# Proteomics of Anopheles gambiae

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# 3

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## 1. Introduction

Mosquitoes are a family of around three and a half thousand species and belong to the order of Diptera. They are found throughout the world except in places permanently frozen. Three quarters of all mosquito species live in the humid tropics and subtropics, where the climate is warm and moist, well adapted to the development of all stages and allows adults survival. Mosquitoes are classified in three subfamilies (*Toxorhynchitinae*, *Anophelinae*, *Culicinae*). *Anopheles* mosquitoes are members of subfamily *Anophelinae*. Like all mosquitoes, its life cycle consists in four stages: egg, larva, pupa, and adult. Female mosquitoes lay 30-150 eggs every 2-3 days in water. *Anopheles* favors as breeding places, quiet water, like permanent or temporary ponds, or quite cove of rivers. Each egg is protected by an eggshell. Mosquitoes can develop from egg to adult in as little as 5 days but usually take 10–14 days in tropical conditions. *Anopheles* can be distinguished from other mosquitoes by their palps, which are as long as the proboscis. Most *Anopheles* mosquitoes are not exclusively anthropophilic or zoophilic. *Anopheles* mosquitoes are more frequent in tropical and subtropical countries but are also found in temperate climates. They are not found at altitudes above 2000-2500 m.

### 1.1 Pathogens transmitted by *Anopheles* mosquitoes

*Anopheles* mosquitoes are vectors of several pathogens. They can transmit O'nyong-nyong virus as well as parasitic nematodes that cause lymphatic filariasis. Of the approximately 460 known species of *Anopheles*, less than 100 can transmit human malaria in nature. The primary malaria vectors in Africa, *A. gambiae* and *A. funestus*, are strongly anthropophilic and, consequently, are two of the most efficient malaria vectors in the world.

Malaria is a parasitic disease that affects 200 million people worldwide and causes 1.5 to 2.7 million deaths per year. Of the 300–500 million clinical cases annually, nearly 90% are in the sub-Saharan countries of Africa where the malaria parasite, *Plasmodium falciparum*, is primarily transmitted by the mosquito *Anopheles gambiae*. The increasing resistance of the parasite to inexpensive drugs and the resistance of mosquitoes to insecticides have created an urgent need for innovative methods that block parasite transmission during its development within the insect. Strategies for the development of malaria vaccine candidates target the stages found within humans e.g. the asexual erythrocytic stages. However vaccine candidates have also been developed against both the sexual stages of malaria in the mosquito (reviewed in [1] and against the mosquito vector itself (reviewed in [2]). The

*Anopheles* mosquito not only carries the parasite from infected to uninfected people, but also plays a vital role in the parasite life cycle [3; 4]. The development of *Plasmodium* within the mosquito is a very complex process and represents a tight coevolved system in which genetic features of both vector and parasite characterize the potential of the parasite to develop and be transmitted. Identification of promising candidate antigens for a mosquito-based transmission blocking vaccine or interference requires a good knowledge of both partners and of proteins implied in cross-talk between them.

### 1.2 *Plasmodium* life cycle in mosquito

When a female *Anopheles* sucks the blood of an infected human host, it receives red blood cells (RBCs) containing different stages of erythrocytic cycle, including gametocytes. In its gut, all stages except the gametocytes are digested (Figure 1). These hatch out from RBCs into the lumen of host's midgut and become active to start the sexual cycle. After a process termed exflagellation, the male microgametes detach from the parent gametocyte.

