Transcriptome profiling of *Rhipicephalus annulatus* reveals differential gene expression of metabolic detoxifying enzymes in response to acaricide treatment

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Research Article

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Abstract

Background

Ticks are hematophagous ectoparasites of economic consequence by virtue of being carriers of infectious diseases damaging for livestock and agro-industry. A widely prevalent tick variety, *Rhipicephalus (Boophilus) annulatus* is recognized as a prime vector of tick-borne diseases in Southern Indian conditions. Tick control using chemical acaricides over time has helped it to evolve resistant properties to widely-used compounds through metabolic detoxification. Identifying detoxification genes is extremely important because it helps to detect valid insecticide targets and will aid to develop novel control strategies for effective insect control.

Methods and Results

In this study, high quality RNA-seq data of untreated and amitraz treated *R.* (*B.*) *annulatus* were assembled, annotated and analyzed. The annotated unigenes were significantly enriched in the pathways of metabolism, signal transduction, cellular community, transport, and catabolism. Overall, 38472 pathways were identified based on Gene ontology enrichment and pathway analysis. Differential gene expression analysis followed by the annotation of differentially expressed genes revealed significant expression of 70 detoxifying genes in response to amitraz treatment. Further, examination of expression level of candidate genes was across differential development stages of *R.* (*B.*) *annulatus* as performed using real time PCR. The results revealed significant differences in the gene expression level across stages

Conclusion

The high expression of CYP and GST genes in *R. (B) annulatus* during acaricide stressed condition may implicate their role in resistance development and increased survival rate of *R. (B) annulatus.* Identified gene targets from this study may be used as valid targets for developing gene-based management strategies.

1 Introduction

Ticks are vectors that have a worldwide distribution and are of huge economic relevance owing to the direct harm they inflict on their hosts. Ticks acquire pathogens from infected vertebrate hosts and transmit diseases to other animals through blood meal [1]. These ticks have an impact on public health due to several human diseases that are transmitted by them like Lyme disease, Kyasanur forest disease, viral borne encephalitis, rickettsial diseases and several other zoonotic illnesses [2, 3].

Due to increasing cases of cattle tick infestations in tropical and subtropical regions, tick control is a priority for developing countries [4]. Tick infestation depletes milk production and is among the most harmful vectors of lethal pathogens, second only to mosquitos [5]. Farm animals can be infected by a

number of tick species, which multiply inside the host and cause damage either through direct injuries or *via* diseases they transmit.

Rhipicephalus (Boophilus) annulatus (*R. (B) annulatus*) is a widespread exophilic tick species that live and feed on the host for several days; ingest blood meal by attaching their hypostome to the skin of the cattle. Once fed, ticks commence engorgement, moulting, reproduction and finally die [6]. *R. annulatus* infestation conduces negative impact on milk productivity through stress, immune dysfunction, blood loss, transmission of pathogen like *B. bovis* and *B. bigemina* [7, 8]. *R. (B) annulatus*, is a member of the family *Ixodidae*, poses a threat to livestock development in South India owing to its capacity to spread many variants of tick-borne diseases including anaplasmosis and babesiosis in cattle [9, 10]. The incidence and prevalence of the ixodid vector species were reported in domestic animals in the South Indian states of Kerala [11, 12] and Tamil Nadu [13]. Exclusively obligate hematophagous ectoparasites ticks infect approximately 80 percent of world's cattle population, and are among the leading causes of production loss in the livestock sector and dairy industry [14]. The outbreaks of tick-borne infectious diseases in Indian states of Kerala, Gujarat and Punjab underline the importance of controlling ticks [15–18].

In the field, commercially available vaccines are effective in enhancing immunity in cattle against ticks [19]. However, the vaccine efficacy varies due to strain variation; for instance, a vaccine developed from the Australian tick strains might not be possible to target Brazilian tick strains. Similarly, the recombinant Bm86 vaccine induced different immunological responses in *R. microplus and R. (B) annulatus* [20, 21] which indicates that genetic and/or physiological differences might be the possible factors for such type of variation in vaccine efficacy. Despite the effort that have been dedicated for many years to screen the tick specific antigen for tick vaccines, only limited number of vaccines is developed from *ixodide* [22–25]. This functional variability and limited vaccine availability indicate its incompetency in providing full protection to cattle and abolishing cattle infestation.

In this context, chemical acaricides remains a control method for cattle producers to mitigate the tick infestation. In addition, the availability and ease of handling made acaricide treatment dominant in tick eradication methods. While the application of acaricide is effective in eradication efforts, the environment is contaminated by their residues [26]. The long-term use of chemicals increased the survival ability of ticks by establishing, developing, and emerging resistance through the dominance of genetic and biological factors [27–29].

The main reason for the enhanced pest status of *R.* (*B) annulatus*, a one-host tick species, is its potential to develop resistant properties to many classes of acaricides. *R.* (*B) annulatus* is constantly challenged in its environment by repeated use of chemical acaricides, but they employ a vast array of defences to counter them: biochemical defenses such as AchE insensitivity, Sodium Channel insensitivity and GABA receptor (Cl - channel) insensitivity.

In many cases, Ticks express different set of genes at high level to cope with acaricide treatment, that further can be used as a target to develop efficient tick control approaches [30–32]. The detoxification of

acaricides via enhanced activity of enzyme families of cytochrome P450, esterases, GST, and ABC transporters are well reported in *Ixodes scapularis* [33], *Boophilus microplus* [34], *Dermacentor variabilis* [35] and *Rhipicephalus sanguineus* [36] using RNA-seq approach. But extensive research has not gone into metabolic enzyme mediated detoxification mechanism and corresponding resistance in *R. (B) annulatus.* Exploring the interaction of detoxification genes with insecticide resistance will help develop integrated pest management programs, thus, defining the precise role of these genes in acaricide resistance of *R. (B) annulatus*.

A formamidine acaricide, Amitraz was initially introduced alongside synthetic pyrethroids in 1986 to restrain organophosphate resistant ticks, but its usage was limited due to high costs. The resistance mechanism of *R. microplus* [37–39, 30] and *R. decoloratus* against amitraz [40] has been experimentally investigated across the world. Those studies demonstrated a substantial increase in the activity of metabolic detoxifying enzymes such as Cytochrome P450s and Glutathione-S-transferase against amitraz. While there are ample reports highlighting the amitraz resistance mechanism, such as molecular information in *R. (B) annulatus* populations is scarce, barring molecular studies throwing light on the cytotoxic effects of amitraz [40] and in-vitro efficacy of the compound in various tick populations [41].

In this study, we leveraged transcriptomics approach to identify and quantify the transcripts in *R. (B) annulatus* larvae following amitraz treatment and compared with the non-treated control. The top most candidate differentially expressed detoxifying genes were considered for validation using qRT-PCR. We have demonstrated the differences in expressed genes/transcripts across different developmental stages of *R. (B) annulatus.* In addition, a comparison of gene expression profiles treatment of amitraz and flumethrin, (a commonly used pyrethroid) treatment, was performed. The molecular information revealed through the study would enhance our understanding and help develop potential strategies for controlling this menacing pest.

