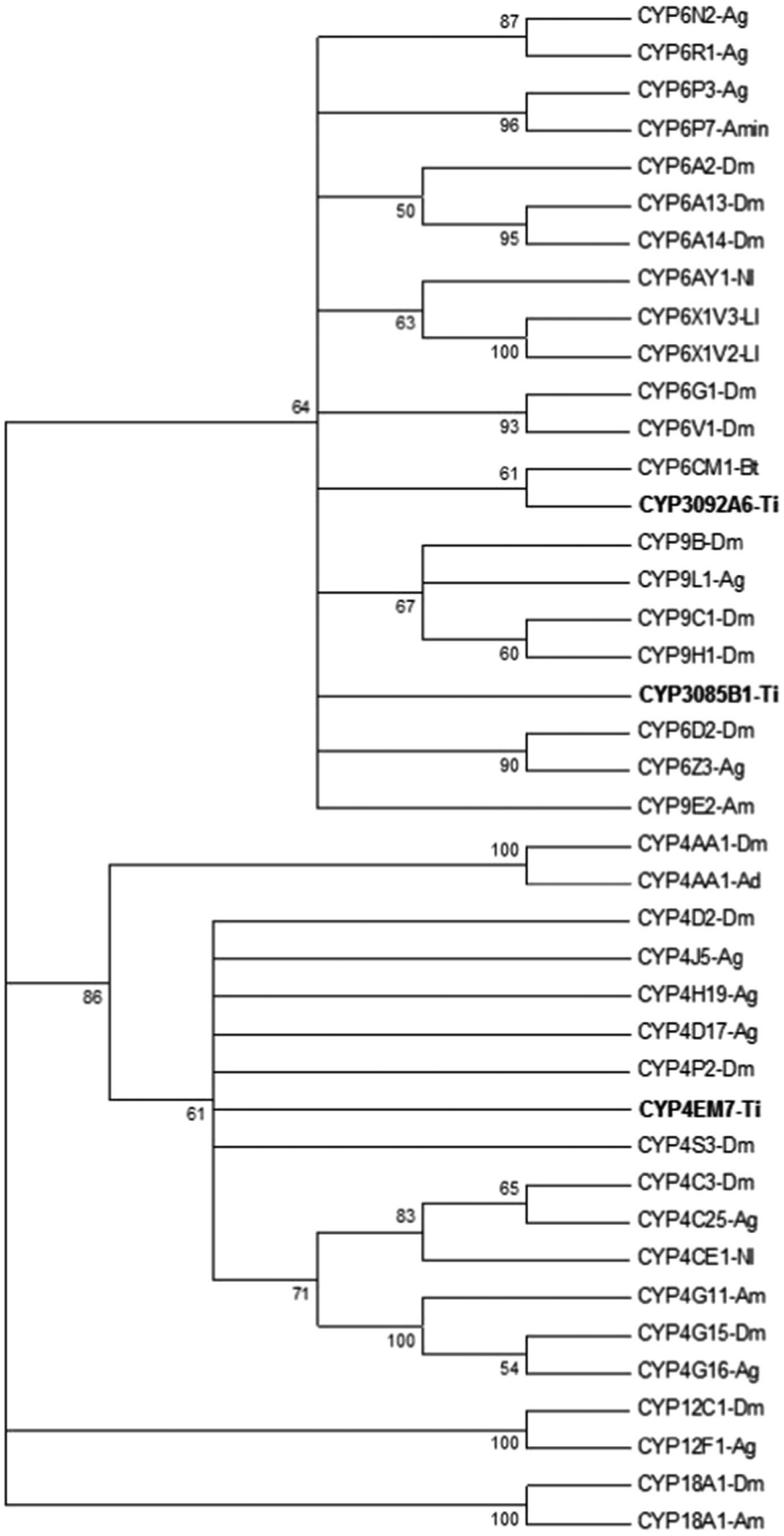



**Fig. 2.** Neighbor-joining phylogeny of *Triatoma infestans* (Ti) P450s deduced amino acid sequences (in bold) and selected P450s from *Anopheles gambie* (Ag), *Anopheles minimus* (Amin), *Drosophila melanogaster* (Dm), *Nilaparvata lugens* (Nl), *Lygus lineolaris* (Ll), *Bemisia tabaci* (Bt), *Apis mellifera* (Am), and *Anopheles darling* (Ad). Bootstrap values next to nodes represent the percentage of 1000 replicate trees that preserved the corresponding clade. Only values > 50% are reported. The tree was constructed with the partial-length sequences (helix I to heme-binding regions) of the P450s.

amplifications using specific primers designed from these 3′ fragments and unspecific or degenerate primers designed from conserved regions of cytochrome P450 sequences of other insects, allowed to obtain longer cDNA partial sequences of three cytochrome P450 genes for *T. infestans*. These genes were named CYP4EM7, CYP3085B1, and CYP3092A6 (GenBank accession numbers: KU291576, KU291577, KU291578, respectively) by the P450 Nomenclature Committee (Dr. D. Nelson personal communication).