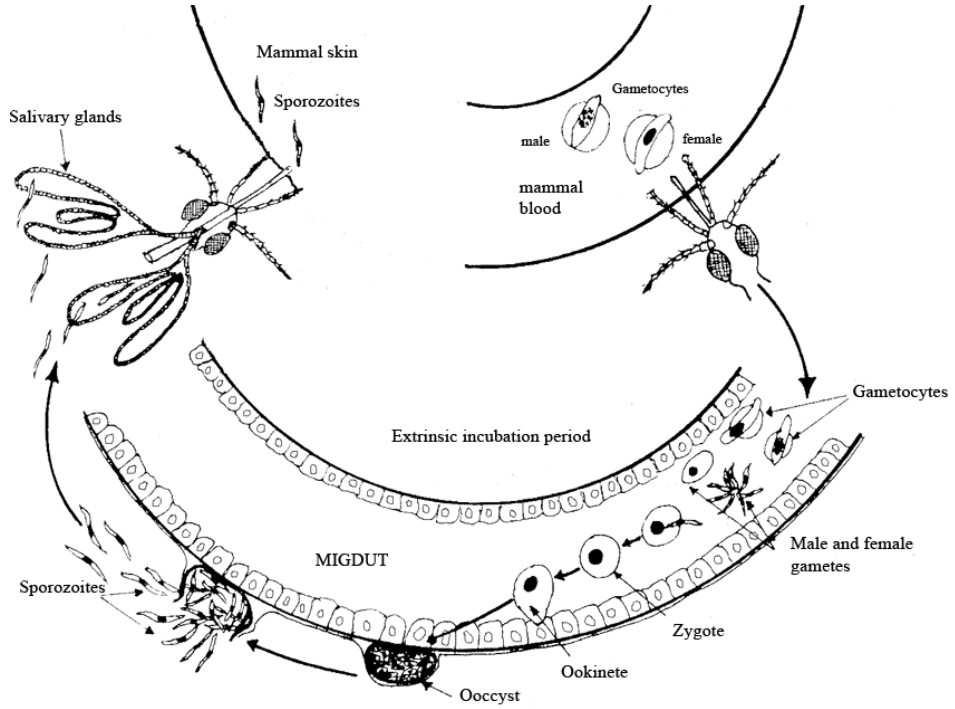



Fig. 1. *Plasmodium* species life cycle in *Anopheles* mosquitoes

The female, macrogametocytes undergo oogenesis and becomes a haploid macro-gamete. Zygotes are formed by fusion of male and female gametes. The zygotic stage of *Plasmodium* species develops to form ookinete. The ookinete passes through the epithelium of the midgut and forms an oocyst on the exterior wall of the midgut. The oocyst matures to form an enlarged structure, after which several nuclear divisions take place. Oocyst ruptures and

releases hundreds of sporozoites. Eventually, these sporozoites migrate to the salivary gland of the female anopheles mosquito. The interval between the acquisition of an infectious agent by a vector and the vector's ability to transmit the agent to other susceptible vertebrate hosts is called the extrinsic incubation period. This parameter is dependent on host, parasite and environmental factors. Estimates are on the order of 10-14 days in areas of high malarial transmission [5].

During the successive stages of parasite development within the mosquito vector, it is faced to several barriers that are decisive for the fate of the parasite and the potential of its transmission. The proteome analysis of these barriers constitutes a first step to better understand the vector/pathogen interactions.

### 1.3 The genome of *Anopheles gambiae*

*Anopheles gambiae* sensus stricto. was selected for full genome sequencing from the 60 or so anopheline mosquito species that transmit malaria, largely because of the large number of malaria fatalities attributed to bites from this mosquito [6; 7]. At 278 Mb, *A. gambiae* genome is considerably larger than the genome size of *Drosophila melanogaster* [8](180 Mb), but smaller than the size of many other mosquito disease vectors such as *Aedes aegypti,* which at approximately 1376 million base pairs is about 5 times the size of the genome of the malaria vector [9]. The difference in size between *A. gambiae* and *D. melanogaster* is largely due to intergenic DNA. Despite evolutionarily diverging 250 million years ago, the two insects reveal remarkable similarities in their DNA sequences. However, there are also important differences in their genomes. Almost half of the genes in both insect genomes are presently identified as orthologs and show an average sequence identity of 56% [6]. This could be explained by successive adaptation of these insects to different life strategies and/or ecological niches, or to the evolutionary adaptation of pathogens to insect vectors or vice versa. Studies have led to the identification of mosquito genes that regulate malaria parasite infection in the mosquito ([10] and those involved in the ability to find and feed on blood of human hosts [11]. In *Aedes aegypti* genome, an increase in genes encoding odorant binding, cytochrome P450, and cuticle domains relative to *A. gambiae* suggests that members of these protein families support some of the biological differences between the two mosquito species [9].

With the genome of *A. gambiae* in hand, scientists possess molecular information to understand the biology of this arthropod vector better, and to possibly fight malaria transmission and other mosquito-borne human illnesses.

### 1.4 Genome versus proteome

Actually, DNA is a chemical instruction manual for everything a plant or animal does, while proteins are the dynamic components. DNA or mRNA sequences cannot sufficiently describe the structure, function and cellular location of proteins. Moreover, some important functional, post-translational modifications, such as glycosylation and phosphorylation, may not even be seen at the genome level. Hence, analysis of the human proteome revealed that it is much more complex than the human genome. While scientists are still deciphering the function of most of the 30,000 human genes, researchers estimate that each gene can give instructions for as many as 100 different proteins. On top of that, every organ has a different

distribution of proteins, and the distribution may change frequently according to physiological modifications. The term "proteome" constitutes the total of proteins expressed by the genome. The technical improvements of the past decade have enhanced proteomic analyses and thereby enabled quantitative analysis of protein expression inside cells. The proteome of several *A. gambiae* organs were deciphered using various proteomic approaches and interesting insights in their function as well as in their putative interaction with pathogens were thereby gathered.