2 Methodology

2.1 Collection and Rearing of Ticks

The adult engorged female ticks were obtained from Instructional Farm, Kerala Veterinary and Animal Sciences University, Wayanad, Kerala. The ticks were collected from all possible infested areas of cattle and maintained in a Biochemical Oxygen Demand (BOD) cabinet with 80 ± 5 per cent relative humidity. Under this incubation condition, ticks were allowed to oviposit and hatch to larvae. It took roughly 20 days for egg-laying, 10–15 days for the hatching of eggs into larvae, and another 20 days for it to mature into an adult. Larvae (8–10 days old) or newly engorged adult ticks were used for further experiments.

2.2. Larval Packet Test

The Larval packet test (LPT) was performed based on FAO's standardized protocol [42] with slight modifications. For dose-dependent bioassay, stock solution was prepared by dissolving the active ingredient of amitraz and flumethrin in methanol. This formulation was sequentially diluted in methanol

to generate series concentrations in a range of 0.146 ppm to 300 ppm for amitraz and 0.024 ppm to 25 ppm for flumethrin. Three replicates were used for the estimation of the acaricidal outcomes of the relevant concentration of chemical acaricides by LPT. Larvae had been exposed to the acaricide carefully impregnated on filter paper of triangular packet shape and allowed to dry. In the region of 100 live larvae aged 10–12 days were kept into each impregnated packet and placed in BOD incubator at 28 ± 10°C, 85 ± 5 per cent RH for 24 hours. The packets were opened following the incubation period and the mortality percentage calculated. The 50 per cent (LC_{50}) lethal concentrations of both acaricides were assessed in *R. (B) annulatus* using Abbott formula.

2.3 Adult Immersion Test

The stage specific expression analysis was performed by comparing acaricide treated larval and adult stages of tick population. The optimum acaricide concentration for the treatment of adult ticks was obtained through Adult Immersion Test (AIT). The AIT was adopted based on the procedure detailed by Drummond et al. [43]. Fully engorged adult female *R. (B) annulatus* were doused in distilled water, drained on absorbent paper before being used for AIT. The different concentrations of amitraz (200 ppm – 350 ppm) and flumethrin (20 ppm – 100 ppm) were prepared using methanol. Four replicates, containing six ticks apiece, were immersed in the respective concentration for two minutes. After two minutes, ticks were transferred to plastic tubes and kept in a BOD incubator having 80 per cent relative humidity at 28°C. The specimen tubes were observed for oviposition, and mortality up to 15 days assessed. The egg mass was continuously monitored by keeping them in a BOD incubator under the same incubation state for the next 30 days.

2.4 Total RNA Extraction and mRNA Sequencing

Total RNA extraction was performed from the pooled untreated and treated (amitraz at 0.56 ppm) *R. (B) annulatus* larvae using the trizol RNA isolation protocol (Life Technologies, Carlsbad, CA). The purity and concentration of total RNA of *R. (B) annulatus* were determined by Nanodrop spectrophotometer 2000 (Thermo Fisher Scientific, Wilmington, DE). In order to obtain transcriptome data, cDNA library construction was performed in accordance with the procedure for the Illumina TruSeq stranded mRNA library prep kit and cDNA library sequencing was conducted on the Illumina HiSeq 500 platform at the Genotypic technology's Genomics facility (Genotypic Inc., Bangalore). The RNA quantity was measured using a Qubit fluorometer and the quality was validated using a high sensitivity Bioanalyzer chip (Agilent Technologies, USA). Then, cDNA libraries were prepared for paired-end sequencing on the Illumina HiSeq 500 platform. The raw RNA-seq data of *R. (B)* annulatus were deposited into the SRA database (NCBI) with the accession number of SRP235650.

2.5 Transcriptome Assembly

Quality check of the raw data (reads) was achieved by using FASTQC. Low quality reads were filtered out using NGS QC Toolkit [44] with stringent Phred-score and high-quality reads were selected. These high-quality sequences were mapped to form longer contigs by Trinity Assembler [45]. In order to fill the intra-scaffold gaps, the paired-end reads were used for de novo assembly. The assembled sequences were

further processed to obtain unigenes using CD-hit. Similar transcripts and assembled transcripts were annotated against the arthropod's protein sequences from UniProt. The transcripts with > = 300 bps were considered for further analysis.

2.6 Annotation of Sequenced Transcriptome Data

For annotation, BLASTX was performed against arthropods protein sequences from UniProt followed by NCBI non-redundant databases. Standalone Gene ontology (GO) was performed employing BLAST2GO program [46] and pathways were mapped using KAAS server (Kyoto Encyclopedia of Genes and Genomes (KEGG) Automatic Annotation Server) [47]. GO terms were assigned to each *R. (B) annulatus* transcript and functionally classified into different categories mainly cellular component, biological process, and molecular function. In addition, *R. (B) annulatus* transcriptome data were screened to identify simple repeat sequences (SSRs) using MISA [48].

2.7 Differential Expression Analysis Using RNAseq Method

Expression profiling was performed to offer insight into the genes driving acaricide resistance by classifying the DEG between amitraz treated and untreated *R. (B) annulatus.* To gauge the expression level of each unigene (amitraz treated and untreated), the CD-hit program was employed, which clustered quality reads from each set of samples and compared the gene expression. Differentially expressed genes were identified in treated *Vs* untreated samples and were analysed using DESeq software and Bioconductor (http://bioconductor.org/) R-packages [49]. Clustered heatmap analysis was performed to visualize gene expression levels of specific contigs by comparing R plot. Finally, the expression levels were quantified between treated and untreated libraries. Additionally, differentially expressed contigs were manually scanned to map the detoxifying genes.

2.8 Real Time PCR

Some of the candidate genes were selected for expression validation using real time PCR. The RT-qPCR was performed with Roche-Light Cycler 480 System, which is a high-performance high-throughput PCR platform. The versatile Light cycler 480 instrument provides fast, highly sensitive, and reproducible gene expression results. We selected 7 CYP450 genes and 2 GST genes: CYP 30980, CYP 27576, CYP 51361, CYP 38633, CYP 41222, CYP 48268, CYP 36267, GST 34065 and GST 55171 based on the fold differences pattern from the transcriptome data for the real time PCR assay. Expression analysis of nine identified detoxifying genes in two developmental stages (larvae and adult) in response to two separate groups of acaricides at its median lethal concentration (amitraz and flumethrin) were tested by quantitative real time PCR. The primer pairs used in qRT-PCR reactions are listed in Table 1. The expression of each sample was normalized by a reference gene Beta actin. Three biological replicates were developed for each gene, and all samples were assayed in technical triplicate. The abundance of expressed genes in the acaricide treated and untreated ticks was measured using the $2^{-\Delta\Delta Ct}$ method [50] and significant expression of the target gene was statistically calculated by Unpaired *T*-tests.

