INTERNATIONAL TRENDS IN IMMUNITY VOL.4 NO.2 APRIL 2016
ISSN 2326-3121 (Print) ISSN 2326-313X (Online) http://www.researchpub.org/journal/iti/iti.html

Anopheles gambiae and *A. stephensi* Immune Response during *Plasmodium berghei* Infection Uncovered by Sialotranscriptomic Analysis

Couto J. [1] ***MSc*, Ferrolho J.**[1] ***DVM, MScVetSc, MScRes*, de la Fuente J.** [2,3] ***PhD* and Domingos A.**[1,4*]**, *BSc, PhD***

Abstract*— Malaria is caused by parasites of the genus *Plasmodium* that are transmitted through the bite of female mosquitoes of the genus *Anopheles*. Responsible for high mobility and mortality rates around the world, this protozoan disease is most common in the tropical and sub-tropical regions. Herein, using a pure transcriptomic data analysis approach on mosquito salivary glands we have identified, compiled and compared immune-related transcripts and their levels of expression in *A. gambiae* and *A. stephensi* after *P. berghei* infection. Focusing in immune mechanisms such as recognition of the parasite, signal modulation by serine protease cascades and effector mechanisms, several subclasses of proteins were investigated, including thioester-containing proteins, leucine-rich domain-containing proteins, C-type lectins, galactoside-binding lectins, clip-domain serine proteases, serine protease inhibitors, and antimicrobial peptides. The anti-vector vaccine potential of key-molecules that have exert an action in regulating parasite development have been considered thus, targeting highly conserved antigenic molecules can be effective to control arthropod-borne diseases, including malaria. This study constitutes the first comparative sialotranscriptomic analysis between these two mosquito vectors upon pathogen invasion, focusing solely specific subclasses of immune-related transcripts. Lastly, in order to search for new targets with potential to become pan-arthropod vaccines, we provide potential candidate genes with interest to be further investigated for malaria control

***Keywords* — Malaria, *Anopheles gambiae*, *Anopheles stephensi*, sialotranscriptome, RNA-seq, innate immune response.**

This paragraph of the first footnote will be completed by the Editor and will contain the date on which you submitted your paper for review.

[1]Instituto de Higiene e Medicina Tropical (IHMT), Rua da Junqueira 100, 1349-008 Lisboa, Portugal

[2]SaBio - Instituto de Investigación de Recursos Cinegéticos, IREC-CSIC-UCLM-JCCM, Ciudad Real, Spain

[3]Department of Veterinary Pathobiology, Center for Veterinary Health Sciences, Oklahoma State University, Stillwater, USA

[4]Global Health and Tropical Medicine - Instituto de Higiene e Medicina Tropical (GHMT-IHMT), Rua da Junqueira 100, 1349-008 Lisboa, Portugal

*Correspondence to Domingos A. (e-mail: adomingos@ihmt.unl.pt).

DOI: 10.18281/iti.2016.2.4

I. Introduction

Malaria is a protozoan disease caused by parasites of the genus *Plasmodium* mostly spread in the tropical and sub-tropical regions, transmitted through the bite of female mosquitoes from the genus *Anopheles* [1, 2]. At present, five species of the genus *Plasmodium* are known to cause malaria in humans, resulting in 198 million cases and 584000 deaths in 2013 [2]. In areas such as sub-Saharan Africa, the majority of the infections are caused by *P. falciparum*, responsible for cerebral malaria, the most malignant form of the disease [3, 4]. *P. vivax* and *P. ovale*, together with *P. falciparum*, are the most widespread species but *P. malariae* and *P. knowlesi* have also been associated with cases of malaria in Southeast Asia [5, 6].

The biological cycle of these protozoa is very complex, including several developmental stages that have to sequentially occur both in the invertebrate definitive host, the anopheline vector, and in the vertebrate intermediate host, humans or others [3]. During its life span, a female *Anopheles* mosquito feeds on an infected vertebrate host repeatedly for protein and ion intake to promote egg development [7]. During the blood meal, gametocytes are ingested and mobile zygotes, the ookinetes, penetrate the mosquito midgut further developing into oocysts [8]. The disruption of the oocysts origins up to thousands of new sporozoites that are released and spread to the haemocoel [9]. Subsequently, these parasitic stages migrate to the salivary glands (SGs) and, during a new blood meal, the parasite, in its infectious form, is transmitted along with the saliva to the vertebrate host [8, 10]. Through the bloodstream, sporozoites rapidly reach the liver, forming the schizonts, which rupture releasing merozoites into the blood stream [11]. This form is able to adhere to and infect red blood cells (RBC), developing into the trophozoite stage [11]. After dividing asexually into mature schizonts (erythrocytic schizogony), RBC lysis occurs and the merozoite form is released into the blood stream infecting other RBC, thus perpetuating the infection [12]. Some of the merozoites may develop into male and female gametocytes, the sexual erythrocytic stages [13].

The mosquito SGs play an important role in transmission of malaria due to the close contact and interaction with *Plasmodium* sporozoites. This interaction is likely to be

ISSN 2326-3121 (Print) ISSN 2326-313X (Online) http://www.researchpub.org/journal/iti/iti.html

receptor-mediated [14, 15] reinforcing the importance of the identification of the membrane proteins, the genes encoding these molecules and investigation of their effect on parasite transmission. New molecular advances in areas such as transcriptomics and proteomics, alongside with complete genome sequencing, and a robust method for interference RNA (RNAi) in adult *Anopheles* mosquitoes [16] have brought the possibility of investigate the role of genes and proteins that might be involved in pathogen survival and transmission mechanisms [17].

Invertebrates, as mosquitos, do not possess an adaptive immune system but, instead, a potent innate immune system [18]. Invading microorganisms must overcome several barriers that are initially physical, such as the midgut wall, and after the immune responses triggered by this epithelium, including those activated systemically by the hemocytes and the fat body culminating in the release of immune effectors into the haemolymph [19]. The few microorganisms that successfully evade these immune defenses are able to progress their developmental cycle and become infective, to be later transmitted to another host during a new blood meal. One such example is the interaction between the *Plasmodium* parasite and its invertebrate host. After the female mosquito blood meal, the ookinetes have to penetrate the midgut epithelium to develop into oocysts in the haemocoel [20]. Thus, successful parasite transmission is dependent on several factors at the molecular and cellular level and these interactions occur at different stages of the invertebrate host and the parasite life cycle. Sequencing of the complete *A. gambiae* genome has identified 242 genes potentially encoding components of the mosquito innate immune system [21] and recently, several specific mosquito gene products that function as antagonists or agonists of parasite development related to the immune response have been successfully identified [19, 22].

Comparative transcriptomics can expand our understanding on malaria vectors bionomics by revealing differences on the expression of genes depending of the *Plasmodium* infection. Mohien and colleagues (2013) developed an integrated approach for transcriptome assembly that facilitated an analysis of the midgut brush borders of the two malaria vectors *A. gambiae* and *A. albimanus* [23]. Another comparative analysis was performed correlating *Drosophila melanogaster* and *A. gambiae* transcriptomes revealing an overall strong and positive correlation of developmental expression between orthologous genes [24].

Since *Anopheles* immune response clearly determines its vectorial capacity, understanding how vector and parasite interact at different stages of parasite development is vital. Identification of the molecules that have an important role in regulating parasite development will inform and direct the design of new strategies to control and block malaria parasite progress in the mosquito. These elements have been targeted to evaluate their potential role as anti-vector vaccines, once they can be species-specific or conserved between species. Targeting highly conserved antigenic molecules can cause cross-reactions against several species [25]. Based on this, pan-arthropod vaccines can be useful to control arthropod-borne diseases, being for this reason a more amplified approach. In order to search for new targets with potential to become pan-arthropod vaccines, we have analyzed and compared the sialotranscriptome of *A. gambiae* and *A. stephensi* during *P. berghei* infection focusing solely in the transcripts that participate in the immune response. A refined shortlist of potential candidate genes was further examined to guide for new approaches to malaria control.

II. Materials and Methods

Sialotranscriptome analysis of *A. gambiae* and *A. stephensi* were carried out as described by Pinheiro-Silva et al. (2015) [26] and Couto et al. (2015) [27]. RNA sequencing (RNA-seq) data can be found in the database: ArrayExpress (www.ebi.ac.uk/arrayexpress) with the Accession no. E-MTAB-3415 and E-MTAB-3964, for *A. gambiae* and *A. stephensi*, respectively.