The partial cDNA sequence of the CYP4EM7 gene comprises 1372 bp with 1194 bp encoding for 398 amino acids and 178 bp 3′-untranslated region (3′-UTR). The cDNA fragment obtained for the CYP3085B1 gene is of 796 bp, which includes 598 bp encoding for 199 amino acids and 198 bp 3′-UTR. A total of 1267 bp of cDNA corresponding to the CYP3092A6 gene was sequenced and shown to comprise 1181 nucleotides that encoded 393 amino acids and 86 bp 3′-UTR. Alignment of the amino acid sequences deduced from the three isolated cDNA fragments allowed to detect salient characteristic or signature motifs of P450s, which include the heme-binding decapeptide (FXXGXXXCXG) and putative "meander"-binding sequences (EXXR and PXRF) in Helix K (Fig. 1). Moreover, in CYP4EM7 was detected a sequence (EVDTFMFEGYDTT) in Helix I, which differs only by one amino acid from that described for CYP4 family; while CYP3085B1 and CYP3092A6 present the sequence AGXXT in that region. In the phylogenetic tree, based on partial amino acid sequences of *T. infestans* P450s and selected P450s from other insects, CYP4EM7 was unambiguously clustered with members of the CYP4 family with strong bootstrap value (86%). On the other hand, although with moderate support (64%), CYP3085B1 and CYP3092A6 were clustered with members of the CYP6 and CYP9 families (Fig. 2). Since it has been described that above the family level, CYP6 and CYP9 are included in the CYP3 clade (Feyereisen, 2006), the position of CYP3085B1 and CYP3092A6 would indicate that they also belong to this clade.

### 3.2. Comparative expression analysis of P450 genes in susceptible and resistant strains of Triatoma infestans

Comparative analysis of transcriptional expression of the P450 genes, CYP4EM7, CYP3085B1, and CYP3092A6, of groups of fifth instar nymphs from the different strains considered for this study (CRV-susceptible, CRV-resistant, CIP-susceptible, and CIP-resistant strains) revealed that the mRNA levels of the CYP4EM7 gene in individuals of the CIP-susceptible and CIP-resistant strains were significantly higher than in insects of the CRV-susceptible and CRV-resistant strains (Fig. 3). In addition, it was observed that constitutive expression of the gene was significantly higher in individuals from the CIP-resistant strain than in individuals from the CIP-susceptible strain. Contrary to what was observed in the CYP4EM7 gene, it was not detected differences in the constitutive expression of the CYP3085B1 and CYP3092A6 genes among the populations analyzed (Fig. 3).

The expression comparative analysis of P450 genes of fifth instar nymphs and adults of both sexes from the CRV-susceptible strain is shown in Fig. 4. The level of constitutive expression of the CYP4EM7 gene was significantly higher in adult individuals than in the nymphal stage. In addition, it was observed that the level of mRNA in adult males was significantly higher than in adult females. On the other hand, no differences of expression of the CYP3085B1 and CYP3092A6 genes between different sexes and stages analyzed were observed.

### 3.3. Expression of P450 genes following treatment with deltamethrin in susceptible and resistant strains of Triatoma infestans

The mRNA levels of the cytochrome P450 genes identified were determined at different interval times after the application of deltamethrin in insects of the four laboratory strains (CRV-susceptible, CRV-resistant, CIP-susceptible, and CIP-resistant strains).

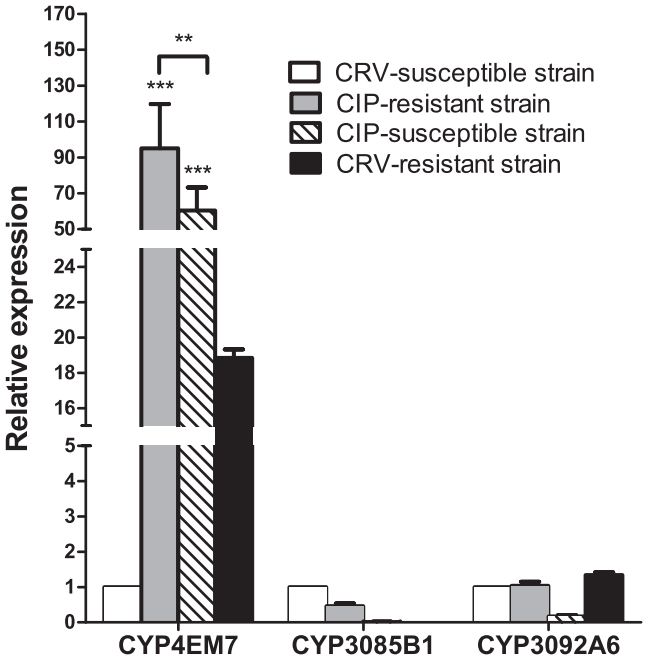



**Fig. 3.** Relative P450 expression (mRNA) of CYP4EM7, CYP3085B1, and CYP3092A6 in the fat body of *Triatoma infestans* nymphs from the CRV-susceptible, CRV-resistant, CIP-susceptible, and CIP-resistant strains. The error bars represent the standard deviation of the mean. Three asterisks on the standard error bar indicate significant difference between the mean of insects from the CIP-resistant and CIP-susceptible strains and the mean of the CRV-resistant and CRV-susceptible strains at $P < 0.001$. Two asterisks between the CIP-resistant and CIP-susceptible strains indicate significant difference between them at $P < 0.01$.