Table 1
Differential gene expression of detoxifying genes in *R. (B) annulatus*

Gene names	No of Upregulated Genes	No of Downregulated Genes	No of Neutrally Regulated Genes	No of Genes Expressed only in Untreated Condition	No of Genes Expressed only in Treated Condition
Cytochrome P450	11	11	16	1	1
Glutathione–S-Transferases	18	1	8	0	0
Esterases	0	0	3	0	0

3 Results

3.1 Larval Packet Test

LC_{50} value of amitraz and flumethrin against *R. (B) annulatus* was calculated using LPT. The concentrations ranging from 300 ppm–0.146 ppm were used to find the lethal concentration of the amitraz to *R. (B) annulatus* larvae. Bioassays on *R. (B) annulatus* with amitraz revealed 100% mortality at 300 ppm and 150 ppm, whereas mortality observed in the untreated sample was far from significant (Table S1). Then, the mortality observed was corrected with natural mortality in the untreated vials using Abbott's [51] formula. Corrected mortality data were transformed into their corresponding probits. The dosage-mortality curve LC was plotted after probit analysis [52], and LC_{50} value was estimated (Fig. 1A). At 0.56 ppm, 50% mortality was observed in *R. (B) annulatus* when treated with amitraz. The ticks that survived the treatment at LC_{50} concentration were taken for RNA isolation and transcriptome analysis.

In the case of flumethrin, *R. (B) annulatus* responded highly even at the lowest concentration. The toxic interaction of flumethrin with *R. (B) annulatus* showed 100 per cent mortality when the dose concentration was between 25 ppm − 1.5625 ppm (Table S2). A graph was plotted using the mortality data against the dosage of acaricide, and the LC_{50} value was calculated using probit analysis (Fig. 1B). The concentration of flumethrin to kill half of the test population was 0.044 ppm. After determining the median lethal concentration, larvae were exposed to each acaricide at its LC_{50}. Then, the survived larvae at LC_{50} dose were used for gene expression studies.

3.2. Adult Immersion Test and Pattern of Mortality

Two acaricides, amitraz and flumethrin, were tested for their acaricidal activities against the adult stage of *R. (B) annulatus*. The higher concentrations of 200 ppm to 350 ppm were used for amitraz and 20 ppm to 100 ppm for flumethrin. At a concentration used in field conditions, amitraz (300 ppm) showed 16.6625 ± 6.8 percentage mortality and 75.80 per cent inhibition of fecundity and flumethrin (80ppm) showed 12.49 ± 4.165 percentage mortality and 100 per cent inhibition of fecundity against *R. (B)*

annulatus (Table S3 and Table S4). Adult ticks treated at 300 ppm for amitraz and 80 ppm for flumethrin were further used for gene expression studies.

3.3. *De Novo* Transcriptome Assembly Statistics

Following quality analysis, high-quality reads from the two libraries (untreated and amitraz-treated) with an average length of 151 bp were clustered using the short-read assembling program Trinity. the assembled contigs produced 50591 (untreated) and 71711 (treated) unigenes of 662 and 542 bp average length respectively. The entire identified transcripts were 76491 in the untreated sample and 52471 in the treated sample. The maximum contig lengths obtained in untreated and treated were 15075 and 15881 respectively, and the minimum contig length obtained was 201 for both the samples. The N50 values were 1169 for untreated and 731 for treated samples. The summary of the *De novo* Assembly Statistics and Clusters of Orthologous Groups (COGs) Statistics is presented in Table S5.

3.4 Transcriptome Annotation

To understand the putative protein functions, the COGs database was used to forecast and categorize the potential functional involvement of the identified unigenes. Since the reference genome was not available, a sequence similarity search was performed with all available arthropod protein sequences (UniProt Database) to identify homologous genes in other species putatively. Annotation of transcripts was done by BLAST ALL program and only those transcripts with more than 30% identity were considered for analysis. A total of 92341 transcripts were generated, among them, total annotated transcripts were 35585, and unannotated transcripts were 56756. BLAST searching of non-redundant databases gave important matches, and those gene families most likely to be encoding gene products engaged in xenobiotic sequestration and detoxification were curated and annotated.

Annotation with BLAST2GO offers a functional classification of the identified *R. (B) annulatus* unigenes based on sequence homology. The GO database was used to classify the functions of *R. (B) annulatus* unigenes into three main categories involved in molecular function, cellular component formation, and the biological process. Those were further categorized into nine biological processes, seven cellular components and ten molecular functions, respectively (Fig. 2). Among the nine subcategories of biological process, DNA integration occupied the highest (6%) followed by the transmembrane transport (1.49%), regulation of transcription (1.16%) and intracellular signal transduction (1.16%). The major subcategories of molecular function included, nucleic acid binding (19.26%) followed by ATP binding (8.41%) and zinc ion binding (8.32%). In the cellular component domain, the majority of the GO terms were shown to be specific for the integral component of the membrane (10.75) followed by the nucleus (4.3%). Our GO of the *R. (B) annulatus* shows high - percentage of genes in the category of molecular function and about 19.26% involved in nucleic acid binding.

3.5 KEGG Pathway Analysis

The KEGG database is a gathering of manually drawn pathway maps that permits pathway-based analysis in understanding gene interactions and biological functions. Using KASS server, identified

unigenes were functionally assigned to several different pathways involved in *R. (B) annulatus* tick species and a total of 38472 pathways were identified. These unigenes were highly represented in metabolic pathways, signal transduction pathways, cellular community, transport and catabolism pathways (Fig. 3). These annotations provided a treasured archive for inquiring into particular cellular processes, molecular functions and metabolic pathways in the *R. (B) annulatus*.

3.6 Identification of Simple Sequence Repeats

Owing to the high degree of polymorphism, abundance and ease of development [53], Simple Sequence Repeats (SSRs) have been routinely used as molecular markers for establishing genome diversity across species. To identify SSRs, the assembled results of transcripts were taken for screening with MISA search tool. A total of 9234 sequences were examined with a total sequence size of 58009214 bp, and 18749 potential SSRs were identified from the *R. (B) annulatus* transcriptome data. The identified SSRs were predominantly mononucleotide and tetranucleotide repeats representing about 63 per cent (11769) and 18 per cent (3457) of the SSRs respectively. A small fraction of dinucleotide repeats (12%) and pentanucleotide repeats (7%) were also identified in the *R. (B) annulatus* transcriptome. Features of SSRs identified in the *R. (B) annulatus* transcriptome is given in Table S6.

