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Exploring data on CYP450 genes expression in *Anopheles aquasalis* infected with *P. vivax*

Abstract

Background: Hematophagy is an essential adaptation in Anopheles aquasalis, a primary vector of *Plasmodium vivax*, and the most widespread human malaria parasite. Blood digestion generates oxidative stress due to the release of free heme, leading to reactive oxygen species (ROS) production. To counteract these effects, mosquitoes rely on detoxification mechanisms, including cytochrome P450 (CYP450) enzymes, which are involved in xenobiotic metabolism, insecticide resistance, and immune modulation. This study aimed to investigate the differential expression of CYP450 genes in An. aquasalis in response to P. vivax infection.

Methods: High-throughput transcriptome sequencing data were analyzed, and differentially expressed genes were identified using edgeR. Gene expression comparisons between *P. vivax*-infected, non-infected, and unfed mosquitoes revealed the overexpression of CYP450 genes, particularly from the CYP4G subfamily, which plays a role in hydrocarbon biosynthesis and desiccation resistance.

Results: The results suggest that CYP450 enzymes contribute to mitigating oxidative stress induced by Plasmodium infection, potentially influencing mosquito immune defense and metabolic adaptation. Given the well-documented role of CYP450 genes in insecticide resistance, their involvement in mosquito-Plasmodium interactions could have implications for malaria vector control strategies.

Conclusion: This study provides novel insights into the metabolic responses of An. aquasalis to P. vivax infection, supporting the need for further investigations to clarify the regulatory networks governing CYP450 gene expression and their broader impact on mosquito physiology and malaria transmission.

Keywords: cytochrome P450, differential expression, malaria transmission

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Rafael Miranda de Souza[1,2]

[1]Doutor (PPGBIO-Interação) Instituto Leônidas e Maria Deane – ILMD Fiocruz Amazônia, Manaus, Amazonas, Brasil

[2]Docente do Centro Universitario do Norte- UNINORTE, Manaus, Amazonas, Brasil

Correspondence: Rafael Miranda de Souza, Doutor (PPGBIO-Interação) Instituto Leônidas e Maria Deane – ILMD Fiocruz Amazônia, Manaus, Amazonas, Brasil, Tel +55-31-988838228

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Introduction

Hematophagy is a nutrient acquisition strategy found in various organisms, with many hematophagous species belonging to the insect class. Due to their blood-feeding behavior on hosts, numerous species have become important vectors of pathogens. The hematophagous process in insects is associated with several significant diseases that affect humans and domestic animals.[1]

One of the common physiological events in the life of these blood-feeding insects is the hydrolysis of host hemoglobin in the digestive tract, leading to the release of heme, a well-known pro-oxidant molecule. Heme plays a crucial role in several essential biological processes. However, free heme is a potent pro-oxidant, promoting the formation of reactive oxygen species (ROS), which can damage a variety of biological molecules.[2]

Oxidative stress is defined as an imbalance between the presence of reactive species, including ROS and others such as reactive nitrogen species (RNS), and the organism's ability to neutralize them through its antioxidant system. These reactive species share a common characteristic: an unpaired electron in their orbital, which results in increased instability and reactivity. Due to this property, they are referred to as free radicals.[3,4]

The degradation of hemoglobin results in the release of four heme molecules, which serve as the prosthetic group of this protein. Although the production of hydroxyl radicals through a Fenton-type reaction (Figure 1) has been reported for free heme, evidence suggests that lipid peroxidation is enhanced by free heme, thereby increasing oxidative stress. Heme facilitates the conversion of hydroperoxides (ROOH), which are species with low reactivity, into highly reactive molecules, such as alkoxyl (RO•) and peroxyl (ROO•) radicals.

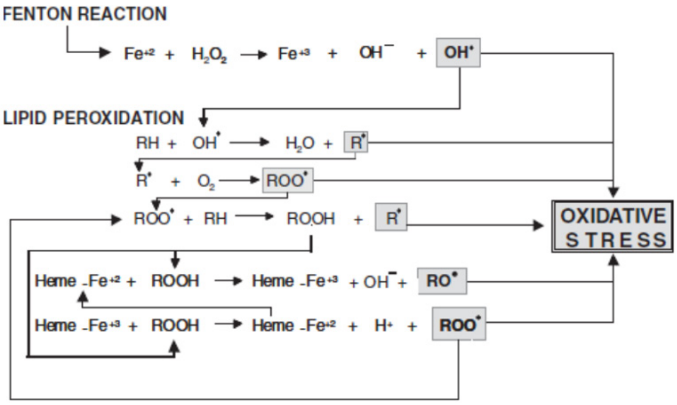

Figure 1 Schematic representation of the heme toxicity mechanism.