Both groups of RNA-seq data were analysed and organized into subclasses according to their functions in the immune response and transcripts that are known to have a crucial impact in the innate immune response of *Anopheles* mosquitoes were selected. Seven subclasses were then selected for further analysis and comparison: thioester-containing proteins (TEPs), leucine-rich immune proteins (LRRs), C-type lysozymes or lectins (CTLs), galectins (GALEs), Clip-domain serine proteases (CLIPs), serine protease inhibitor - serpins (SRPNs) and antimicrobial peptides (AMPs).

III. Results

Effector molecules of the mosquito innate immune system that are involved in the elimination of *Plasmodium* parasites are potential targets for disease control [28], and their function can be determined by transcriptomic and RNAi-based studies [29]. Immune reactions start when microorganisms are detected and recognized by the host germline-encoded pattern recognition receptors (PRRs) that can be cell-bound or circulating in the haemolymph [30]. PRRs include several classes of molecules [31], and from these we have focused on the TEPs, LRRs, C-type lectins CTLs and GALEs, whose transcripts that correspond to the genes encoding these proteins were found in the sialotranscriptomes. Our selection was based on the importance of each of these molecules in the mosquito innate immune response and their role during pathogen infection. Furthermore, for the same reason key components of the serine protease cascades that act in signal modulation, such as CLIPs and SRPNs, and those recognized as AMPs, were also selected for comparison and analysis.

During the *A. gambiae* and *A. stephensi* transcriptomic analysis, a total of 154 and 79 transcripts belonging to the immunity class were found differentially expressed, respectively (Fig. 1-A). Our results have shown that both anophelines had more transcripts upregulated (63.6% in *A. gambiae* and 77.2% in *A. stephensi*) than downregulated (36.4% in *A. gambiae* and 22.8% in *A. stephensi*) (Fig. 1-B). However, within the immunity class only 104 and 39 of these transcripts of *A. gambiae* and *A. stephensi* belonged to the aforementioned selected subclasses (Fig. 1-B and 1-C). Levels

ISSN 2326-3121 (Print) ISSN 2326-313X (Online) http://www.researchpub.org/journal/iti/iti.html

of expression for each of the transcripts can be observed in Appendix I - Table 1.

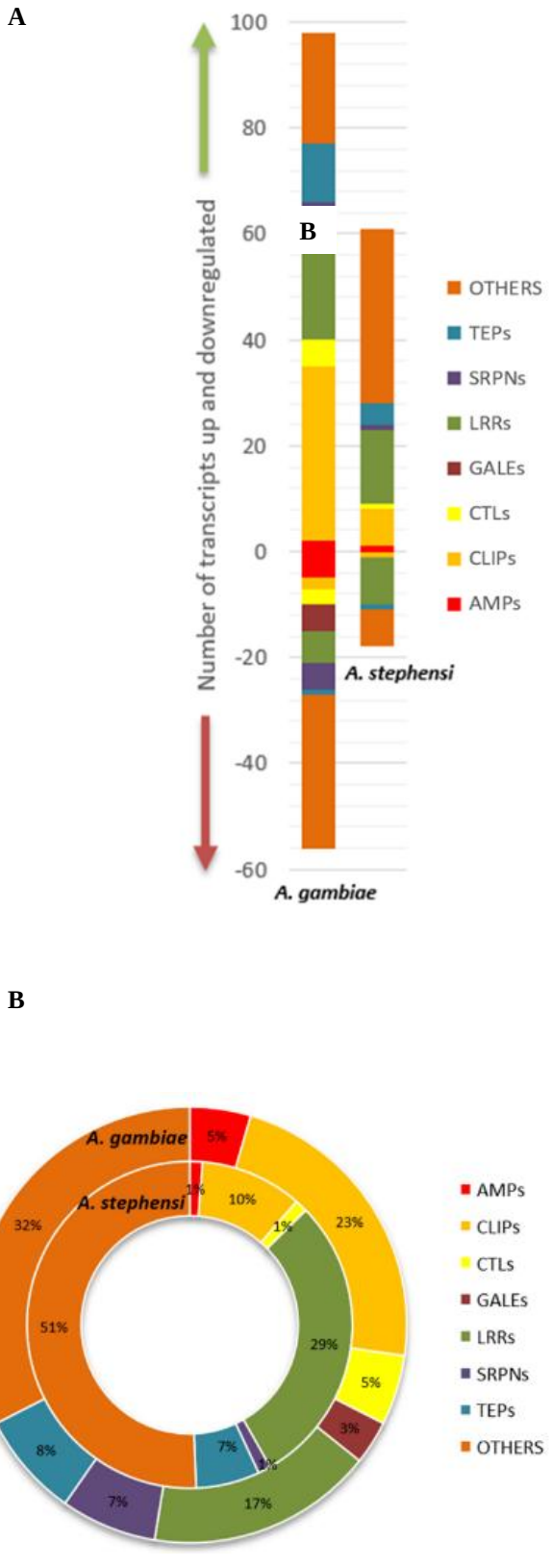

Fig. 1. Global comparative sialotranscriptome profiles of *Anopheles gambiae* and *Anopheles stephensi* during *Plasmodium berghei* infection. **(A)** Distribution of differentially expressed transcripts per functional class. Green and red arrows indicate genes up and downregulated, respectively, of each functional class. **(B)** Functional classification and comparison of *A. gambiae* and *A. stephensi* transcripts. Each area represents the percentage (%) of transcripts within each functional class per mosquito species. **(C)** Number of transcripts significantly differentially expressed for *A. gambiae* and *A. stephensi*. The area of intersection corresponds to sequences with ⩾80% of homology between these two species. Green and red arrows indicate genes up and downregulated, respectively, of each functional class. **TEPs** - Thioester-containing proteins; **SRPNs** - Serpins; **LRRs** - Leucine-rich domain-containing proteins; **GALEs** - Galactoside-binding lectins; **CTLs** - **C**-type lectins; **CLIPs** - Clip-domain serine proteases.

A. Pattern recognition receptors

When triggered by the pathogen, PRRs recognize the invader and can induce direct anti-pathogen defense mediating microbial destruction by encapsulation or phagocytosis, or indirect triggering of intracellular signaling pathways leading to gene activation [32, 33]. Contrasting with vertebrates, the invertebrates' innate immune system seems to be deficient in a memory and adaptive response, which is thought to be compensated by specific activation of anti-pathogen immune response pathways by class-specific PPRs [32]. To date, only approximately 150 predicted PPRs genes have been found in the *A. gambiae* genome [21]; but interestingly the wide variety of these receptors gives the insects the capacity and plasticity of recognizing a number of different of pathogens originated by V(D)J recombination, as well as somatic hyper-mutation of the antibody immunoglobulin (Ig) domains [32]. One such example is the response of *A. gambiae* mosquitoes against the challenge with *P. berghei* or *P. falciparum* with the splice variants of the *A. gambiae* Down syndrome cell adhesion molecule that is coded by *AgDscam* gene [32]. Our data analysis regarding the *A. gambiae* sialotranscriptome alone has revealed 51 transcripts differentially expressed that encode PRR proteins, suggesting an intense immune response by this mosquito species. From these, 26 were genes encoding LRRs, 12 related to TEPs functions, 8 and 5 were representative of CTLs and GALEs (Fig. 1-A). In the *A. stephensi*

sialotranscriptome 29 transcripts were found, and this lower number might be due to its weaker immune response, as already described [34]. From these, 23 transcripts were related to LRRs, 5 were genes encoding TEPs, and only one was included in CTLs subclass (Fig. 1-A). Each group of proteins will be characterized in detail below.

A.1. Thioester-containing proteins

Leucine-rich repeat domain-containing proteins are a family of proteins of the mosquito immune system with an important role against pathogens, including *Plasmodium* parasites [29]. Two putative PRRs of this family, leucine-rich immune protein 1 (LRIM1) and *Anopheles-Plasmodium*-responsive leucine-rich repeat 1 (APL1C), present in the mosquito haemolymph in a form of a complex were found to be necessary for *P. berghei* destruction during midgut invasion of susceptible *A. gambiae*. After gene knockdown, a parasite infection intensity increase was observed [35]. Dimopoulos and colleagues (2002) have found that upon *P. berghei* infection LRIM1 gene was strongly upregulated [36]. In a different study, it was found that LRIM1 plays a role as an antagonist of ookinetes development *P. berghei* in *A. gambiae* midgut. The absence of LRIM1 induced approximately a fourfold increase in the number of parasites in susceptible mosquitoes [19]. In this study, we have found that the family of LRRs was one of the most represented subclass within this immunity subclass in both mosquito species, with 16.8% and 29.1% of transcripts found in *A. gambiae* and *A. stephensi*, respectively (Fig. 1-B). Also, it was observed that in *A. gambiae* and *A. stephensi* the genes encoding these proteins were mostly upregulated, corresponding to 76.9% and 60.9% of transcripts from the LRRs subclass (Fig. 1-B and Appendix I - Table 1). Our findings reinforce that LRRs are present and expressed to antagonize the malaria parasite infection as part of a complement-like system [37]. RNA-seq results are in line with previous findings regarding the silencing of LRIM1 during a medium-level infection of *P. falciparum* in *A. gambiae* mosquitoes, which resulted in a 3.1-fold increase of infection [38]. The overall results suggest a crucial role of these proteins in the intensity of infection.