3.7 Transcripts Encoding Detoxification Enzymes and Insecticide Targets

A salient feature of metabolic resistance to acaricides is the highest transcriptional expression of detoxifying enzymes, which usually results in increased activity and significant overexpression of CYP450s, esterases (ESTs) and Glutathione S-transferases (GSTs) genes. Previously published insect genome data of *Anopheles gambiae, T. castaneum and Drosophila melanogaster* [54–56] were mined for retrieving genes encoding various detoxification enzymes, which were then used in homology search in *R. (B) annulatus*. In our study, a total of 70 detoxifying genes were identified from the *R. (B) annulatus* transcriptome including 44 CYP450, 23 GST, and 3 ESTs encoding detoxifying enzymes. The identified detoxifying genes expressed differentially when triggered by amitraz (Fig. 4). The genes differentially upregulated after feeding were selected and discussed as potential antigen candidates for tick vaccines.

3.8 Comparative Transcriptomic Analysis of Acaricide Treated and Untreated *R. (B) annulatus* Larvae

RNA-seq has cemented its reputation as a very seminal technology to gauge the gene expression response of samples at tissue, organism and whole-genome level. Differentially expressed contigs between the amitraz treated and untreated *R. (B) annulatus* tick larvae were determined using the DESeq and Bioconductor (http://bioconductor.org/) R-packages (Fig. 5). To examine whether amitraz treatment against *R. (B) annulatus* larvae resulted in statistically considerable changes in gene expression, the quantum of gene expression was appraised after normalizing the gene abundance from each library to reads per kb per million reads (RPKM). Comprehensive DEseq computational analysis facilitated the recognition of differentially expressed transcripts vis-à-vis amitraz. Overall, 16635 transcripts exhibited

upregulation, 15539 exhibited down regulation while 56864 transcripts were neutral. Notably, 1898 transcripts showed expression only in untreated conditions, whereas 1403 showed expression only in treated conditions. The complete transcriptome data revealed substantial differences in the expression patterns among amitraz treated and untreated *R. (B) annulatus* larva. We also compared the differentially expressed detoxifying genes upon amitraz treatment revealing 29 upregulated genes, 12 down-regulated genes and 27 genes that were neutral (Table 1). Compared to the untreated sample, one CYP450 gene was expressed only in treated condition. One CYP450 gene had an expression in untreated condition but did not show any expression in response to amitraz treatment.

3.9 Gene expression profile of metabolic detoxifying genes in acaricides treated *R. (B) annulatus* larvae and adult using RT PCR

Several metabolic detoxifying genes identified from RNAseq data were further used for expression studies using Real-Time PCR. The genes were named with their respective family name followed by the transcriptome data ID. We selected nine detoxifying genes (Table S7) for extensive studies on their up-regulation/down-regulation in various developmental stages of *R. (B) annulatus* mediated by different insecticides. Genes such as CYP450–30980 and 41222 (Fig. 6A) and GST 34065 (Fig. 6B) were significantly upregulated in response to amitraz treatment in both adult and larval stages making them important candidate genes for drug resistance against amitraz. Whereas, CYP450–51361 and 38633 (Fig. 6C) showed significant overexpression in response to flumethrin treatment in both adult and larval stages. A few genes were only upregulated in adult stages of the ticks in response to flumethrin such as CYP450–41222 and 36267. The GST gene 55171 showed higher expression in flumethrin treated larvae (Fig. 6D). In our study, RT-PCR results were consistent with the transcriptomic data obtained from the tick larvae treated with amitraz.

4 Discussion

Rhipicephalus (Boophilus) annulatus ticks are adept in transmitting a large variety of infectious diseases such as anaplasmosis and babesiosis and causing huge impediments in cattle industry. *R.(B) annulatus* occupy large areas of Southern India [10, 41]. *R. (B) annulatus* voraciously sucks blood meal, inject toxins, transmits pathogens, and finally modifies immune responses in cattle, which negatively impacts cattle productivity and results in significant economic losses [57, 58]. Tick control through acaricide treatment remains a basic strategy, although progress toward vaccine development or alternative tick control tools has been made. Amitraz, a formamidine pesticide predominantly used in ectoparasite control in several geographic areas of South India due to its inhibitory effect on tick fecundity and the impairment of oocytes [40, 41]. However, the high efficacy of amitraz against ticks has diminished due to the development of acaricidal resistance. Consequently, *R. annulatus* with a high resistance level is insensitive to certain amitraz formulations, rendering this acaricide ineffective for tick control. Thus, it is

necessary to understand the mechanism through which *R. (B) annulatus* counteract the effect of insecticide. Ongoing research and development initiatives are geared to find a successful management strategy for this pest.

Previous studies for the underlying resistance mechanism in ticks led to the finding that behavioral, biochemical and genetic mechanisms are involved in neutralizing acaricide [59, 60, 31]. The genetic mechanisms accelerating resistance evolution that may involve target site insensitivity or increased metabolic enzymes such as cytochrome P450s, GSTs, CCEs, and ABC transporters, which sequester, catalyze or aid the elimination of the acaricide molecules, thus inhibiting them from binding their target [61]. The extensive scientific background on metabolic enzymes [62, 63, 40] encouraged us to assess the expression levels of metabolic detoxifying enzymes in response to amitraz. In this study, the transcriptomics approach was employed to obtain the differential expression level of genes related to metabolic detoxification. In addition, using qRT-PCR the expression level of CYPs and GSTs in *R. (B)annulatus* at the sublethal doses of flumethrin and amitraz was examined.

In-vitro bioassays such as larval packet test and adult immersion test was done to compare the acaricidal effects of amitraz and flumethrin against *R. (B) annulatus*. Larval toxicity experiments revealed that *R. (B)annulatus* showed slight susceptibility difference against Amitraz and flumethrin, indicating that flumethrin is comparatively more effective in larval mortality than amitraz. The LC_{50} value observed for amitraz was 100 ppm and 80 ppm for flumethrin against *R. (B) annulatus*. Based on this result, flumethrin seems to be comparatively effective than amitraz. This result is in parallel with the results of acaricidal efficacy of flumethrin in *R. microplus* collected from Northern India, which showed high efficacy compared with other compounds [64]. The present result and previous data advocate that a comparatively lower concentration of flumethrin is required to produce 50% mortality than amitraz. This might be due to the late introduction of flumethrin into the Indian market [64]. Several authors have studied the efficacy of amitraz and flumethrin and revealed different spectrum of susceptibility in tick populations [65, 66, 41]. This susceptibility may be influenced by many factors such as application method, dose, and frequency of acaricides, genetic variation of the tick, geographic locations, defence mechanism in cattle and breed of cattle [67, 68]. Keeping these factors into consideration, livestock farmers can make a logical decision to adopt a particular chemical for tick control. To date, several studies have assessed the acaricidal efficacy of different compunds against *R. (B) annulatus* [40, 41, 64]. Yet, research headways in understanding the defence mechanism at the genetic level have been few and far between due to the lack of genomic information of this ectoparasite. Therefore, to better understand their defence molecular mechanism and association of the metabolic enzymes with acaricide resistance, we conducted a comparative transcriptome analysis of Amitraz treated and untreated *R. (B) annulatus.* We used larval stage of *R. (B) annulatus* for de novo sequencing, assembly, annotation and downstream analysis. According to the annotated results, 70 genes were found to be involved in the detoxification mechanism in *R. (B) annulatus.* Among the 70 genes, 44 were annotated as CYP450, 23 as GSTs and 3 as ESTs. Considering the abundance of detoxifying genes, more than half of the genes belonged to the Cytochrome P450 monooxygenase family. Further, to investigate gene expression changes associated