Insects have evolved antioxidant systems to counteract oxidative stress induced by free heme. The most important protective mechanisms include heme aggregation, production of antioxidant enzymes, synthesis of heme-binding proteins, generation of low-molecular-weight antioxidant molecules, enzymatic degradation of heme, and heme binding to the peritrophic matrix.[1,5,6]

Among the multiple enzyme species involved in detoxification are cytochrome P450 monooxygenases (P450s). P450s are remarkable due to their multiple clades and diverse functions. With the increasing

number and diversity of insect transcriptomes and genomes being reported, the diversity of insect P450 genes can be better identified. These enzymes are also associated with insecticide resistance in various insect species.[7]

Maintaining a balance between ROS generation and detoxification reactions is essential for homeostasis, highlighting the critical role of detoxification enzymes such as the P450 protein family. The aim of this study is to investigate, through in silico analysis, the CYP450 genes involved in the infection of *Anopheles aquasalis* by *Plasmodium vivax*.

Material and methods

To identify P450 genes in *Anopheles aquasalis*, the expression profile of *Anopheles aquasalis* was analyzed using high-throughput transcriptome sequencing in response to *Plasmodium vivax* invasion of the midgut, based on data from the NCBI (BioProject Accession: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE124997). To validate the transcriptome analysis, differentially expressed genes were analyzed between three groups of mosquitoes: *P. vivax*-blood-fed group (Pv), non-infective group (Bl; blood-fed using *P. vivax*-blood in which the gametocytes were inactivated), and unfed group (Unf; unfed mosquitoes). The lists of differentially expressed genes were obtained from the supplementary material of Santana et al.,[8] and filtered to retain only sequences identified as CYP450.

Differential expression (DE) analysis was performed using the GLM test in the edgeR v.3.16.5 package in R. Pairwise comparisons were conducted between the different experimental groups. A transcript was considered differentially expressed if it's adjusted P-value, after controlling for the false discovery rate (Benjamini–Hochberg adjustment), was less than 0.05 and if the log fold change (logFC) was higher than 1.

To visualize gene expression patterns, hierarchical clustering was performed, and a Heatmap was generated using the gplots v.3.0.1 package in R. Additionally, a Volcano Plot was constructed to illustrate the distribution of differentially expressed genes based on statistical significance and fold-change values. These analyses were conducted for the following comparisons: (i) *P. vivax*-blood-fed group (Pv) vs. unfed group (Unf) and (ii) unfed group (Unf) vs. non-infective group (Bl; blood-fed using *P. vivax*-blood in which gametocytes were inactivated).

Results

Differential gene expression analysis

The differential expression analysis was conducted using the edgeR package, applying the Trimmed Mean of M-values (TMM) normalization method. The comparisons were made between: (i) *P. vivax*-blood-fed group (Pv) vs. unfed group (Unf), and (ii) unfed group (Unf) vs. non-infective group (Bl; blood-fed using *P. vivax* blood in which gametocytes were inactivated). Genes were considered differentially expressed (DE) when the false discovery rate (FDR) was below 0.05 and the log fold change (logFC) was greater than 1 (up-regulated) or lower than -1 (down-regulated).

Gene expression patterns in P. vivax-infected mosquitoes

In the comparison between the *P. vivax*-blood-fed (Pv) and unfed (Unf) groups, a total of 88 features were analyzed. Of these, 14 genes were differentially expressed (FDR < 0.05), with 4 up-regulated and 10 down-regulated genes.

To visualize the expression patterns, a heatmap was generated, showing hierarchical clustering of differentially expressed genes across samples (Figure 2).

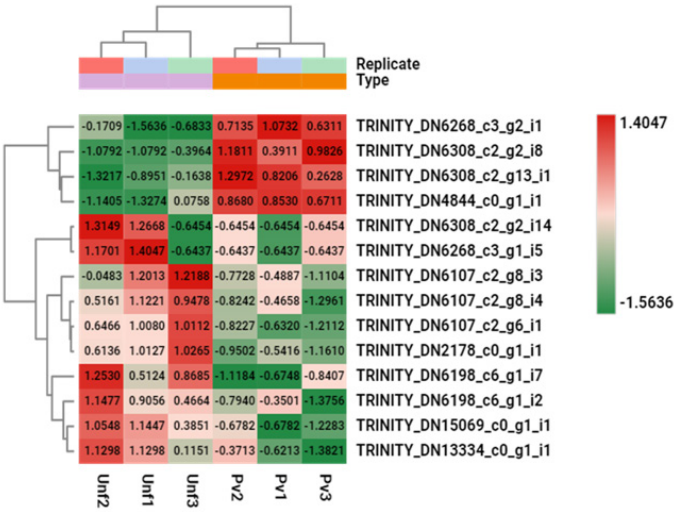

Figure 2 Heatmap of differentially expressed genes between *P. vivax*-blood-fed and unfed groups.