The relative expression levels of the genes CYP4EM7, CYP3085B1, and CYP3092A6 in fifth instar nymphs of both sexes of the CRV-susceptible strain of reference are shown in Fig. 5. The transcript levels of the

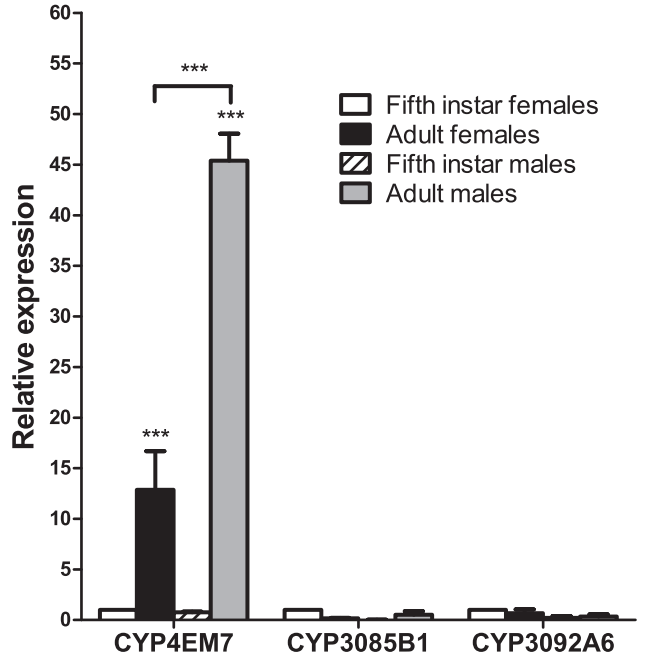
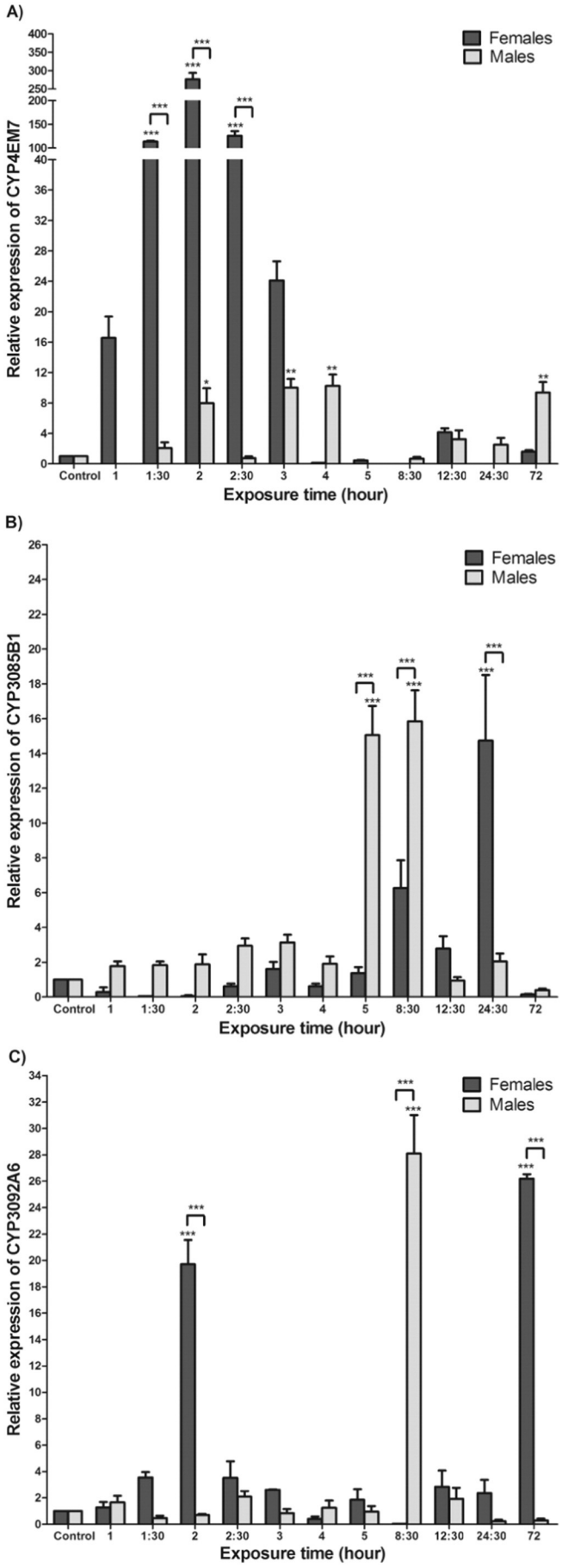



**Fig. 4.** Relative P450 expression (mRNA) of CYP4EM7, CYP3085B1, and CYP3092A6 in the fat body of nymphs and adults of *Triatoma infestans* from the CRV-susceptible strain. The error bars represent the standard deviation of the mean. Three asterisks on the standard error bar indicate significant difference between the mean of adults and the mean of nymphs at $P < 0.001$. Three asterisks between adult males and adult females indicate significant difference between them at $P < 0.001$.




three P450 genes identified in *T. infestans*, varied significantly among different insecticide exposure times. The results show that the levels of P450 mRNAs were increased after insecticide application in relation to those detected in individuals not exposed to deltamethrin (controls). The level of CYP4EM7 mRNA in female nymphs was increased after insecticide application, reaching significant levels between 1:30 and 2:30 h, with a maximum of expression at 2 h of exposure to deltamethrin. In male nymphs, a significant increase in gene expression at 2, 3, 4, and 72 h after exposure was observed. However, the relative expression levels in female nymphs were significantly higher than those observed in male nymphs in the early hours after insecticide application (1:30, 2, and 2:30 h) (Fig. 5A). Similarly, although in adult females the level of CYP4EM7 transcripts was increased significantly (8 fold) 4 h after insecticide application, the gene expression in female nymphs was extremely higher than in adults (Fig. A.1A). Significant increments of expression of the CYP3085B1 and CYP3092A6 genes were also observed in female and male nymphs at different times after the application of deltamethrin (Fig. 5B, C). Besides, the induction of both genes was higher in nymphs than in adults (Fig. A.1B, C).