with the amitraz treatment in *R. (B) annulatus*, differentially expressed contigs were compared using DEseq. There was a total of 16635 transcripts exhibited upregulation, 15539 exhibited down regulation. Among results obtained with Deseq analysis, we compared differentially expressed detoxifying genes upon amitraz treatment. Of these, 29 upregulated (11 CYP450 and 18 GST) and 12 (11 CYP450 and 1 GST) downregulated detoxifying genes were identified. Further, a total of nine differentially expressed genes obtained from the Illumina analysis were selected to examine the expression changes in various developmental stages of *R. (B) annulatus* mediated by amitraz and flumethrin by qRT-PCR. Among the 9 genes, 3 CYP genes (CYP41222; CYP27576 & CYP 309890) and 1 GST gene (GST34065) were highly expressed in amitraz treated adult and larva of *R. (B) annulatus*. Similarly, in the case of flumethrin treated samples, 3 CYP genes (CYP38633; CYP51361 & CYP30980) were significantly upregulated in both adult and larvae. Cytochrome P450 Monooxygenases are remarkable in their ability to oxidize widely diverse substrates and are capable of producing an array of molecular reactions [69]. The existence of the cytochrome P450 system has been established in different arthropod species including ticks. Study on metabolic resistance showed that highest expression of cytochrome P450-like transcript assists in growing coumaphos resistant Mexican populations of *R. (B) microplus* [70]. Different expression patterns or expressional variation of transcripts encoding CYP450 genes in multiple tissues and in different developmental stages of an insect indicate the CYP450 catalytic activity [71]. As a result, populations of cattle ticks, *R. (B) microplus*, have developed tolerance to nearly all synthetic and chemical acaricides over the past decades due to their intensive use [72]. Comprehensive analysis of the expression of P450 cytochrome oxidases in *R. microplus* showed the association of pyrethroid acaricide resistance with the level of CYP expression [31]. Studies [73, 40, 62, 63] have also reported the association of acaricide resistance in ticks with the increased level of the expression of P450s genes and their role in xenobiotic detoxification. Apart from *CYP* protein, high expression of GSTs have also been identified, which play a role in conferring resistance to ticks [74, 75]. In our study, the high expression of CYP and GST genes in *R. (B). annulatus* during acaricide stressed condition may implicate their role in resistance development and increased survival rate of *R.(B) annulatus*. Those genes could be used as targets for pest control by inhibiting their action through sequence-specific gene silencing via RNA interference [76]. Also, the downregulation of certain genes in response to these acaricides could be due to other novel feedback mechanisms. Understanding these pathways could open up new avenues to improve tick management strategies.

The larval insensitivity to flumethrin may indirectly highlights the efficiency as a drug to target the larval stage compared to Amitraz. This is consistent with the larval packet and adult immersion tests where flumethrin that caused more lethality than Amitraz. This could be attributed to the unaltered gene expression pattern of various genes involved in the metabolic resistance in the larval stages of the ticks. This provides an evidence that overexpression could confer acaricide resistance, possibly in the absence of target site insensitivity.

Overall, our research exploited a combination of transcriptome analysis, differential gene expression along with gene expression validation for the study of resistance mechanism. The results from this study showed that our approach relying on the Illumina Hi-Seq platform enabled a comprehensive

representation of the *R. (B) annulatus* transcriptome. Identified gene targets from this study may be used as valid targets for evolving gene-based management strategies. These outcomes are geared to evaluate the possible molecular mechanisms involved in tick-borne diseases, thus contributing positively to the benefit of medical and veterinary professionals, farmers, and the common public. The knowledge creation under the aegis of this study provides ample scope for practical application, especially in generating important insights for developing new strategies to control this menacing pest.

5 Conclusion

Improved understanding of the detoxifying genes in *R. (B) annulatus* is indispensable for construing the functional analysis and understanding the complex processes involved in resistance mechanism, thus providing valuable insights into the various pathways and processes that are crucial for the survival of ticks. This study quantified the relative expression of detoxifying genes in insecticide-treated and untreated *R. (B) annulatus* and their correlation between qRT-PCR assay and by Illumina sequencing to discern the genetic and molecular basis of insecticide resistance. Having realized the overriding significance of these genes in *R. (B) annulatus*, we have analyzed their expressional variations in two stages, including larval and adult stages in response to two different acaricides. Increasing access to Next-generation Sequencing technology and transcriptome technology can accelerate research on multiple aspects of acaricide resistance and could play a significant role in developing novel control tools. Together this will propel further research in the field of toxicology and insecticide resistance, and advance our understanding of the role of environmental adaptation and microevolution as vital processes underlying resistance development.

Declarations

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Author's Contribution

Amritha Achuthkumar: Conceptualization, Investigation, Formal analysis, Writing and Visualization of the research project. **U. Shamjana:** Manuscript Writing, Review, Editing and Visualization. **Kumar Arvind:** Data Curation, Analysis and Editing. **Sincy Varghese:** Sample Collection and Technical Support. **Dr. Reghu Ravindran:** Conceptualization, Methodology and Resources for the research project. **Dr. Tony Grace:** Supervision, Conceptualization, Formal analysis and Editing. All authors read and approved the final manuscript.

Ethics declarations

Conflict of interest

The authors declare that they have no conflict of interest.

Informed consent

All authors informed consent.

Consent to publish

All authors approved the version to be published.