A.2. C-type lectin-like and galactoside-binding lectin proteins

Dependent of Ca^{2+} C-type lectins like (CTLs) can recognize and bind to a pathogen through the C-terminal carbohydrate recognition domain [29]. This family of extracellular or membrane-bound proteins, act as regulators of the mosquito immune response focused in pathogen recognition [29]. Osta and colleagues (2004) identified via gene silencing two important CTLs, CTL4 and CTLMA2, as genes whose products protect *P. berghei* ookinetes against an *A. gambiae* innate immune response, namely melanization [20]. This process is part of the humoral immune response of insects, that takes place in the extracellular space between the midgut epithelial cells and the basal lamina, and one of its purposes is the sequestration of invading microorganisms in a dense melanin coat [20]. These vector proteins seem to have been somehow modified to protect the parasite, once their functional silencing in a susceptible (G3) strain lead to the melanization of the parasites. The knockout of CTL4 caused practically a complete melanization of the developing ookinetes (97%) on the basal site of the midgut epithelium, whereas the knockout of CTLMA2 led to a partial melanization (48%). Nonetheless, the total number of *Plasmodium* parasites that had penetrated the epithelium was not significantly affected [20]. This partial melanization can be justified by a possible inactivation of LRIM1 that might defend the *Plasmodium* parasite from the melanization response, leading to parasite development [39]. Our analysis has shown that the genes encoding CTLs were mostly upregulated in both mosquito species after *P. berghei* infection (Fig. 1-A and Appendix I - Table 1); although, more transcripts that were found were expressed in *A. gambiae* (8 transcripts) in comparison with *A. stephensi* (1 transcript) (Fig. 1-A).

As members of the lectins family, GALEs have a C-terminal carbohydrate recognition domain [40]. By recognizing specific glycoconjugates, it is now suggested that they might act in pathogen recognition or in phagocytosis [41], although the knowledge regarding their precise role and abundance in insects is still limited [42]. A novel putative secreted GALE has been identified from the SGs transcriptome and proteome of *A. stephensi*, showing an 81% identity at the amino acid level with a protein of similar size from *A. gambiae* (agCP6926) [43]. A putative infection-responsive GALE (IGALE20) was found to be expressed in the midgut of *A. gambiae* after *P. berghei* challenge, revealing their important role in insect innate immunity during midgut invasion [44]. After a deep analysis of both sialotranscriptome catalogues, we were able to verify that genes encoding for GALEs (5 transcripts) were only present in *A. gambiae* (Fig. 1-A) and, interestingly, these genes were found downregulated (Fig. 1-A and Appendix I - Table 1). This decrease in the expression level can be explained by the ability of some parasites to alter the capacity of *A. gambiae* GALEs to recognize them, enabling the attachment and invasion by *Plasmodium* [40]. On the other hand, an absent differential expression of GALE genes in the SG of *A. stephensi* during *P. berghei* infection does not invalidate the presence of these genes in this vector [42].

B. Signal modulation

The recognition of invading microorganisms activates proteolytic cascades of serine proteases to trigger effector responses, for example, the synthesis of AMPs to eliminate the attacker [45, 46]. Serine proteases belong to a wide family of endopeptidases which have a residue of serine that is catalytically active. Some of these proteins have a particular domain between 30 to 60 amino acids that can be identified by their N-terminal, and due to their configuration, these are named as CLIPs [47]. Phylogenetic studies have showed that *Anopheles* and *Drosophila* genomes present four sub-families of CLIPs (CLIPs A, B, C and D) [21], that can be divided in two categories, positive regulators and negative regulators [48]. During the serine protease cascade, the signal amplification is regulated by SRPNs, serine protease inhibitors. SRPNs inhibit serine proteases through an irreversible covalent bind to the center of these enzymes [19]. In terms of the immune response, serine proteases play an essential role through melanization. For this process to occur, the proteolytic activation of inactive

prophenoloxidases (PPOs) zymogens to active phenoloxidases (POs) is necessary and closely regulated through a balance between positive and negative regulators, CLIPs, and their inhibitors, SRPNs [19]. In our sialotranscriptomic results, *A. gambiae* expressed more transcripts related to serine proteases (46 transcripts) than *A. stephensi* (9 transcripts), which represents 29.9 and 11.4% of differential expressed genes within the immunity class, respectively (Fig. 1-B). From this 46 *A. gambiae* transcripts, 35 were related to CLIPs functions and 11 encoded for SRPNs. In the *A. stephensi* mosquito, 8 and 1 transcripts corresponded to CLIPs and SRPNs, respectively, included in serine proteases category.

B.1. CLIP-domain serine proteases

The melanotic encapsulation of parasites can be the cause of mosquito refractoriness against *Plasmodium*. In *Aedes aegypti*, CLIPA14 and CLIPB6 are homologous of the PPO activation cofactors, enriched by different transcriptional factors such as REL2 and REL1, respectively [49]. Interestingly, these two CLIPs can be found both upregulated in susceptible strains of *A. gambiae* [50]. Christophides and colleagues (2002) have found that *A. gambiae* CLIPB14 was persistently upregulated when the midgut invasion by *Plasmodium* ookinetes occurs [21]. Also, that these infections led to a transient induction of CLIPB15. A different study has characterized molecular and functionally *A. gambiae* CLIPB14 and CLIPB15 expressed in the hemocytes [51], demonstrating that, after gene knockdown, these two CLIPs are involved in parasite destruction. In susceptible and refractory strains of mosquitoes it was observed that the number of developing oocysts and melanized ookinetes had a significant increase in comparison with the controls and with the individual CLIP knockdowns [51]. Nevertheless, when silencing CLIPB14 or CLIPB15 in refractory mosquitoes, ookinete melanization was not prevented [51]. These results suggest that CLIPB14 and CLIP15 play a role as negative regulators of ookinete melanization. The same negative feedback of suppressing melanization process is also mediated by CLIPA2, 5 and 7, allowing the development of malaria parasites in *A. gambiae* mosquitoes [52]. On the other hand, different CLIPs, such as CLIPB17 and CLIPA8, have been found to be positive regulators of mosquito melanization. In *A. gambiae*, the depletion of CLIPB17 and CLIPA8 led to a higher or absolute elimination of melanization, respectively [48]. When analyzing the *A. gambiae* sialotranscriptome alone, it was observed that the majority of CLIPs found and that were differentially expressed were included within the sub-families A and B. CLIPA2, 5 and 7 serine proteases associated with the melanization suppression [52] were found upregulated in our results, suggesting a similar mediations process to the one that occurs in the mosquito midgut. The gene encoding CLIPA8 was found upregulated, probably because is acting in the stimulation of melanization process. Although our targets were the SGs, the result is in agreement with previous findings reported for the midgut in that CLIPA8 activates the PO cascade and its knockdown blocks melanization, promoting the oocysts development [48, 52]. Similarly, CLIPB14 and CLIPB15 were also found upregulated in our analysis. Studies conducted in the midgut reported that CLIPB14 is only found enriched 28 hours *post*-infection, particularly during midgut invasion; whilst CLIPB15 is continuously upregulated having a mild increase during *Plasmodium* infection [21]. When analyzing the *A. stephensi* sialotranscriptome, we have found that the sub-families A and B were less represented within the CLIPs subclass. Only the three transcripts encoding for CLIPA4, CLIPA14 and CLIPB6 were found and were related to these two sub-families. Interestingly, CLIPA14 and CLIPB6 were exclusively found in this mosquito data and they have been implicated in the PPO activation [49]. Finally, we have found that TEPs, LRIMs and CLIPs transcripts in both mosquitoes SGs infected by *P. berghei* were predominantly upregulated (Fig. 1-A and Appendix I - Table 1). It has been found that during midgut pathogen invasion, these proteins form the complex LRIM1/APL1C/TEP to interact with the ookinetes, being therefore, as in our results, their genes also upregulated [46]. The same was verified previously with APL and LRIM in *A. funestus* [53]. Moreover, previous studies indicate that in the midgut, silencing of a determined TEP leads to the inhibition of CLIP cleavage activity. This confirms that the accumulation of TEP on malaria parasites and bacteria is regulated by CLIPs, which leads to distinct defense reactions, including lysis and melanization of the pathogen [54].