Additionally, a Volcano Plot was constructed to illustrate the distribution of differentially expressed genes, highlighting those that were significantly up- and down-regulated (Figure 3).

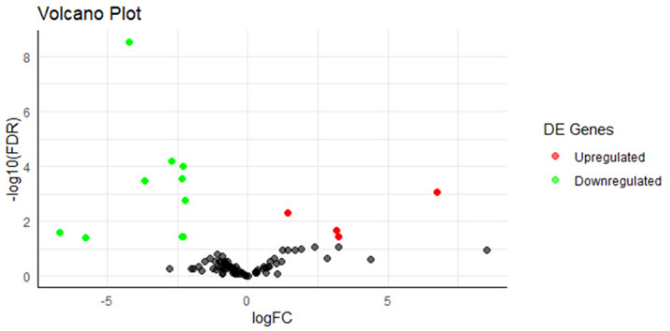

Figure 3 Volcano plot of differentially expressed genes between *P. vivax*-blood-fed and unfed groups.

Gene expression changes in response to inactivated P. vivax blood meal

In the comparison between the unfed (Unf) and non-infective (Bl) groups, 88 features were analyzed, and 6 genes were found to be differentially expressed (FDR < 0.05). Among them, 4 were up-regulated and 2 were down-regulated. A heatmap depicting the expression profile of these genes is presented in Figure 4.

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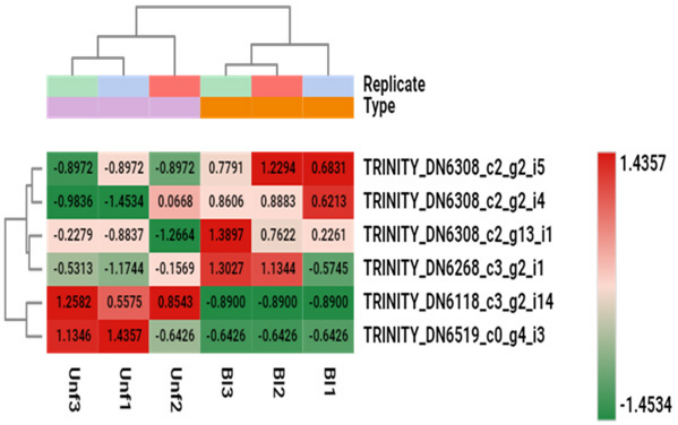

Figure 4 Heatmap of differentially expressed genes between unfed and non-infective groups.

The Volcano Plot, highlighting significant changes in gene expression, is shown in Figure 5.

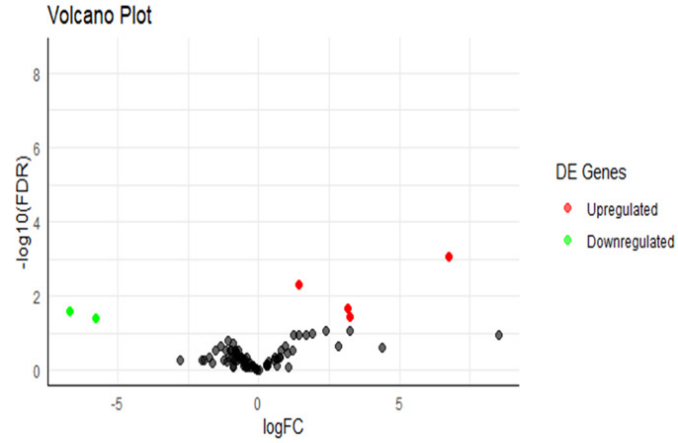

Figure 5 Volcano plot of differentially expressed genes between unfed and non-infective groups.

Discussion

The gene expression results between groups demonstrated the overexpression of the following transcripts: *P. vivax*-Blood-Fed and Unfed Groups (TRINITY_DN4844_c0_g1_i1; TRINITY_DN6268_c3_g2_i1; TRINITY_DN6308_c2_g2_i8; TRINITY_DN6308_c2_g13_i1) and Unfed and Non-Infective Groups (TRINITY_DN6268_c3_g2_i1; TRINITY_DN6308_c2_g2_i4; TRINITY_DN6308_c2_g2_i5; TRINITY_DN6308_c2_g13_i1).