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Figures

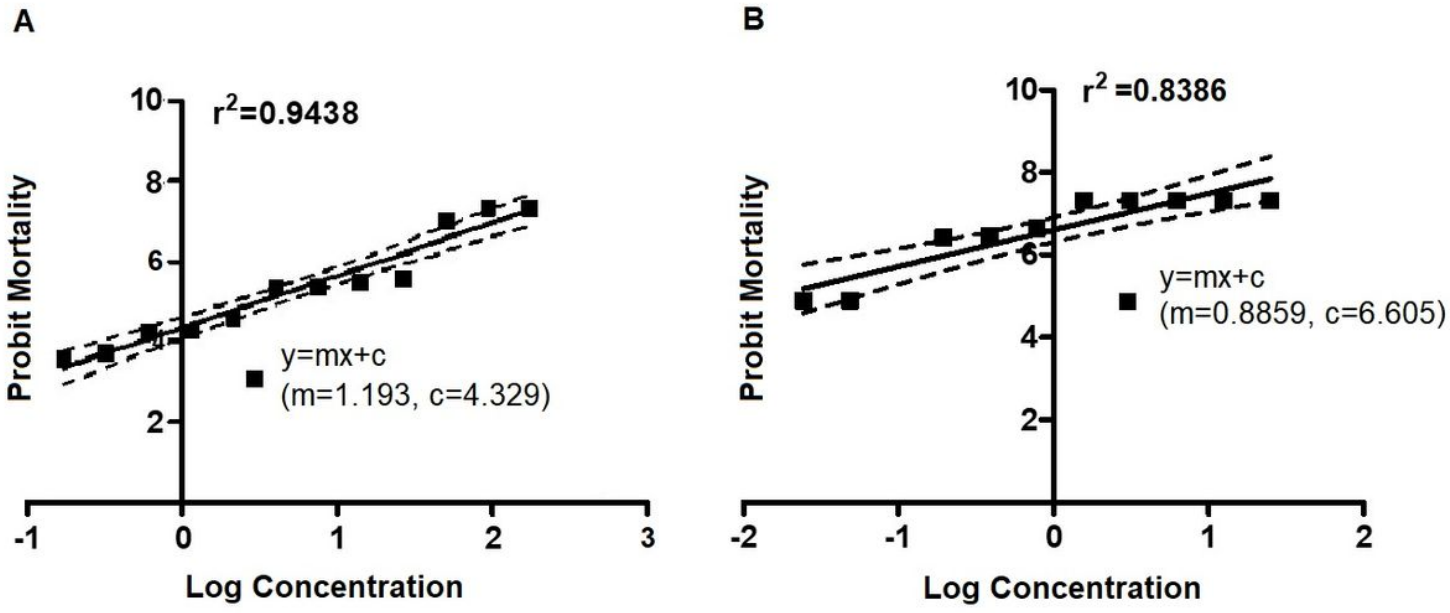

Figure 1

Mortality data of *R.(B) annulatus* larvae in response to different instecicides. **A**. Dose response graph of *R.(B) annulatus* larvae against amitraz using LPT. **B.** Dose response graph of *R.(B) annulatus* larvae against flumethrin using LPT.

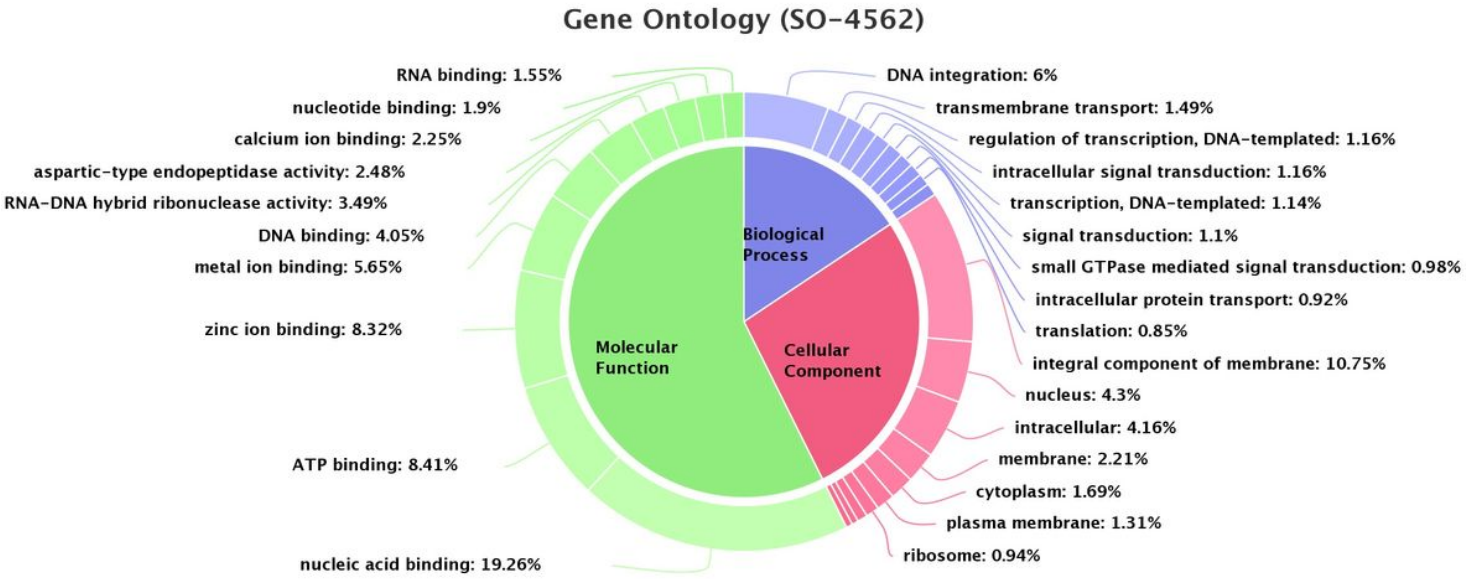

Figure 2

Summary of the Gene ontology classification from the *R.(B) annulatus* transcriptome