B.2. Serpins

The haemolymph of insects is rich in serine protease inhibitors. As an important class of negative regulators, SRPNs belong to this large family of proteases with an intra or extra-cellular conserved structure [31]. In insects, SRPNs can be produced in response to physiological or pathological stimulus and regulate immune protease pathways that prevent damaging effects of uncontrolled immune responses [21, 54, 55]. They are substrate-specific and their inhibitory function is mediated by a reactive center loop (RCL) [31]. The first inhibitory SRPN has been reported and designated as SRPN2, and its presence was found required for successful *P. berghei* oocyst development in *A. gambiae*. The knockdown of SRPN2 affected the invasion of *A. gambiae* midgut by the parasite through oocyst number reduction, due to increased ookinete lysis and melanization [31]. The serpin SRPN6 has been studied in *A. stephensi* and *A. gambiae* after infection with *P. berghei*, revealing that its gene expression is strongly induced in both mosquitos' midguts during ookinete invasion. This SRPN has been associated with the outcome of infection in *A. stephensi* in that its gene knockdown increased the number of oocysts; whereas the knockdown of *A. gambiae* SRPN6 had no effect on the oocyst load or prevalence of infection in the midgut [9]. A different study has suggested that the transcripts encoding four intracellular serine protease inhibitors of SRPN10 isoforms (KRAL, RCM, FCM and CAM) in *A. gambiae* females are induced as a response to midgut invasion by *P. berghei* ookinetes and might regulate the apoptosis of invaded cells that leads to epithelial damage [56, 57]. The upregulation of each isoform, under different physiological conditions, suggests that alternative splicing has a fundamental role for the regulation of serpins' function [56]. Our data analysis has shown that the number of transcripts encoding for SRPNs was higher for *A. gambiae* (11 transcripts) in comparison with *A. stephensi* (1 transcript) (Fig. 1-B), of which the genes differentially expressed included SRPN2, SRPN10

and SRPN6. Levels of expression for each of the transcripts can be observed in Appendix I - Table 1. In *A. gambiae*, SRPN2 was upregulated, while SRPN10 was downregulated (Appendix I - Table 1). The transcript corresponding to the protein SRPN6 was not found in this mosquito. In the *A. stephensi* sialotranscriptome, only SRPN6 was identified and was upregulated (Appendix I - Table 1).

C. Effector mechanisms

Mosquito immunity includes several effector mechanisms, such as AMPs, melanization, phagocytosis and cellular immune response [46]. In this study the main focus will be on the AMPs because after pathogen invasion, the synthesis of small mostly positively charged AMPs is the last step of the mosquito inducible humoral response by different pathways [19]. In our sialotranscriptome analysis most of them were found in *A. gambiae* (7 transcripts) in comparison with *A. stephensi* (1 transcript) (Fig. 1-B).

C.1. Antimicrobial peptides

After microorganism recognition by PRRs, AMPs are quickly synthesized by the fatty body, hemocytes and epithelia, and secreted into the haemolymph where they accumulate in high concentrations [58-60]. In *A. gambiae* they are also synthesized locally by the barrier epithelia [36, 61, 62]. The *A. gambiae* full genome sequencing has revealed genes that encode for several of these proteins, including defensins (DEFs), cecropins (CECs), attacins and gambicin (GAM) [21]. As reported by Luna and collegues (2006), the expression of some AMPs can be influenced by *Toll* and *Imd* pathways [63]. After silencing components of the *Imd* pathway, such as the NF-κB transcription factor REL2, the expression of DEF1 and a GAM1 was reduced in *A. gambiae* cell lines. Besides, the over expression of either NF-κB transcription factor REL1 or REL2 induced the expression of CEC1, GAM1 and DEF1, implicating them in the expression of AMPs. In contrast, in the *A. stephensi* cell line MSQ43 the transcription factors were downregulated [63]. In other studies, AMPs such as CEC1, CEC3, GAM1, are REL2-influenced in *A. gambiae* cells lines [64, 65]. Kim and colleagues (2004) have shown *in vivo* that CEC1 transgene expression in transgenic *A. gambiae* reduced the number of *P. berghei* oocysts in the midgut [66]. Other authors demonstrated that CEC1 was found upregulated in mosquitoes infected by *Plasmodium* sp. [21], and that CEC2 is significantly enriched in the transcriptome of midgut, especially in a compartment named cardia [67]. At the moment, DEF3, DEF2 and CEC2 still have unidentified functions; though, clustering studies suggest that DEF3 exerts an action in immunity during blood meal inducing cuticle expansion, and perhaps against fungal infection [68]. In our results, AMPs-related transcripts that were identified in *A. gambiae* sialotranscriptome included CEC1, CEC2, CEC3 and GAM1. These revealed a low expression at the transcriptional level (Appendix I - Table 1) probably because the mosquito immune response is being affected by the parasite. The transcripts referring to the proteins DEF1 and DEF3 were also found. Finally, when the SG transcriptome of *A. stephensi* was analyzed, only DEF1 was found in the AMPs subclass. The absence of others AMPs transcripts in the analyzed data may be related to a non-differential expression of AMP genes by different pathways, such as *Imd* and *Toll* pathway.

Our overall results have identified the transcripts that codify for IGL-1, TEP4, TEP12, TEP14, CLIPA4, CLIPA14 and CLIPC4 (Fig. 2) as good targets for future studies to evaluate their potential as pan-*Anopheles* vaccines, due to the high homology and conservation between of these genes in the two species. Fig. 2 shows the conserved immunity genes that are common between *A. gambiae* and *A. stephensi* with at least 80% homology, and the ones only found in one of the species. Also, the immune mechanism in which they are involved is highlighted. Interestingly, with a similar expression and high homology of sequences between both species we were able to identify IGL-1, TEP4, TEP12, TEP14, CLIPA4, CLIPA14 and CLIPC4.

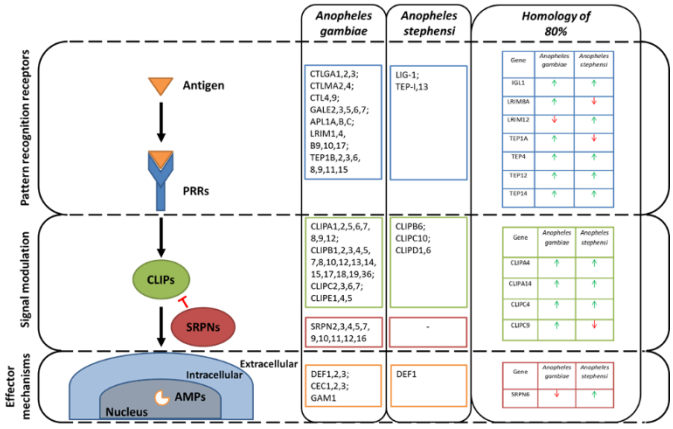

Fig. 2. Immune-related transcripts of *Anopheles gambiae* and *Anopheles stephensi* salivary glands during *Plasmodium berghei* infection. Selected subclasses of proteins involved in pattern recognition, signal modulation, and effector mechanisms. **AMPs** - Antimicrobial peptides; **APLs** - *Anopheles-Plasmodium*-responsive Leucine-Rich Repeat; **CECs** - Cecropins; **CLIPs** - Clip-domain serine proteases from signal modulation; **CTLs** - C-type lectins; **DEFs** - Defensins; **GALEs** - galectins; **GAMBs** - Gambicins; **LRIMs** - Leucine-rich immune protein; **PRRs** - Pattern recognition receptors; **SRPNs** - serpins; **TEPs** - Thioester containing proteins. Differential expression is represented by the green and red arrows, indicating up or downregulated, respectively.