Significant increase of CYP4EM7 and CYP3085B1 transcript levels in female fifth instar nymphs from the CRV-resistant strain was detected between 2:30 and 3 h after the insecticide application, with a second peak of expression at 5 and 72 h of the exposure to the insecticide, respectively (Fig. 6A, B); while for the CYP3092A6 gene was observed induction only to 2 h after exposure (Fig. 6C). In female fifth instar nymphs from the CIP-susceptible strain, the deltamethrin induced the expression of the CYP4EM7 and CYP3085B1 genes at the exposure times of 72 and 24:30 h, respectively (Fig. 7A, B), and for CYP3092A6 at 12:30 and 24:30 h (Fig. 7C).

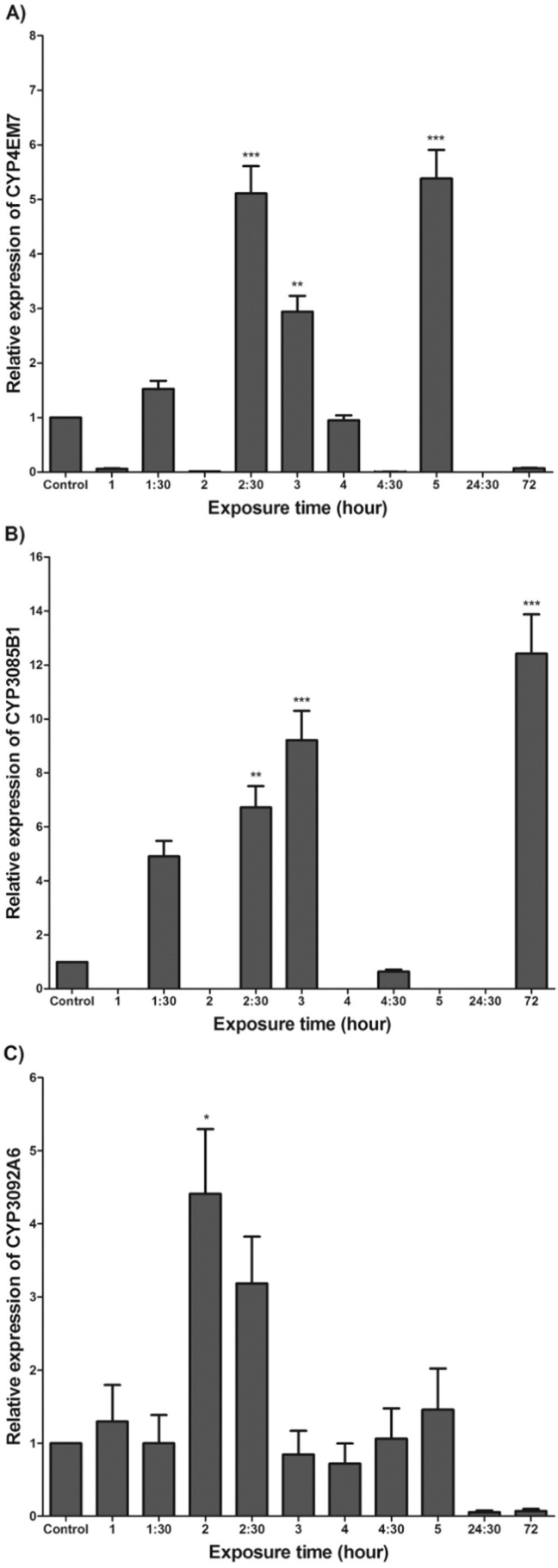
For the CIP-resistant strain, the relative expression of the three P450 genes was analyzed only four times after the application of deltamethrin because the low number of individual available from this population. The mRNA levels of female fifth instar nymphs from this strain are shown in Fig. 8. The results show that the mRNA level of CYP4EM7 remained similar to the control in the first hours after insecticide application and increased significantly (10 fold) relative to the level detected in individuals not exposed to deltamethrin (control) at 72 h of exposure to the insecticide (Fig. 8A). Similarly, a significant induction in the expression of the CYP3092A6 gene of 7.55 fold was observed at 72 h after insecticide exposure (Fig. 8C). In contrast, the CYP3085B1 mRNA level decreased significantly compared to the control in the subsequent hours of the insecticide application (Fig. 8B).

## 4. Discussion

Increased metabolic detoxification of insecticides, primarily by activities of cytochrome P450 monooxygenases (P450s), is the most frequent type of resistance mechanism occurring in insects (Li et al., 2007). It has been proposed that induction and/or constitutive overexpression of P450s is linked to the adaptation of insects to their environment (Terriere, 1983, 1984). As part of this adaptation, it has been hypothesized that both constitutively increased expression (overexpression) and induction of P450s are thought to be responsible for increased levels of detoxification of insecticides (Zhu et al., 2008).