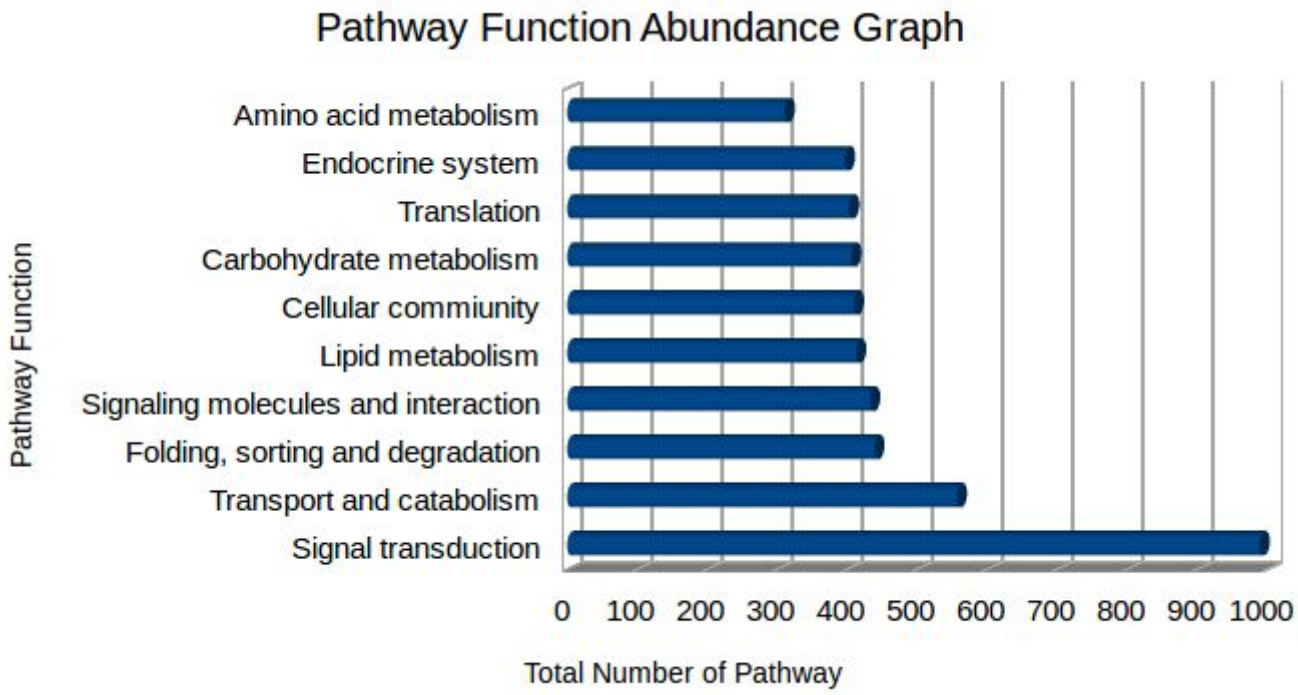

Figure 3

Pathway function abundance graph from the *R.(B) annulatus* transcriptome

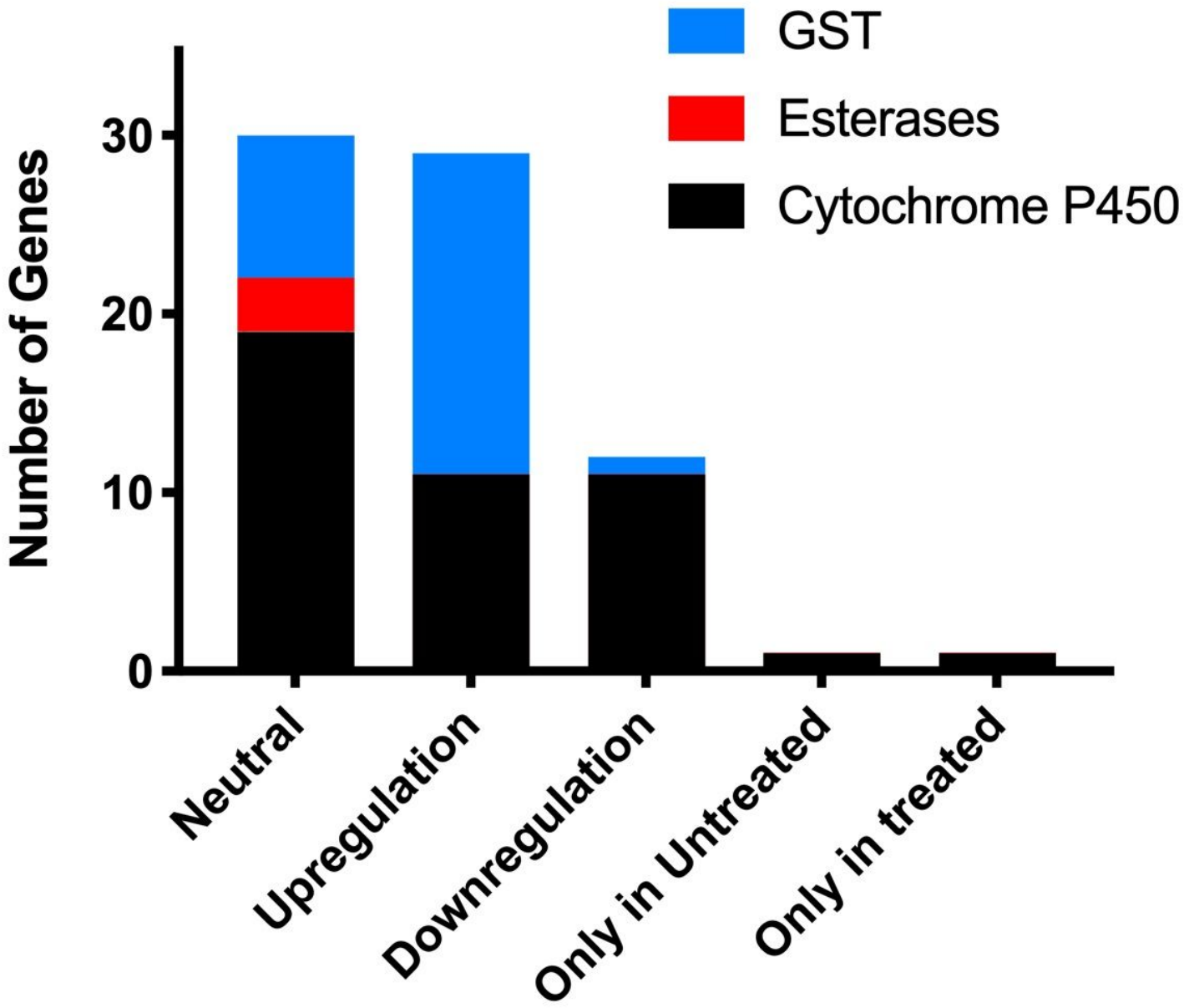

Figure 4

Number of differentially expressed detoxifying genes identified from the *R.(B) annulatus transcriptome*