IV. CONCLUSIONS

To date, several efforts have been made to control human malaria worldwide but, nonetheless, this disease burden is still increasing, not only due to socio-economic and politic factors that are not positively contributing for its reduction, but also because the control measures that have been directed to the mosquito vector with insecticides, environment control and insecticide-impregnated bed nets are not completely effective. These strategies have faced major drawbacks regarding insecticide-resistance of the vector and drug-resistance of the parasite, which led to the malaria reassurance. For this, new approaches and the design of new strategies are urgently needed. One strategy is to unravel the key-molecules and mechanisms, particularly the ones present in the mosquito immunity that are essential for the *Plasmodium* successful development in the mosquito vector and cause their blockage to interrupt parasite transmission to the vertebrate host. Here, we

have compared sialotranscriptome of *A. gambiae* and *A. stephensi* during *P. berghei* infection, focusing in transcripts that are known to have a crucial impact in innate immune response of *Anopheles* mosquitoes. In this study, we conclude that *A. gambiae* and *A. stephensi* have similarities and differences in the expression of genes related to innate immune system, since initial immune response to the production of antimicrobial peptides. The understanding of this interplay may be conducted for the development of the new strategies of malaria control.

ACKNOWLEDGMENTS

The authors would like to acknowledge Renato Pinheiro-Silva for the scientific advice and constructive comments regarding the *A. gambiae* transcriptomic analysis.

The authors declare no competing personal or financial interests.

References

[1] CDC, *CDC - Malaria*. **2014, Available at:** http://www.cdc.gov/malaria/. [Accessed: 18-Feb-2015].

[2] World Health Organization, *WHO*, World Malaria Report 2014, **2014, Available at:** http://www.who.int/malaria/publications/world_malaria_report_2014/report/en/. [Accessed: 18-Feb-2015].

[3] Thera MA, Plowe CV, Vaccines for malaria: how close are we?, *Annual review of medicine*, **2012;** 63(345-57),

[4] World Health Organization, *WHO | Malaria*. **2014, Available at:** http://www.who.int/topics/malaria/en/. [Accessed: 19-Feb-2015].

[5] Kantele A, Jokiranta TS, Review of cases with the emerging fifth human malaria parasite, *Plasmodium knowlesi*, *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, **2011;** 52(11): 1356-62,

[6] Kawamoto F, Win TT, Mizuno S, Lin K, Kyaw O, Tantulart IS, Mason DP, Kimura M, Wongsrichanalai C, Unusual plasmodium malariae-like parasites in southeast Asia, *The Journal of parasitology*, **2002;** 88(2): 350-7,

[7] Winzerling JJ, Pham DQD, Iron metabolism in insect disease vectors: mining the *Anopheles gambiae* translated protein database, *Insect biochemistry and molecular biology*, **2006;** 36(4): 310-21,

[8] Miller LH, Baruch DI, Marsh K, Doumbo OK, The pathogenic basis of malaria, *Nature*, **2002;** 415(6872): 673-9,

[9] Abraham EG, Pinto SB, Ghosh A, Vanlandingham DL, Budd A, Higgs S, Kafatos FC, Jacobs-Lorena M, Michel K, An immune-responsive serpin, SRPN6, mediates mosquito defense against malaria parasites, *Proceedings of the National Academy of Sciences of the United States of America*, **2005;** 102(45): 16327-32,

[10] Wang S, Jacobs-Lorena M, Genetic approaches to interfere with malaria transmission by vector mosquitoes, *Trends in biotechnology*, **2013;** 31(3): 185-93,

[11] Bray RS, Garnham PCC, The life-cycle of primate malaria parasites, *Br. Med. Bull.*, **1982;** 38(2): 117-122,

[12] Ferrolho J, Domingues N, Domingos A, The Role of Regulatory CD4+CD25+ T Cell Subset in Host Homeostasis during Protozoan Infection: An Overview, *researchgate.net*,

[13] Liu K, Dong Y, Huang Y, Rasgon JL, Agre P, Impact of trehalose transporter knockdown on *Anopheles gambiae* stress adaptation and susceptibility to *Plasmodium falciparum* infection, *Proceedings of the National Academy of Sciences of the United States of America*, **2013;** 110(43): 17504-9,

[14] Brennan JD, Kent M, Dhar R, Fujioka H, Kumar N, *Anopheles gambiae* salivary gland proteins as putative targets for blocking transmission of malaria parasites, *Proceedings of the National Academy of Sciences of the United States of America*, **2000;** 97(25): 13859-64,

[15] Sidjanski SP, Vanderberg JP, Sinnis P, *Anopheles stephensi* salivary glands bear receptors for region I of the circumsporozoite protein of *Plasmodium falciparum*, *Molecular and Biochemical Parasitology*, **1997;** 90(1): 33-41,

[16] Barnard A-C, Nijhof AM, Fick W, Stutzer C, Maritz-Olivier C, RNAi in Arthropods: Insight into the Machinery and Applications for Understanding the Pathogen-Vector Interface, *Genes*, **2012;** 3(4): 702-41,

[17] Ménard R, Malaria: methods and protocols, **2013;**

[18] Clayton AM, Dong Y, Dimopoulos G, The *Anopheles* innate immune system in the defense against malaria infection, *Journal of innate immunity*, **2014;** 6(2): 169-81,

[19] Osta MA, Christophides GK, Vlachou D, Kafatos FC, Innate immunity in the malaria vector *Anopheles gambiae:* comparative and functional genomics, *The Journal of experimental biology*, **2004;** 207(Pt 15): 2551-63,

[20] Osta MA, Christophides GK, Kafatos FC, Effects of mosquito genes on *Plasmodium* development, *Science (New York, N.Y.)*, **2004;** 303(5666): 2030-2,

[21] Christophides GK, Zdobnov E, Immunity-related genes and gene families in *Anopheles gambiae*, *Science*, **2002;**

[22] Dong Y, Das S, Cirimotich C, Souza-Neto JA, McLean KJ, Dimopoulos G, Engineered *Anopheles* Immunity to *Plasmodium* Infection, *Plos Pathogens*, **2011;** 7(12):

[23] Ubaida Mohien C, Colquhoun DR, Mathias DK, Gibbons JG, Armistead JS, Rodriguez MC, Rodriguez MH, Edwards NJ, Hartler J, Thallinger GG, Graham DR, Martinez-Barnetche J, Rokas A, Dinglasan RR, A bioinformatics approach for integrated transcriptomic and proteomic comparative analyses of model and non-sequenced anopheline vectors of human malaria

parasites, *Molecular & cellular proteomics : MCP*, **2013;** 12(1): 120-31,

[24] Koutsos AC, Blass C, Meister S, Schmidt S, MacCallum RM, Soares MB, Collins FH, Benes V, Zdobnov E, Kafatos FC, Christophides GK, Life cycle transcriptome of the malaria mosquito *Anopheles gambiae* and comparison with the fruitfly *Drosophila melanogaster*, *Proceedings of the National Academy of Sciences of the United States of America*, **2007;** 104(27): 11304-9,

[25] de la Fuente J, Kocan KM, Strategies for development of vaccines for control of ixodid tick species, *Parasite Immunology*, **2006;** 28(7): 275-283,

[26] Pinheiro-Silva R, Borges L, Coelho LP, Cabezas-Cruz A, Valdés JJ, Rosario VA, RNA-seq analyses of gene expression changes in the salivary glands of *Anopheles gambiae* elicited by *Plasmodium berghei* infection, *Parasite & Vectors*, **2015;**

[27] Couto J, *Transcriptomic analysis of Anopheles stephensi salivary glands during the infection with Plasmodium berghei*. 2015. **Available at:** http://hdl.handle.net/10773/14639

[28] Liu Y, Zhang F, Liu J, Xiao X, Zhang S, Qin C, Xiang Y, Wang P, Cheng G, Transmission-blocking antibodies against mosquito C-type lectins for dengue prevention, *PLoS pathogens*, **2014;** 10(2): e1003931-e1003931,

[29] Cirimotich CM, Dong Y, Garver LS, Sim S, Dimopoulos G, Mosquito immune defenses against *Plasmodium* infection, *Developmental and comparative immunology*, **2010;** 34(4): 387-95,

[30] Mogensen TH, Pathogen recognition and inflammatory signaling in innate immune defenses, *Clinical microbiology reviews*, **2009;** 22(2): 240-73, Table of Contents,

[31] Michel K, Budd A, Pinto S, Gibson TJ, Kafatos FC, *Anopheles gambiae* SRPN2 facilitates midgut invasion by the malaria parasite *Plasmodium berghei*, *EMBO reports*, **2005;** 6(9): 891-7,

[32] Dong Y, Taylor HE, Dimopoulos G, *AgDscam*, a hypervariable immunoglobulin domain-containing receptor of the *Anopheles gambiae* innate immune system, *PLoS biology*, **2006;** 4(7): e229-e229,

[33] Gross L, A protean insect receptor holds the key to broad-based pathogen recognition, *PLoS biology*, **2006;** 4(7): e246-e246,