In this study, cDNA sequences of three cytochrome P450 genes, CYP4EM7, CYP3085B1, and CYP3092A6, were isolated. The predicted amino acid sequences presented salient characteristic or signature motifs of P450s and CYP4EM7 unambiguously was clustered with

**Fig. 5.** Relative P450 expression (mRNA) of CYP4EM7 (A), CYP3085B1 (B), and CYP3092A6 (C) in the fat body of *Triatoma infestans* nymphs of the CRV-susceptible strain at different times after treatment with deltamethrin at $LD_{50}$ concentration. The error bars represent the standard deviation of the mean. One, two or three asterisks on the standard error bar indicate significant difference between the mean of the treatment with the insecticide and the mean of the control at $P < 0.05$, $P < 0.01$ or $P < 0.001$, respectively. Three asterisks between female and male nymphs within the same exposure time point indicate significant difference between them at $P < 0.001$.



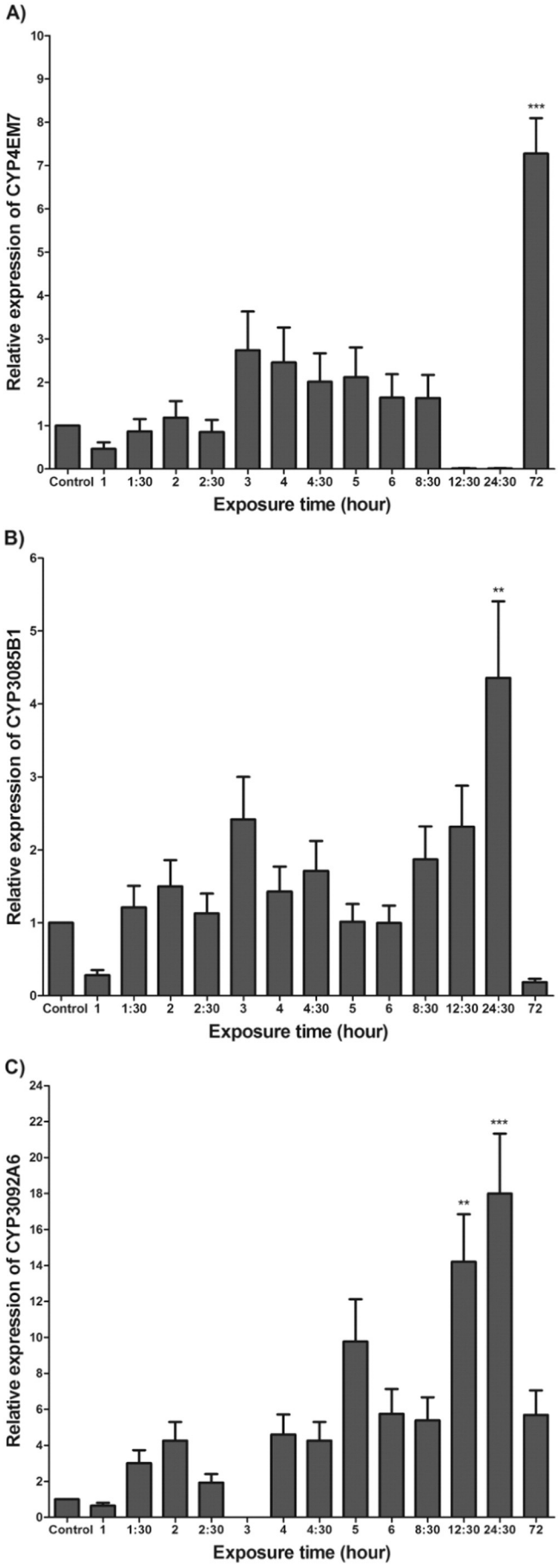

members of the CYP4 family. The transcriptional expression of the P450 genes identified was analyzed in different laboratory strains of *T. infestans*. To minimize the possibility that the results reflect a probable difference between colonies that is not related to insecticide resistance, in this study were used two deltamethrin susceptible strains (CRV-susceptible and CIP-susceptible strains) and two deltamethrin resistant strains (CRV-resistant and CIP-resistant strains). The CRV-resistant strain, which was originated from individuals collected in Mataral (Department of Santa Cruz, Bolivia), showed a lower resistance ratio (RR = 17.38) (Toloza et al., 2008) than the CIP-resistant strain (RR = 233.42) (Germano et al., 2014) that was originated from specimens of La Esperanza (Department of General Güemes, Chaco province, Argentina). In addition, to rule out possible effects of the environment, two colonies that were originated from individuals collected in two populations of the same geographical area, the CIP-resistant and CIP-susceptible colonies, were included in this study.

Results of the comparative analysis of the expression of the P450 genes at transcriptional level in nymphs from the different strains considered for this study support the hypothesis that postulates the involvement of P450 genes in the development of resistance to pyrethroid insecticide in *T. infestans*. In particular, the finding of the highest level of constitutive expression of the CYP4EM7 gene in the CIP-resistant strain (Fig. 3), where the highest degree of insecticide resistance was detected, supports this hypothesis. In contrast, mRNA levels of the CYP3085B1 and CYP3092A6 genes were not significantly different among strains. These results are in agreement with the findings that not all isolated P450s from insecticide-resistant insects are overexpressed (Zhu and Snodgrass, 2003; Gong et al., 2005; Bautista et al., 2007).