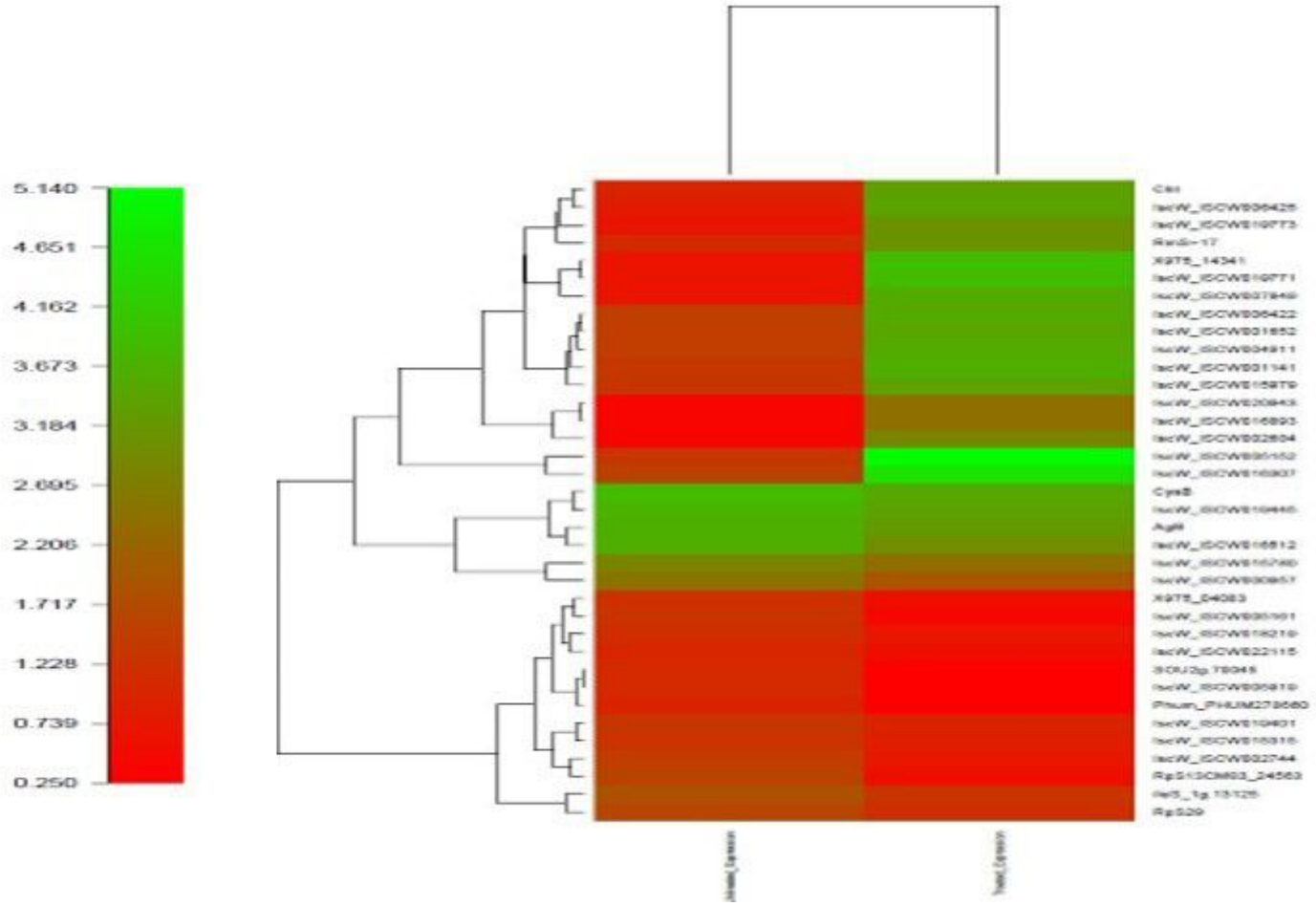

Figure 5

Heatmap of differentially expressed genes in untreated and amitraz treated *R.(B) annulatus* larvae

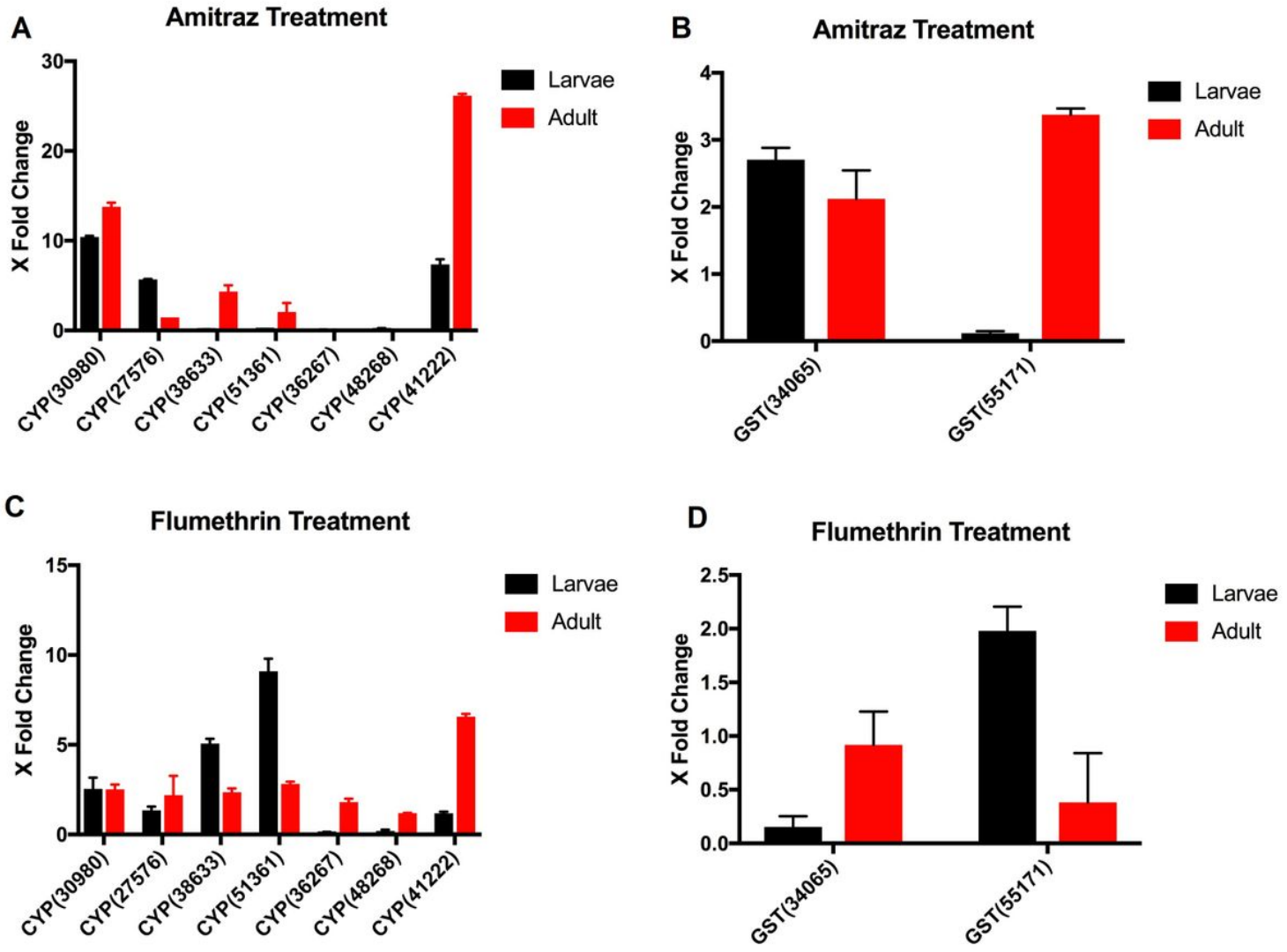

Figure 6

Stage specific expression profiling of detoxifying genes in *R.(B) annulatus* in response to amitraz and flumethrin. **A.** Stage specific expression patterns of CYP450 genes of *R.(B) annulatus* in response to amitraz. **B.** Stage specific expression patterns of GST genes of *R.(B) annulatus* in response to amitraz. **C.** Stage specific expression patterns of CYP450 genes of *R.(B) annulatus* in response to flumethrin. **D.** Stage specific expression patterns of GST genes of *R.(B) annulatus* in response to flumethrin

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTables.pdf