[34] Xu X, Dong Y, Abraham EG, Kocan A, Srinivasan P, Ghosh AK, Sinden RE, Ribeiro JMC, Jacobs-Lorena M, Kafatos FC, Dimopoulos G, Transcriptome analysis of *Anopheles stephensi-Plasmodium berghei* interactions, *Molecular and biochemical parasitology*, **2005;** 142(1): 76-87,

[35] Povelones M, Waterhouse RM, Kafatos FC, Christophides GK, Leucine-rich repeat protein complex activates mosquito complement in defense against *Plasmodium* parasites, *Science (New York, N.Y.)*, **2009;** 324(5924): 258-61,

[36] Dimopoulos G, Christophides GK, Meister S, Schultz J, White KP, Barillas-Mury C, Kafatos FC, Genome expression analysis of *Anopheles gambiae*: responses to injury, bacterial challenge, and malaria infection, *Proceedings of the National Academy of Sciences of the United States of America*, **2002;** 99(13): 8814-9,

[37] Fraiture M, Baxter RHG, Steinert S, Chelliah Y, Frolet C, Quispe-Tintaya W, Hoffmann JA, Blandin SA, Levashina EA, Two mosquito LRR proteins function as complement control factors in the TEP1-mediated killing of Plasmodium, *Cell host & microbe*, **2009;** 5(3): 273-84,

[38] Garver LS, Bahia AC, Das S, Souza-Neto JA, Shiao J, Dong Y, Dimopoulos G, *Anopheles Imd* Pathway Factors and Effectors in Infection Intensity-Dependent Anti-*Plasmodium* Action, *Plos Pathogens*, **2012;** 8(6):

[39] Boëte C, Malaria parasites in mosquitoes: laboratory models, evolutionary temptation and the real world, *Trends in parasitology*, **2005;** 21(10): 445-7,

[40] Vasta GR, Roles of galectins in infection, *Nature Reviews Microbiology*, **2009;**

[41] Pace KE, Baum LG, Insect galectins: roles in immunity and development, *Glycoconjugate journal*, **2004;** 19(7-9): 607-14,

[42] Kamhawi S, Ramalho-Ortigao M, Kumar S, Lawyer PG, Turco SJ, Barillas-Mury C, Sacks DL, Valenzuela JG, A Role for Insect Galectins in Parasite Survival, *Cell*, **2004;** 119(3): 329-341,

[43] Valenzuela JG, Francischetti IMB, Pham VM, Garfield MK, Ribeiro JMC, Exploring the salivary gland transcriptome and proteome of the *Anopheles stephensi* mosquito, *Insect biochemistry and molecular biology*, **2003;** 33(7): 717-32,

[44] Dimopoulos G, Richman A, Müller HM, Kafatos FC, Molecular immune responses of the mosquito *Anopheles gambiae* to bacteria and malaria parasites, *Proceedings of the National Academy of Sciences of the United States of America*, **1997;** 94(21): 11508-13,

[45] Ligoxygakis P, Pelte N, Hoffmann JA, Reichhart J-M, Activation of *Drosophila Toll* during fungal infection by a blood serine protease, *Science (New York, N.Y.)*, **2002;** 297(5578): 114-6,

[46] Yassine H, Osta MA, *Anopheles gambiae* innate immunity, *Cellular microbiology*, **2010;** 12(1): 1-9,

[47] Clements AN, The Biology of Mosquitoes, Volume 3 Transmission of Viruses and Interactions with Bacteria, **2012;**

[48] Volz J, Müller H-M, Zdanowicz A, Kafatos FC, Osta MA, A genetic module regulates the melanization response of *Anopheles* to *Plasmodium*, *Cellular microbiology*, **2006;** 8(9): 1392-405,

[49] Zou Z, Souza-Neto J, Xi Z, Kokoza V, Shin SW, Dimopoulos G, Raikhel A, Transcriptome analysis of *Aedes aegypti* transgenic mosquitoes with altered immunity, *PLoS pathogens*, **2011;** 7(11): e1002394-e1002394,

[50] Simões ML, Gonçalves L, Silveira H, Hemozoin activates the innate immune system and reduces *Plasmodium berghei* infection in *Anopheles gambiae*, *Parasites & vectors*, **2015;**

[51] Volz J, Osta MA, Kafatos FC, Müller H-M, The roles of two clip domain serine proteases in innate immune responses of the malaria vector *Anopheles gambiae*, *The Journal of biological chemistry*, **2005;** 280(48): 40161-8,

[52] Barillas-Mury C, CLIP proteases and *Plasmodium* melanization in *Anopheles gambiae*, *Trends in parasitology*, **2007;** 23(7): 297-9,

[53] Crawford JE, Guelbeogo WM, Sanou A, Traoré A, Vernick KD, Sagnon NF, Lazzaro BP, *De novo* transcriptome sequencing in *Anopheles funestus* using Illumina RNA-seq technology, *PloS one*, **2010;** 5(12): e14202-e14202,

[54] Blandin SA, Marois E, Levashina EA, Antimalarial responses in *Anopheles gambiae:* from a complement-like protein to a complement-like pathway, *Cell host & microbe*, **2008;**

[55] An C, Jiang H, Kanost MR, Proteolytic activation and function of the cytokine Spätzle in the innate immune response of a lepidopteran insect, Manduca sexta, *The FEBS journal*, **2010;** 277(1): 148-62,

[56] Danielli A, Barillas-Mury C, Kumar S, Kafatos FC, Loukeris TG, Overexpression and altered nucleocytoplasmic distribution of *Anopheles* ovalbumin-like SRPN10 serpins in *Plasmodium*-infected midgut cells, *Cellular microbiology*, **2005;** 7(2): 181-90,

[57] Danielli A, Kafatos FC, Loukeris TG, Cloning and characterization of four *Anopheles gambiae* serpin isoforms, differentially induced in the midgut by *Plasmodium berghei* invasion, *The Journal of biological chemistry*, **2003;** 278(6): 4184-93,

[58] Brown KL, Hancock REW, Cationic host defense (antimicrobial) peptides, *Current opinion in immunology*, **2006;**

[59] Gilbert LI, *Insect molecular biology and biochemistry*. 2012.

[60] Meister M, Lemaitre B, Hoffmann JA, Antimicrobial peptide defense in *Drosophila*, *BioEssays : news and reviews in molecular, cellular and developmental biology*, **1997;** 19(11): 1019-26,

[61] Richman AM, Dimopoulos G, Seeley D, Kafatos FC, *Plasmodium* activates the innate immune response of *Anopheles gambiae* mosquitoes, *The EMBO journal*, **1997;** 16(20): 6114-9,

[62] Brey PT, Lee WJ, Yamakawa M, Koizumi Y, Perrot S, Francois M, Ashida M, Role of the integument in insect immunity: epicuticular abrasion and induction of cecropin synthesis in cuticular epithelial cells, *Proceedings of the National Academy of Sciences*, **1993;** 90(13): 6275-6279,

[63] Luna C, Hoa NT, Lin H, Zhang L, Nguyen HLA, Kanzok SM, Zheng L, Expression of immune responsive genes in cell lines from two different *Anopheline* species, *Insect molecular biology*, **2006;** 15(6): 721-9,

[64] Hoa NT, Zheng L, Functional characterization of the NF-κB transcription factor gene REL2 from *Anopheles gambiae*, *Insect Science*, **2007;** 14(3): 175-184,

[65] Meister S, Kanzok SM, Zheng X-L, Luna C, Li T-R, Hoa NT, Clayton JR, White KP, Kafatos FC, Christophides GK, Zheng L, Immune signaling pathways regulating bacterial and malaria parasite infection of the mosquito *Anopheles gambiae*, *Proceedings of the National Academy of Sciences of the United States of America*, **2005;** 102(32): 11420-5,

[66] Kim W, Koo H, Richman AM, Seeley D, Vizioli J, Klocko AD, O'Brochta DA, Ectopic expression of a cecropin transgene in the human malaria vector mosquito *Anopheles gambiae* (Diptera: Culicidae): effects on susceptibility to *Plasmodium*, *Journal of medical entomology*, **2004;** 41(3): 447-55,

[67] Warr E, Aguilar R, Dong Y, Mahairaki V, Dimopoulos G, Spatial and sex-specific dissection of the *Anopheles gambiae* midgut transcriptome, *BMC genomics*, **2007;** 8(1): 37-37,

[68] Maccallum RM, Redmond SN, Christophides GK, An expression map for *Anopheles gambiae*, *BMC genomics*, **2011;** 12(1): 620-620,

APPENDIX

Appendix A

Table 1. List of immune-related transcripts of *Anopheles gambiae* and *Anopheles stephensi* salivary glands during *Plasmodium berghei* infection. Selected transcripts grouped by immune subclasses are identified by their Gene ID from the database: VectorBase (www.vectorbase.org) and gene description. The levels of expression in fold-change are described. AMPs - Antimicrobial peptides; CLIPs - Clip-domain serine proteases from signal modulation; CTLs - C-type lectins; DEFs - Defensins; GALEs - galectins; LRRs - Leucine-rich repeat; SRPNs - serpins; TEPs - Thioester containing proteins.