### 1.5 Proteomics methods

Thanks to the combination of developments in new instrumentation, fragmentation methods, availability of completed genome sequences and bioinformatics, there has been a shift from analysis of one protein at the time to more comprehensive proteome analyses. In the past decade, mass spectrometry (MS) has emerged as the dominant technology for in-depth characterization of the protein components of biological systems {12; 13; 14] but a number of other technologies, resources, and expertise are absolutely required to perform significant experiments. These include protein separation science (and protein biochemistry in general), genomics, and bioinformatics. Proteomics has evolved from 1D and 2D gel electrophoresis (1DE and 2DE)/MALDI TOF (Matrix Assisted Laser Desorption Ionization) MS to gel-free liquid chromatography (LC)-MS/MS (tandem mass spectrometry) approaches. Present-day 1D/2D LC-MS/MS workflows exhibit much higher sensitivity, speed, quantitative dynamic range and ease of use in comparison with gel-based resolving techniques. These various approaches are summarized in Figure 2.

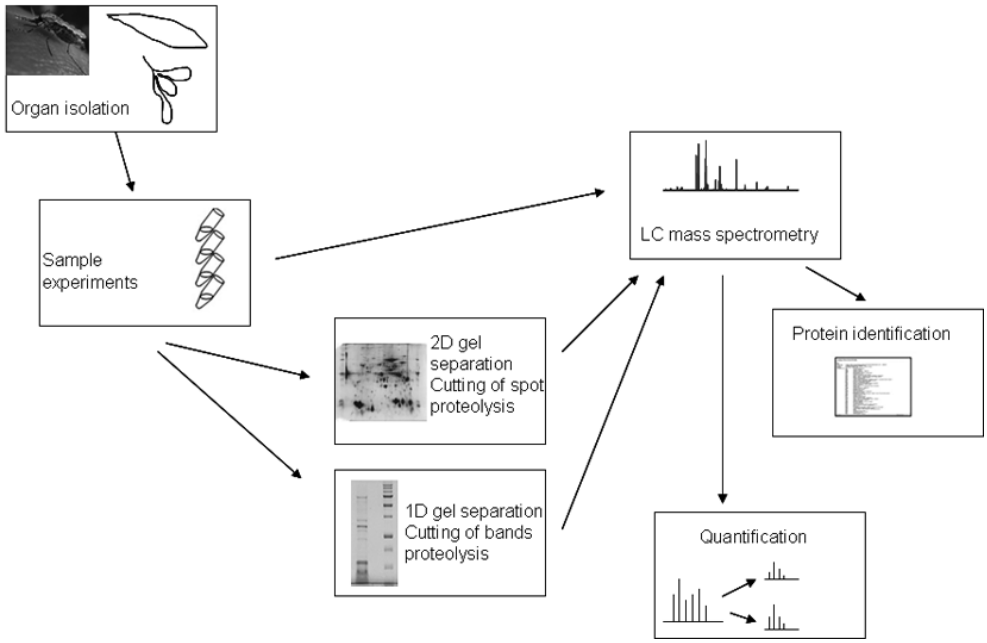



Fig. 2. Proteomic workflow

Beyond protein identification, there are a number of well-established options available for protein quantification. Difference gel electrophoresis (DIGE) following 2DE is one option. Various mass spectrometry based methods have been employed for quantitative analysis of protein expression in a complex protein mixture upon particular modification, such as prior to and after parasite infection. These quantitative approaches utilize the stable isotope-labelling techniques combined with mass spectrometry analysis, including isotope coded affinity tag (ICAT) [15; 16]), isobaric tag for relative and absolute quantitation (iTRAQ) [17], and stable isotope labelling of amino acids in cell culture (SILAC) [18; 19].

## 2. Proteomic analyses of *Anopheles gambiae*

During the last ten years, several approaches were followed to describe the protein content of *Anopheles gambiae*'s organs, hemolymph and saliva. The subsequent paragraphs will present protein data obtained from these various approaches.