The overexpression of CYP450 genes in mosquitoes may play a crucial role in the detoxification and neutralization of metabolites and toxins produced by *Plasmodium* parasites, such as reactive oxygen species (ROS). These parasites may produce various metabolites that interfere with mosquito physiology. CYP450 enzymes in mosquitoes could facilitate the metabolism or modification of these parasite-derived molecules, rendering them less harmful or promoting their excretion. By enhancing the detoxification capacity of mosquitoes, the overexpression of CYP450 genes could potentially mitigate the negative impact of *Plasmodium* toxins on mosquito survival, reproductive fitness, and susceptibility to malaria infection.

Assessments of CYP expression levels in response to infection have also been observed in other models. Cornman et al.,[9] analyzed the transcriptomic profile of candidate genes involved in the immune response of honeybee larvae infected with *Paenibacillus larvae* (the causative agent of American foulbrood disease) 72 hours post-infection. Their data revealed a set of genes potentially involved in the response to infection, including hymenopteran-specific protein tyrosine kinase genes, a hymenopteran-specific serine endopeptidase, and a cytochrome P450 (CYP9Q1). Interestingly, the identified cytochrome P450 genes belong to the CYP E class, a common class of oxidative enzymes in animals that play various roles, including toxin metabolism and pesticide detoxification.

A possible relationship between detoxification responses and infection is suggested by a study in which *Anopheles gambiae* (*An. gambiae*) mosquitoes were infected with the rodent parasite *Plasmodium berghei* (*P. berghei*), and the expression of detoxification enzymes was measured at different time points. Several alterations were observed during ookinete invasion of the midgut and the subsequent release of sporozoites into the hemocoel, most notably the overexpression of CYP6M2 in both the mosquito midgut and fat body.[10]

The findings of Félix et al.,[10] are consistent with those of Baton et al.[11] The authors conducted a transcriptomic analysis using genome-wide microarrays to examine An. gambiae hemocyte responses to bacterial and *P. berghei* infections. Their findings revealed significantly upregulated genes during sporozoite passage through the hemolymph, including genes implicated in phagocytosis and redox metabolism (a cytochrome P450 and a glutathione S-transferase). In contrast, hemocytes from *P. berghei*-infected mosquitoes exhibited downregulation of genes related to redox stress and oxidoreduction 24 hours post-infection, coinciding with the blood digestion phase and ookinete invasion.

Alonso et al.,[12] conducted a genome-wide association study (GWAS) to identify potential SNPs involved in the modulation of *Anopheles darlingi* responses to *Plasmodium vivax* and *Plasmodium falciparum* in samples collected from the Brazilian Amazon. Their results demonstrated a strong association between *An. darlingi* SNPs and CYP450. When evaluating potential similarities among these SNPs, a gene with 73% similarity to CYP6Z2 in *An. gambiae* was identified through a BLASTp search. Moreover, this association was observed exclusively in samples infected with *P. vivax*, suggesting that this metabolically significant gene family—CYP450—is likely related to Plasmodium infection in the Brazilian Amazon.

Although the precise role of CYPs in infection modulation remains unclear, some insights can be inferred from studies on other detoxification enzymes. A study that highlights the role of detoxification enzymes in infection response was conducted by Molina-Cruz et al.[13] Their research on *An. gambiae* mosquitoes infected with *P. berghei* demonstrated increased expression levels of detoxification enzymes, as well as catalase suppression, 24 hours post-infection. The authors suggested that reducing catalase expression during a period of high metabolic activity would elevate intracellular H_2O_2 levels, which are toxic to the parasite. To test this hypothesis, catalase silencing was performed, resulting in reduced infectivity in the mosquito midgut, potentially due to ookinete lysis.

Oliveira et al.,[14] corroborated the role of catalases in ookinete lysis. The invasion of midgut epithelial cells by ookinetes triggers a nitration response through a two-step process, in which nitric oxide synthase (NOS) induction is followed by a peroxidase-mediated reaction involving heme peroxidase 2 (HPX2) and Nox5 as an H_2O_2 source. This epithelial nitration process functions similarly to opsonization, promoting the activation of the mosquito complement cascade.

The external insertion of detoxification enzymes also aids in establishing certain infections, as demonstrated in the following model. MacLeod et al.,[15] evaluated the development of *Trypanosoma brucei brucei* in tsetse flies after feeding them antioxidants (glutathione, cysteine, N-acetylcysteine, ascorbic acid, and uric acid). It was observed that adding glutathione at specific concentrations significantly increased midgut infection rates from 15% to 44% and 92%, compared to the control. This suggests that trypanosome cell death in the midgut is normally induced by ROS.