The CIP-susceptible strain also showed a high level of constitutive expression of the CYP4EM7 gene (Fig. 3). However, the highly resistant individuals from the CIP-resistant strain showed mRNA levels of this gene significantly higher than in insects from the CRV-resistant, CVR-susceptible, and CIP-susceptible strains. Therefore, it is likely that the overexpression of the CYP4EM7 gene in insects from the CIP-resistant strain may contribute to the resistance. As mentioned above, the CIP-susceptible and CIP-resistant colonies were originated from specimens collected in two localities of the same geographical area, Los Quirquinchos and La Esperanza (Department of General Güemes, Chaco province, Argentina), respectively. The increased constitutive expression detected in the CIP-susceptible and CIP-resistant strains would support the hypothesis which states that in this area could exist naturally tolerant populations to pyrethroids, explaining the presence of many localities with very low susceptibility (Mougabure-Cueto and Picollo, 2015). The low susceptibility would not be a consequence of selection mediated by insecticide (real resistance). It would be the consequence of natural tolerance of populations of *T. infestans* from the area by some other environmental factor. This could imply a selection event, which lead to a constitutive increase in enzymes involved in antioxidant defense. In the long-term, this process could contribute to the development of resistance in Los Quirquinchos and other localities of the region, which could compromise the use of insecticides for control of vector populations.

Although substantially less than in the CIP-resistant strain, resistance to deltamethrin was detected in the CRV-resistant strain originated from specimens collected in the locality of Mataral (Bolivia). However, contrary to the observed in individuals of the CIP-resistant and CIP-susceptible strains, the mRNA level of the CYP4EM7 gene detected in individuals of the CRV-resistant strain was not significantly higher than in individuals of the CRV-susceptible strain (Fig. 3).

**Fig. 6.** Relative P450 expression (mRNA) of CYP4EM7 (A), CYP3085B1 (B), and CYP3092A6 (C) in the fat body of *Triatoma infestans* female nymphs of the CRV-resistant strain at different times after treatment with deltamethrin at $LD_{50}$ concentration. The error bars represent the standard deviation of the mean. One, two or three asterisks on the standard error bar indicate significant difference between the mean of the treatment with the insecticide and the mean of the control at $P < 0.05$, $P < 0.01$ or $P < 0.001$, respectively.




Therefore, the constitutive expression of that gene in the CRV-resistant strain was not significant. This may arise in response to different insecticides applied in the places of origin of each population and/or genetic variations between Argentinian and Bolivian populations of *T. infestans*. Vector control of Chagas disease in Argentina is mainly based on the application of deltamethrin, while in Bolivia are used with more frequency other pyrethroids. Differences in treatments with insecticides could have selected different mechanisms that confer resistance in the insect vector (Mougabure-Cueto and Picollo, 2015). Moreover, based in different patterns of resistance to pyrethroids observed between populations of the insect vector of three geographic regions of Argentina and Bolivia, Santo Orihuela et al. (2008) suggested that resistance to pyrethroids based in enzymes would have multiple origins.

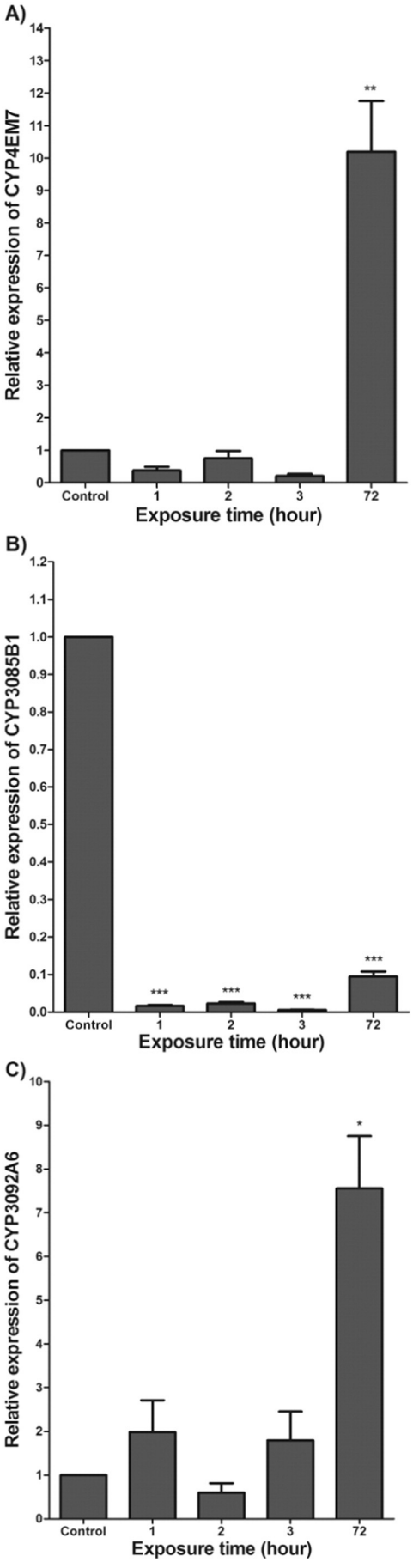
The induction of cytochrome P450 enzyme activity by deltamethrin appears to be due to the increased expression of its gene through a mechanism that is largely controlled at the transcriptional level (Gong et al., 2005). Since induction is time dependent, in this study the expression at transcriptional level of the P450 genes isolated was determined at different times after topical application of the insecticide. On the other hand, as inducibility may vary according to the amount of inductor received by the experimental insects (Vincent et al., 1985), insects with similar weights were selected for being stimulated with the $LD_{50}$ calculated for each stage and strain.