### 2.1 The peritrophic matrix (PM) proteome

The peritrophic matrix (or peritrophic membrane) lines the gut of most insects at one or more stages of the life cycle [20]. This is the only region of the insect in contact with the "external" environment not covered by cuticle. It has important roles in the facilitation of the digestive processes in the gut and the protection of the insect from invasion by microorganisms and parasites. Since it poses a partial, natural barrier against parasite invasion of the midgut, it is speculated that modifications to the PM may lead to a complete barrier to infection. In agreement with this statement, it was showed that thickening of the PM by experimental manipulation resulted in decreased *Plasmodium* infectivity [21]. Within the blood mass, the *Plasmodium* gametes mate and develop into motile ookinetes, a process that takes 16–24 h depending on the *Plasmodium* species. To traverse the gut epithelium, the ookinetes must first cross the PM. For this purpose, the *Plasmodium* parasite secretes its own chitinase, which allows it to penetrate this chitin-containing structure [22]. Detailed characterization of the structure of the PM may help to find out new strategies to block the parasite at the midgut stage. The complete PM proteome of *A. gambiae* was determined using 1D-electrophoresis followed by LC-MS [23]. Out of the 209 proteins identified by mass spectrometry, 123 had predicted signal peptides, of which 17 also had predicted transmembrane domains and were therefore candidate midgut surface proteins that were coextracted from the gut during sample processing (Table 1). Among them were nine new chitin-binding peritrophic matrix proteins, expanding the list from three to twelve peritrophins. As expected, the number of proteins with signal peptides is relatively more abundant than those with transmembrane domains only. Based on their annotations, the proteins fell into different classes, with diverse putative functions ranging from immunity to blood digestion. Only 5% of the detected proteins were either known PM proteins or contained clear chitin binding domains (CBDs). Based on these results, the authors proposed a structural model of *A. gambiae* PM to explain the putative interactions among the proteins identified in their study. During the course of blood ingestion the PM is rapidly secreted by the distended midgut epithelium. The chitin fibrils are assembled into a wide cross-hatched pattern connected by peritrophins containing multiple CBDs. Between 0 and 24 h, the crosshatch is fully stretched and then slowly contracts like an accordion between 36 and 48 h post-blood feeding as blood digestion proceeds to completion. The various classes of

peritrophins that have been identified by proteomic analysis were proposed to assemble together to produce a PM as well as perform several of its functions.

| Peritrophic matrix | Protein/gene ID | Domain/motifs/Known/putative function/comments | Putatively secreted |
|---|---|---|---|
| | Protein with chitin binding domains (CBD) | | |
| | AGAP006795 | 2 CBD | yes |
| | AGAP006796 | 1 CBD | yes |
| | AGAP009830 | A CBD | yes |
| | AGAP010364 | 2 CBD | yes |
| | AGAP001819 | 1 CBD | yes |
| | AGAP010363 | 2 CBD | yes |
| | AGAP011616 | 3 CBD | yes |
| | AGAP006433 | 4-CBD | no |
| | AGAP006434 | 4-CBD | yes |
| | AGAP006432 | 2 CBD; immune-responsive gene | yes |
| | AGAP006414 | 1 CBD; mucin domain; chitinase | yes |
| | Proteins with no predicted CBD | | |
| | AGAP006442 | 12.9 Conserved hypothetical protein; unknown function | yes |
| | AGAP004883 | 19.7 Conserved hypothetical protein; snake toxin-like protein folds/disulfide rich; unknown function | yes |
| | AGAP007860 | 33.8 Conserved hypothetical protein; putative protein binding motifs; unknown function | yes |
| | AGAP007612 | 92.2 Conserved hypothetical protein; snake toxin-like protein folds/disulfide rich; unknown function | yes |
| | AGAP002851 | 16.3 Conserved hypothetical protein; MD2-lipid recognition domain | yes |
| | AGAP001352 | 28.1 Conserved hypothetical protein; odorant/hormone binding domain | yes |
| | AGAP010132 | 52.1 Conserved hypothetical protein; CD36 scavenger receptor Class B domain | yes |
| | AGAP006398 | 31.2 Conserved hypothetical protein; galactose-like binding protein/lectin-like domain | no |
| | AGAP004916 | 35.0 Conserved hypothetical protein; Fibrillin/fibrinogen-like; globular domain; unknown function | yes |

Table 1. PM proteins identified by 1D and LC-MS

The proteins of the PM may provide unique opportunities for the control of insect pests and vector borne diseases. Immunological control of blood and tissue-feeding insects by the effects of binding antibody to the proteins of the PM may be explored as well as other functional genetics approaches based on the understanding of the function of its components.