Kumar et al.,[16] had previously demonstrated a similar process. The authors used a *Plasmodium*-refractory *An. gambiae* strain that exhibited chronic oxidative stress, characterized by elevated H_2O_2 and superoxide levels in the hemolymph. Parasite survival in the mosquito midgut increased after ingestion of antioxidants—ascorbate and uric acid—in the refractory strain, and these compounds blocked the melanotic encapsulation of ookinetes.

As cited above, antioxidants contribute to infection development; however, their modulation concerning CYP450 remains unclear. Certain mechanisms may induce the overexpression of these enzymes, such as blood digestion and ookinete invasion, which cause increased ROS production and other reactive species, consequently upregulating detoxification enzymes. In the midgut, the release of oocysts leads to cytoskeletal rearrangement, which likely also regulates CYP expression.

In contrast to other antioxidants, CYP overexpression occurs 24 hours post-feeding/infection. Other models suggest that a reduction in antioxidants, such as catalase, leads to increased ROS levels, reducing bacterial competitiveness and thereby facilitating *P. vivax* development inside *An. aquasalis* by inhibiting immune pathways to enhance its survival within the vector.[17]

Currently, insect P450 genes are grouped into four major phylogenetic clades: CYP2, CYP3, CYP4, and the mitochondrial P450 clade. The CYP2 family is evolutionarily conserved and plays a role in insect sensory organ development and ecdysone synthesis. The CYP3 clade is divided into two relatively conserved families, CYP6s and CYP9s, which are involved in insecticide resistance and xenobiotic metabolism, some of which can be induced. The CYP4 genes encode constitutive isoenzymes that metabolize pheromones and xenobiotics and are involved in odor production. CYP3 and CYP4 genes are present in large numbers in insects, enabling them to cope with multiple environmental stresses.[7,18]

The data showed that the most overexpressed gene was TRINITY_DN6308_c2_g2_i8, designated as Cytochrome P450 4g15, based on the reference table from which the *in-silico* data for this study were extracted.[8]

The CYP4G subfamily is also remarkable because genes of this subfamily are very highly expressed. The function of the CYP4G genes was first elucidated by Qiu et al.,[19] who showed that survivors of CYP4G1 RNAi are deficient in cuticular hydrocarbons (CHC), highly sensitive to desiccation stress, and impaired in their pheromone mediated courtship behavior. The role of CYP4G enzymes in the synthesis of alkanes and alkenes which serve as waterproofing agents on the insect epicuticle and in many pheromonal functions as well,[20,21] has been confirmed in subsequent studies. Balabanidou et al.,[22] showed that both *Anopheles gambiae* CYP4G genes are highly expressed in the oenocytes and that CYP4G16 is a functional oxidative decarbonylase. Adult oenocytes synthesize sex- and species-specific mixes of cuticular hydrocarbons from VLCFAs via a pathway requiring a cytochrome P450 aldehyde oxidative decarbonylase. Cuticular hydrocarbons are essential for adult desiccation resistance and pheromonal communication.[23,24]

Conclusion

The findings of this study provide valuable insights into the role of CYP450 genes in Anopheles aquasalis responses to *Plasmodium vivax* infection. The differential expression analysis revealed a significant upregulation of CYP450 genes, particularly within the CYP4G subfamily, suggesting their involvement in detoxification, oxidative stress management, and immune defense.

The overexpression of these enzymes in infected mosquitoes supports the hypothesis that CYP450-mediated pathways play a crucial role in mitigating the toxic effects of ROS generated during blood digestion and parasite invasion. Additionally, the observed gene expression patterns indicate potential interactions between detoxification mechanisms and pathogen susceptibility, shedding light on how mosquitoes adapt to infection while maintaining physiological homeostasis.

Given the well-established role of CYP450 enzymes in insecticide resistance, these findings raise important considerations for malaria vector control strategies. Understanding the dual function of these genes in both detoxification and immune response modulation may offer new perspectives for developing targeted interventions to disrupt mosquito-Plasmodium interactions.

Future research should further explore the regulatory networks governing CYP450 expression in response to Plasmodium infection, as well as their broader implications for mosquito survival and malaria transmission. These findings contribute to the growing body of knowledge on vector-pathogen interactions and highlight the potential of metabolic pathways as targets for malaria control strategies.

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Conflicts of interest

The author declares there is no conflict of interest.

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