The expression at transcriptional level of the cytochrome P450 genes isolated was induced by deltamethrin in the different sexes and stages studied, except for CYP4EM7 and CYP3085B1 in adult males of the susceptible colony of reference (CRV-susceptible strain) and the CYP3085B1 gene in fifth instar nymphs from the CIP-resistant strain (Fig. 8B). In relation to the apparent lack of induction of expression of the CYP4EM7 and CYP3085B1 genes by insecticide in adult males of the CRV-susceptible strain, it is important to clarify that the expression of the three P450 genes in adult females were analyzed until 24 h after insecticide treatment, but it was not possible to determine gene expression of adult males after 4 h of application of the insecticide because of there were not enough adult males. Therefore, this result would not be conclusive. On the other hand, in the CRV-susceptible strain the transcript levels of the CYP4EM7 gene induced by the topical application of the insecticide in female nymphs were higher than in males of the same stage and in adult females, while expression of the CYP3085B1 and CYP3092A6 genes in nymphs of both sexes was higher than in adult females. In all cases, gene expression in nymphs was higher than the detected at the times analyzed after insecticide treatment in adult males. These results suggest that different regulatory mechanisms for induction are involved between sexes and the two developmental stages.

In agreement with the findings of Bautista et al. (2007) in *Plutella xylostella*, the induction data in *T. infestans* show that the P450 genes responded differently to exposure to insecticide, and up-regulation was more evident in the susceptible colony of reference (CRV-susceptible strain). The CYP4EM7 gene was inducible in the four strains considered in this study. The transcript level of the CYP3085B1 gene was induced by exposure to deltamethrin in the CRV-susceptible, CRV-resistant, and CIP-susceptible strains. By contrast, in the CIP-resistant strain was detected down regulation after exposure to the insecticide (Fig. 8B). Moreover, it was observed that the CYP3092A6 gene was inducible in the different sexes and stages analyzed in the four strains studied.

Induction of gene expression may reflect a compromise between energy saving (i.e., enhancing the activity of the detoxification system only when a chemical stimulus occurs) and adjustment to a rapidly changing environment (Brattsten, 1979). If the three deltamethrin inducible

**Fig. 7.** Relative P450 expression (mRNA) of CYP4EM7 (A), CYP3085B1 (B), and CYP3092A6 (C) in the fat body of *Triatoma infestans* female nymphs of the CIP-susceptible strain at different times after treatment with deltamethrin at $LD_{50}$ concentration. The error bars represent the standard deviation of the mean. Two or three asterisks on the standard error bar indicate significant difference between the mean of the treatment with the insecticide and the mean of the control at $P < 0.01$ or $P < 0.001$, respectively.




genes, CYP4EM7, CYP3085B1, and CYP3092A6, are involved in the detoxification of the same or other insecticides, such an induction could lead to an elevated tolerance to these insecticides, which consequently contributes to a difficulty in controlling *T. infestans* populations in the field.

In female nymphs from the CRV-susceptible and CRV-resistant strains peaks of expression of the CYP4EM7 gene were observed in the first hours after exposure to deltamethrin (Figs. 5A, 6A). Whereas in the CIP-susceptible and CIP-resistant strains, where a constitutive overexpression of that gene was detected (Fig. 3), it was observed that in the first hours after exposure to the insecticide the expression levels not differed of the control individuals. In addition to the constitutive overexpression observed in both populations, the gene expression was further increased at 72 h after exposure to deltamethrin (Figs. 7A, 8A).

It has been postulated that because P450 expression also varies between developmental stages of an insect, the degree or magnitude of induction by insecticide may be dependent on the basal level of the gene in a particular stage. To this respect, it has been demonstrated in *Helicoverpa zea* that P450s with relatively low basal level exhibit higher degree of induction compared to those with moderate basal level (Sasabe et al., 2004). However, in *Plutella xylostella*, it was observed that even those P450 genes with low basal expression levels were down-regulated by the concentrations of substrate used (Bautista et al., 2007). In the CRV-susceptible strain of reference, the levels of basal expression of the CYP4EM7 gene of fifth instar nymphs were lower than those detected for adults (Fig. 4). In agreement to what has been described for *H. zea*, individuals of nymphal stage showed higher degree of induction. While the expression of the CYP4EM7 gene detected in the nymphal stage is highly induced by the insecticide, in adults the gene expression would be principally constitutive. The constitutive expression of the CYP4EM7 gene in adults may also have relevance in the development of tolerance to insecticides in the vector of Chagas disease *T. infestans*.

In summary, we identified and characterized three cytochrome P450 genes in the Chagas' disease vector *T. infestans*. Since the constitutively increased expression and induction of P450s are thought to be responsible for increased levels of detoxification of insecticides in insects and would be involved in the development of insecticide resistance (Terriere, 1983, 1984; Carino et al., 1994; Kasai et al., 2000; Feyereisen, 2005; Daborn et al., 2002, 2007; Li et al., 2007), the overexpression particularly of CYP4EM7 in the CIP-resistant strain of *T. infestans* and the deltamethrin-inducibility of CYP4EM7, CYP3085B1, and CYP3092A6 in all the strains analyzed, suggest that these genes would be involved in the detoxification of deltamethrin and the evolution of resistance to this insecticide in *T. infestans* populations.