### 2.2 Hemolymph proteome

Insects employ an open circulatory system for the transport of nutrients, wastes, and signalling molecules throughout the body. The insect circulatory system also functions in thermoregulation, promoting ventilation through the tracheal system, and the circulation of humoral immune molecules and immune blood cells (hemocytes) that survey tissues for foreign entities. The primary organ driving hemolymph circulation in mosquitoes is a dorsal vessel that is subdivided into an abdominal heart and a thoracic aorta. Hemolymph plays a very important role in protecting against harmful microorganisms like *Plasmodium*. Two-dimensional SDS–PAGE and microsequencing or peptide mass fingerprinting was used to identify major proteins in the hemolymph of *A. gambiae* [24; 25]. They found about 280 protein spots in hemolymph and identified 28 spots, representing 26 individual proteins (Table 2). Most of these proteins have known or predicted functions in immunity. These include prophenoloxidase 2 subunit, two clip-domain serine proteases (CLIP B4 and A6), a thioester protein (TEP15), two serpins (SRPN2 and SRPN15), a cystatin and apolipophorin III (apoLPIII). Other proteins are implied, in iron transport like ferritin, or lipid biology. Many of the proteins have been found in hemolymph in other insects but one protein is novel: a new member of the MD-2-related lipid-recognition family. Fourteen spots were induced following bacterial injection but not by wounding. Three of the identified proteins increased in spot intensity or appeared de novo following bacterial injection: a phenoloxidase, and two chitinase-like proteins. A subset of proteins decreased following bacterial injections: these included the light and heavy chains of ferritin. Several proteins appeared in hemolymph following any wound or injection. These included two isoforms of phosphoglycerate mutase (PGM), triose phosphate isomerase (TPI), an actin, glutathione S transferase S1-1, and adenylate kinase. Most of these are metabolic enzymes lacking signal peptides that are likely to be released as a result of damage to muscles and other tissues by injury. The map obtained is a useful tool for examining changes in hemolymph proteins following blood feeding and infection by parasites.

Interestingly, antibodies raised against hemolymph proteins were shown to decrease mosquito infection by *Plasmodium vivax* [26]. These antibodies are directed against 11 different antigens, 6 of which were specific of the hemolymph and four were common to midgut and ovary. Identification of these antigens by mass spectrometry could lead to the development of blocking vaccines against malaria.

### 2.3 Proteomics of salivary glands and saliva

Mosquito saliva and salivary glands are central to the interaction between parasite, vector and mammalian host. Sporozoite maturation in the mosquito salivary glands before its transmission to vertebrates is a key stage for the effective transmission to humans since it increases the sporozoite's ability to infect vertebrate hepatocytes [27; 28]. Additionally,

sporozoites are injected into the vertebrate skin with nanoliter volumes of saliva. Saliva is a complex biologically active solution, contains a large number of biomolecules responsible for antihemostatic activity, which assist hematophagous arthropods during the feeding process [29].

| **Hemolymph** | Protein/gene ID | Domain/motifs/Known/putative function/comments | Putatively secreted |
|---|---|---|---|
| Constitutevely expressed proteins | agCP2491 | OBP9, odorant binding protein | yes |
| | agCP1503 | apoLP-III, Apolipophorin IIII | yes |
| | agCP1469 | Ferritin, heavy | yes |
| | agCP1261 | Ferritin, light chain | yes |
| | agCP5831 | AgLLP1, lipocalin-like protein | yes |
| | agCP10937 | D7H1, salivary D-7 like protein | yes |
| | agCP3566 | TEP15, thioester protein | yes |
| | agCP9948 | AgH-1, hemolymph glycoprotein family | yes |
| | agCP9547 | ML3, ML-domain protein | yes |
| | AF007166 | CLIPA6, Clip domain serine protease | yes |
| | agCP9254 | CLIPB4, clip domain serine protease | yes |
| | agCp3768 | Srpn15, putative haplotype of Srpn9 Serpin | yes |
| | ebiP6959 | Srpn2, Serpin | yes |
| | agCP1375 | chitinase | no |
| | agCP1985 | Cystatin | yes |
| | ebiP1964 | aldo/keto reductase | no |
| | AF004915 | PPO2, prophenoloxidase subunit | no |
| | AF513639 | GST-S1-2, glutathione S transferase | no |
| Proteins modulated | | | |
| appearing after any injection | BM624855 | GST-SI-I, glutathione S transferase | no |
| | agCP12756 | Phosphoglycerate mutase | no |
| | agCP12096 | Triosephosphate isomerase | no |
| | BM622046 | Adenylate cyclise | no |
| appearing or altered after bacterial injury | AY496420 | BR-1, chitinase-like | yes |
| | AY496421 | BR-2, chitinase-like | yes |
| | AJ010195 | PO6, phenoloxidase | no |