Mosquito	Subclass	Gene ID	Gene description	Fold-change
Anopheles gambiae	AMPs	AGAP000692	CEC3: cecropin anti-microbial peptide C	-1,124
		AGAP000693	CEC1: cecropin anti-microbial peptide A	-1,005
		AGAP000694	CEC2: cecropin anti-microbial peptide B	-1,111
		AGAP004632	DEF2: defensin anti-microbial peptide 2	1,141
		AGAP007199	DEF3: defensin anti-microbial peptide 3	2,368
		AGAP008645	GAM1: gambicin anti-microbial peptide	-1,030
		AGAP011294	DEF1: defensin anti-microbial peptide 1	-1,052
	CLIPs	AGAP000315	CLIPC6: Clip-Domain Serine Protease	1,259
		AGAP000573	CLIPC4: Clip-Domain Serine Protease	1,083
		AGAP001648	CLIPB17: Clip-Domain Serine Protease	1,639
		AGAP002270	CLIPB7: Clip-Domain Serine Protease	2,270
		AGAP003057	CLIPB8: Clip-Domain Serine Protease	1,175
		AGAP003246	CLIPB2: Clip-Domain Serine Protease	1,508
		AGAP003247	CLIPB19: Clip-Domain Serine Protease	1,198
		AGAP003249	CLIPB3: Clip-Domain Serine Protease	1,833
		AGAP003250	CLIPB4: Clip-Domain Serine Protease	1,270
		AGAP003251	CLIPB1: Clip-Domain Serine Protease	1,256
		AGAP003689	CLIPC7: Clip-Domain Serine Protease	1,700
		AGAP004148	CLIPB5: Clip-Domain Serine Protease	-1,087
		AGAP004317	CLIPC2: Clip-Domain Serine Protease	1,110
		AGAP004318	CLIPC3: Clip-Domain Serine Protease	1,073
		AGAP004719	CLIPC9: Clip-Domain Serine Protease	1,230
		AGAP004855	CLIPB13: Clip-Domain Serine Protease	1,107
		AGAP008091	CLIPE1: Clip-Domain Serine Protease	-1,463
		AGAP009215	CLIPB18: Clip-Domain Serine Protease	1,643
		AGAP009217	CLIPB12: Clip-Domain Serine Protease	2,267
		AGAP009844	CLIPB15: Clip-Domain Serine Protease	1,405
		AGAP010530	CLIPE4: Clip-Domain Serine Protease	1,289
		AGAP010547	CLIPE5: Clip-Domain Serine Protease	1,093
		AGAP010731	CLIPA8: Clip-Domain Serine Protease	1,240
		AGAP010833	CLIPB14: Clip-Domain Serine Protease	1,303
		AGAP010968	CLIPA9: Clip-Domain Serine Protease	1,052
		AGAP011780	CLIPA4: Clip-Domain Serine Protease	1,077
		AGAP011781	CLIPA12: Clip-Domain Serine Protease	1,338

Mosquito	Subclass	Gene ID	Gene description	Fold-change
		AGAP011787	CLIPA5: Clip-Domain Serine Protease	1,268
		AGAP011788	CLIPA14: Clip-Domain Serine Protease	1,277
		AGAP011789	CLIPA6: Clip-Domain Serine Protease	1,244
		AGAP011790	CLIPA2: Clip-Domain Serine Protease	1,369
		AGAP011791	CLIPA1: Clip-Domain Serine Protease A1	1,302
		AGAP011792	CLIPA7: Clip-Domain Serine Protease	1,315
		AGAP013184	CLIPB36: Clip-Domain Serine Protease	1,234
		AGAP013442	CLIPB10: Clip-Domain Serine Protease	1,064
	CTLs	AGAP000007	IGL1: contactin-like putative cell adhesion molecule	1,087
		AGAP002625	CTL9: C-type lectin enzyme	-1,218
		AGAP005334	CTLMA2: C-Type Lectin (CTL) - mannose binding	1,075
		AGAP005335	CTL4: C-type lectin enzyme	1,093
		AGAP006430	CTLGA2: C-Type Lectin (CTL) - galactose binding A2	1,557
		AGAP007407	CTLMA4: C-Type Lectin (CTL) - mannose binding	1,310
		AGAP010193	CTLGA3: C-Type Lectin (CTL) - galactose binding A3	-1,099
		AGAP010196	CTLGA1: C-Type Lectin (CTL) - galactose binding A1	-1,552
	GALEs	AGAP000341	GALE2: galectin2	-1,171
		AGAP004806	GALE6: galectin6	-1,155
		AGAP004807	GALE7: galectin 7	-1,216
		AGAP004934	GALE3: galectin3	-1,130
		AGAP011287	GALE5: galectin5	-1,078
	LRRs	AGAP001127	P37NB protein	1,157
		AGAP003878	n/a	1,086
		AGAP004405	glucose-repressible alcohol dehydrogenase transcriptional effector homolog	1,522
		AGAP005496	LRIM12	-1,256
		AGAP005693	LRIM17/LRRD7	1,256
		AGAP005962	n/a	1,134
		AGAP006183	slit protein	1,529
		AGAP006348	LRIM1	1,457
		AGAP006408	n/a	-1,158
		AGAP006643	n/a	-1,070
		AGAP007033	APL1C: *Anopheles Plasmodium*-responsive Leucine-Rich Repeat 1C	1,383
		AGAP007035	APL1B: *Anopheles Plasmodium*-responsive Leucine-Rich Repeat 1B	1,688
		AGAP007036	APL1A: *Anopheles Plasmodium*-responsive Leucine-Rich Repeat 1A	1,641
		AGAP007039	LRIM4	1,274
		AGAP007453	LRIM9: leucine-rich immune protein (Short)	1,263
		AGAP007454	LRIM8A: leucine-rich immune protein (Short)	1,186
		AGAP007455	LRIM10	1,304
		AGAP007456	LRIM8B	1,220
		AGAP007758	n/a	1,156
		AGAP008927	protein TILB homolog	2,734
		AGAP009924	n/a	-1,121

Mosquito	Subclass	Gene ID	Gene description	Fold-change
		AGAP010180	centrosomal protein CEP97	2,734
		AGAP012317	n/a	-1,261
		AGAP012425	n/a	-1,070
		AGAP013059	n/a	1,145
		AGAP013186	n/a	1,164
	OTHERS	AGAP000016	SCRB10: Class B Scavenger Receptor	-1,211
		AGAP000536	PGRPS1: Peptidoglycan Recognition Protein (Short) 1	1,130
		AGAP000631	integrator complex subunit 6	1,189
		AGAP000999	TOLL5A: Toll-like protein 5A	1,261
		AGAP001182	n/a	-1,141
		AGAP001212	PGRPLB: peptidoglycan recognition protein (Long)	1,088
		AGAP003141	Insulin-related peptide binding protein	1,179
		AGAP003354	Venom allergen	-1,322
		AGAP003428	speckle-type POZ protein	1,167
		AGAP004170	n/a	-1,348
		AGAP004455	GNBPB1: 3-Glucan Binding Protein	-1,091
		AGAP004774	host cell factor	1,137
		AGAP004845	scavenger receptor class B member	1,120
		AGAP004846	SCRB9: Class B Scavenger Receptor	-1,074
		AGAP004847	SCRB7: Class B Scavenger Receptor 7	1,094
		AGAP004916	n/a	-1,023
		AGAP004920	CASPS6: caspase (short class) 6	-1,181
		AGAP004921	CASPS5: caspase (short class) 5	-1,082
		AGAP004922	CASPS11: caspase (short class) 11	-1,401
		AGAP004977	PPO6: Prophenoloxidase enzyme	-1,056
		AGAP005203	PGRPLC: Peptidoglycan Recognition Protein (Long) Transcript 2	-1,050
		AGAP005252	MYD: TOLL pathway signalling	-1,128
		AGAP005716	SCRB16: Class B Scavenger Receptor	-1,159
		AGAP005725	SCRB3: Class B Scavenger Receptor 3	-1,583
		AGAP005933	NFkappaB essential modulator	-1,128
		AGAP006342	PGRPS3: Peptidoglycan Recognition Protein (Short) 3	-1,115
		AGAP006343	PGRPS2: Peptidoglycan Recognition Protein (Short) 2	-1,068
		AGAP006419	Venom allergen	1,256
		AGAP006421	Venom allergen	1,178
		AGAP006974	TOLL9: Toll-like protein 9	-1,081
		AGAP007293	IAP7: Inhibitor of Apoptosis 7	-1,275
		AGAP007294	IAP1: Inhibitor of Apoptosis 1	-1,111
		AGAP007343	LYSC2: C-Type Lysozyme	3,365
		AGAP007347	LYSC1: Lysozyme c-1	1,119
		AGAP007385	LYSC4: C-Type Lysozyme	1,177
		AGAP007386	LYSC7: C-Type Lysozyme	-1,089
		AGAP007394	protein AATF/BFR2	1,237