## Acknowledgments

We thank the Centro de Referencia de Vectores, Servicio Nacional de Chagas de Córdoba, Argentina, for providing insects used in our studies. This research was supported by grants from Agencia Nacional de Promoción Científica y Tecnológica (PICT-0293, FONCyT-Argentina), Consejo Nacional de Investigaciones Científicas y Técnicas (PIP 11220120100361, CONICET-Argentina), and Secretaría de Ciencia y Tecnología, Universidad Nacional de Córdoba (203/14-103/15, SECyT-UNC-Argentina). The experiments of this work were performed during the tenure of a CONICET fellowship awarded to Carla G. Grosso and Maria J. Blariza. Gastón Mougabure-Cueto, María I. Picollo, and Beatriz A. Garcia are Career Investigators of CONICET.

**Fig. 8.** Relative P450 expression (mRNA) of CYP4EM7 (A), CYP3085B1 (B), and CYP3092A6 (C) in the fat body of *Triatoma infestans* female nymphs of the CIP-resistant strain at different times after treatment with deltamethrin at $LD_{50}$ concentration. The error bars represent the standard deviation of the mean. One, two or three asterisks on the standard error bar indicate significant difference between the mean of the treatment with the insecticide and the mean of the control at $P < 0.05$, $P < 0.01$ or $P < 0.001$, respectively.

## Appendix A

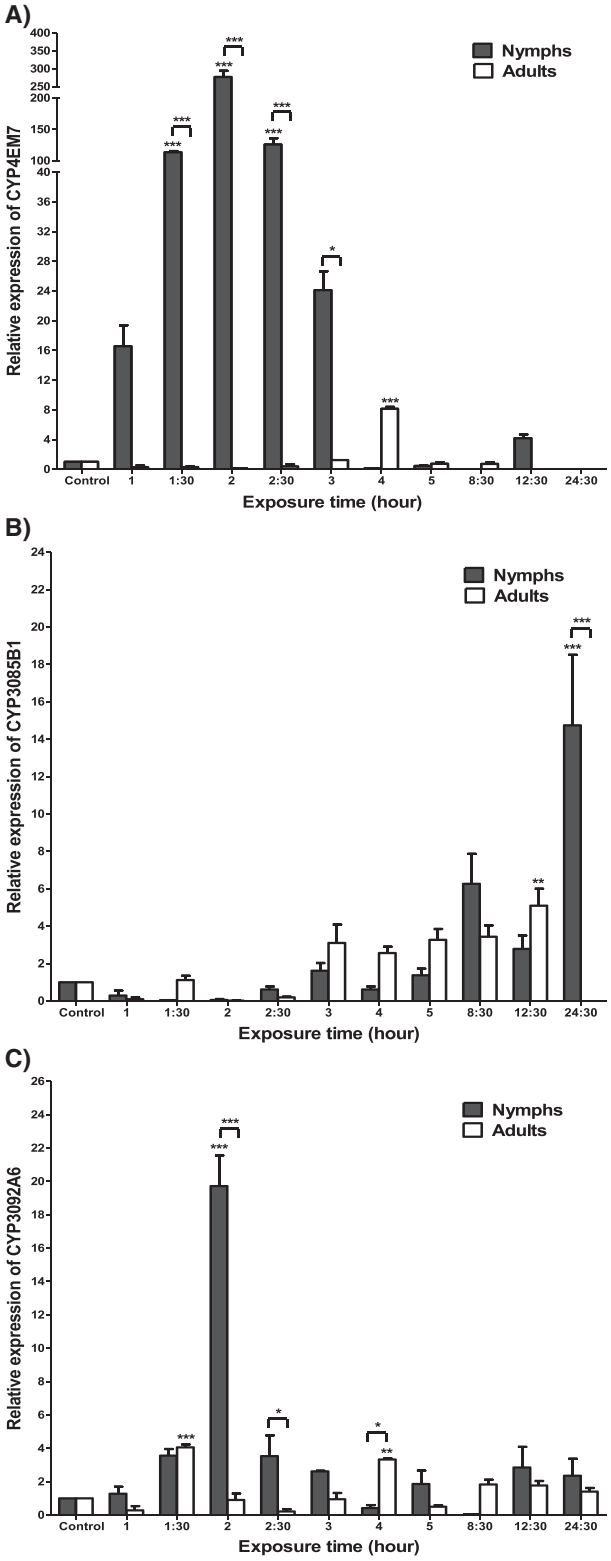



**Fig. A.1.** Relative P450 expression (mRNA) of CYP4EM7 (A), CYP3085B1 (B), and CYP3092A6 (C) in the fat body of female nymphs and adults of *Triatoma infestans* from the CRV-susceptible strain at different times after treatment with deltamethrin at $LD^{50}$ concentration. The error bars represent the standard deviation of the mean. Two or three asterisks on the standard error bar indicate significant difference between the mean of the treatment with the insecticide and the mean of the control at $P < 0.01$ or $P < 0.001$, respectively. One or three asterisks between nymphs and adults within the same exposure time point indicate significant difference between them at $P < 0.05$ or $P < 0.001$, respectively.

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