Mosquito	Subclass	Gene ID	Gene description	Fold-change
		AGAP007612	n/a	-1,172
		AGAP007679	n/a	-1,081
		AGAP007684	Tubulointerstitial nephritis antigen	1,202
		AGAP007821	n/a	-1,124
		AGAP008004	n/a	1,114
		AGAP008113	n/a	-1,022
		AGAP009166	IKK1: IMD pathway signalling IKK-beta	1,127
		AGAP009651	CD63 antigen	-1,036
		AGAP009653	n/a	-1,038
		AGAP010186	echinoid	1,227
		AGAP010773	n/a	1,933
		AGAP011693	CASPL1 : caspase (long class) 1	1,162
		AGAP011952	CASPS3: caspase (short class) 3	-1,276
	SRPNs	AGAP001375	SRPN12: serine protease inhibitor (serpin) homologue - unlikely to be inhibitory	-2,637
		AGAP001377	SRPN11: serine protease inhibitor (serpin) homologue - unlikely to be inhibitory	1,236
		AGAP003139	SRPN9: serine protease inhibitor (serpin)	-1,092
		AGAP005246	SRPN10: serine protease inhibitor (serpin) homologue - unlikely to be inhibitory	-1,153
		AGAP006910	SRPN3:serine protease inhibitor (serpin)	1,182
		AGAP006911	SRPN2: serine protease inhibitor (serpin)	1,221
		AGAP007693	SRPN7: serine protease inhibitor (serpin)	1,115
		AGAP009212	SRPN6: serine protease inhibitor (serpin)	-1,226
		AGAP009213	SRPN16: serine protease inhibitor (serpin)	1,164
		AGAP009221	SRPN5: serine protease inhibitor (serpin)	-1,111
		AGAP009670	SRPN4: serine protease inhibitor (serpin)	1,068
	TEPs	AGAP008364	TEP15: thioester-containing protein	1,103
		AGAP008366	TEP2: thioester-containing protein	-1,093
		AGAP008368	TEP14: thioester-containing protein	1,262
		AGAP008654	TEP12: thioester-containing protein	1,585
		AGAP010812	TEP4: thioester-containing protein	1,375
		AGAP010814	TEP6: thioester-containing protein	1,601
		AGAP010815	TEP1 (CM000358 11202090..11205737) thioester-containing protein	1,522
		AGAP010815	TEP1 (CM000358 11206189..11206882): thioester-containing protein	1,830
		AGAP010816	TEP3: thioester-containing protein	1,518
		AGAP010818	TEP11: thioester-containing protein	1,651
		AGAP010830	TEP9: thioester-containing protein	1,597
		AGAP010831	TEP8: thioester-containing protein	2,618
Mosquito	**Subclass**	**Gene ID**	**Gene description**	**Fold-change**
Anopheles stephensi	AMP	AGAP011294	DEF1: defensin anti-microbial peptide 1	1,860
	CLIP	AGAP000572	CLIPC10: Clip-Domain Serine Protease	1,053
		AGAP000573	CLIPC4: Clip-Domain Serine Protease	3,478
		AGAP002422	CLIPD1: Clip-Domain Serine Protease	2,905

Mosquito	Subclass	Gene ID	Gene description	Fold-change
		AGAP002813	CLIPD6: Clip-Domain Serine Protease	2,123
		AGAP003252	CLIPB6: Clip-Domain Serine Protease	5,220
		AGAP004719	CLIPC9: Clip-Domain Serine Protease	-0,840
		AGAP011780	CLIPA4: Clip-Domain Serine Protease	3,617
		AGAP011788	CLIPA14: Clip-Domain Serine Protease	1,241
	CTL	AGAP000007	IGL1: contactin-like putative cell adhesion molecule	1,360
	LRR	AGAP000601	n/a	1,892
		AGAP001127	P37NB protein	-1,874
		AGAP002007	n/a	-1,906
		AGAP004405	glucose-repressible alcohol dehydrogenase transcriptional effector homolog	-2,277
		AGAP004458	n/a	-1,081
		AGAP005496	LRIM13	1,074
		AGAP005693	n/a	-0,957
		AGAP005744	n/a	1,995
		AGAP006644	n/a	1,161
		AGAP006647	n/a	-1,074
		AGAP007059	n/a	-2,449
		AGAP007384	n/a	2,061
		AGAP007454	LRIM8A: leucine-rich immune protein (Short)	-1,435
		AGAP008611	n/a	0,963
		AGAP008785	n/a	2,953
		AGAP009839	n/a	1,216
		AGAP009924	n/a	2,055
		AGAP010770	n/a	1,556
		AGAP011292	n/a	2,618
		AGAP012317	n/a	2,647
		AND_003345	Leucine rich repeat containing protein	1,915
		AND_005520	Membrane glycoprotein LIG-1	-3,871
		AND_006574	Leucine rich repeat protein	3,052
	OTHERS	AGAP000247	n/a	3,509
		AGAP000536	PGRPS1: Peptidoglycan Recognition Protein (Short) 1	3,619
		AGAP000904	n/a	3,181
		AGAP000999	TOLL5A: Toll-like protein 5A	1,256
		AGAP001212	PGRPLB: peptidoglycan recognition protein (Long)	2,277
		AGAP001954	psidin: Phagocyte signaling-impaired protein	1,218
		AGAP002731	n/a	1,452
		AGAP002738	SCRB5	1,658
		AGAP002790	n/a	-1,822
		AGAP003428	speckle-type POZ protein	1,056
		AGAP003521	n/a	3,510
		AGAP004455	GNBPB1: 3-Glucan Binding Protein	-0,973
		AGAP004643	SCRB6: Class B Scavenger Receptor 6	-2,613

Mosquito	Subclass	Gene ID	Gene description	Fold-change
		AGAP004845	scavenger receptor class B member	-1,803
		AGAP004847	SCRB7: Class B Scavenger Receptor 8	1,749
		AGAP004918	n/a	-2,205
		AGAP005252	MYD: TOLL pathway signalling	2,352
		AGAP005713	n/a	3,079
		AGAP005901	n/a	1,485
		AGAP005901	n/a	2,217
		AGAP006328	n/a	4,216
		AGAP006747	REL2	2,117
		AGAP006771	n/a	1,506
		AGAP006974	TOLL9: Toll-like protein 10	2,688
		AGAP007294	IAP1: Inhibitor of Apoptosis 2	1,385
		AGAP007563	n/a	1,828
		AGAP007684	Tubulointerstitial nephritis antigen	-1,568
		AGAP007809	n/a	1,024
		AGAP008412	n/a	3,249
		AGAP008813	n/a	2,381
		AGAP009143	SCRAC1	2,378
		AGAP009166	IKK1: IMD pathway signalling IKK-beta	1,733
		AGAP010186	echinoid	1,271
		AGAP011119	Lysozyme i-1	2,395
		AGAP013186	n/a	2,621
		AND_003269	Pellino	2,076
		AND_004871	Interleukin enhancer binding factor	0,989
		n/a	Immune response-related protein	-1,271
		n/a	Prophenol oxidase (EC 1.14.18.1)	1,743
		n/a	Atlastin	2,297
	SRPN	AGAP009212	SRPN6: serine protease inhibitor (serpin)	1,496
	TEP	AGAP008368	TEP14: thioester-containing protein	1,071
		AGAP008407	TEP13	3,230
		AGAP008654	TEP12: thioester-containing protein	2,943
		AGAP010812	TEP4: thioester-containing protein	2,287
		AGAP010815	TEP-I	-0,882