Table 2. Proteins identified in *Anopheles gambiae* hemolymph

Following SDS-PAGE of *A. gambiae* salivary proteins, under denaturing non-reducing conditions and Edman sequencing, 12 saliva proteins were identified [30]. Among these proteins were *A. gambiae* D7-related proteins 1-3, similar to *Aedes aegypti* D7. The D7 proteins are among the most abundant salivary proteins in adult female mosquitoes and sand flies [31]. D7 sequences were identified in *Culex quinquefasciatus*, *An. arabiensis, An. stephensi, An. darlingi* mosquitoes and *Lutzomyia longipalpis* and *Phlebotomus papatasi* sand flies.

The other identified proteins were GSG6, hypothetical protein 8 (CB1), similar to hypothetical protein 9 (bB2) and herein called HP 9-like, SG1-like 2, putative 5′ nucleotidase and SG1. Edman degradation for other bands was reported to be unsuccessful, either because the protein's amino terminus was blocked, or because PTH-amino acids could not be reliably identified.

A proteomic analysis of salivary glands from female *A. gambiae* mosquitoes was carried out [32]. Salivary gland extracts were hydrolyzed with trypsin using a 1-DE in-gel and an in solution digestion and analyzed by LC-MS/MS. This led to identification of 69 unique proteins, 57 of which were novel. A large proportion of the identified proteins were involved in protein, carbohydrate and nucleic acid metabolism, transport or energy pathways. Almost 25% of the proteins could be not be ascribed any biological function. A subcellular localization was assigned to each protein either based on the literature or the presence of particular domains/motifs. The majority of the proteins were classified as extracellular proteins. D7 family proteins, apyrases and proteins of the salivary gland-like (SGlike) family were the commonest extracellular proteins. Proteins involved in translation and protein folding were the predominant cytoplasmic proteins with a small number of proteins classified as nuclear, vesicular or lysosomal proteins. Approximately 40% of proteins could not be assigned any specific localization because of lack of any distinctive features and lack of homology to other known proteins.

To further describe *A. gambiae* salivary gland and saliva contents, several techniques: 1-DE, 2-DE and LC-MS/ MS were combined to characterize the protein content of 8 year-old and 21 year-old mosquito salivary glands [33]. Overall, this study has identified five saliva proteins and 122 more proteins from the salivary glands, including the first proteomic description for 89 of these salivary gland proteins. Proteomic analysis of 8 year-old blood-fed mosquitoes allowed the identification of 55 proteins. LC-MS/ MS and 2-DE-MS identified a similar number of proteins and both appear more effective than 1-DE-MS. Thirty percent of the proteins identified are secreted (Table 3).

2D-E profiles suggested that several secreted proteins may present sequence divergence or be extensively processed and/or post-translationally modified in *A. gambiae* salivary glands.

Since the invasion and sporozoite maturation take place during the process of salivary glands ageing, the effect of salivary gland age on salivary component composition was examined. LC MS/MS profiling of young *versus* old salivary gland proteomes suggests that there is an over-representation of proteins involved in signalling and proteins related to the immune response in the proteins from older mosquitoes.

A comparative proteomic analysis of salivary gland samples from infected or *Plasmodium berghei*-free mosquitoes was performed using the iTRAQ labeling.

The expression levels of five secreted proteins were altered when the parasite was present. The levels of GSG6, apyrase, D7 related-1 protein precursor and D7 precursor allergen AED A2 are decreased whereas the level of gVAG is increased in infected salivary glands. Apyrase, GSG6 and D7 precursor allergen AED A2 were shown to be implied in blood feeding [34; 35; 36]. In addition, GSG6 was found to be a serological indicator of exposure to Afrotropical malaria vectors [37].

| **Secreted salivary gland proteins** | Protein/gene ID | Domain/motifs/Known/putative function/comments |
|---|---|---|
| | GI:18389879 | 30 kDa protein, anti-platelet |
| | AGAP011971-PA | Alpha-amylase, carbohydrate digestion |
| | AGAP008278-PA | Apyrase, anti-aggregation |
| | AGAP008284-PA | D7 precursor allergen AED A2 |
| | AGAP008282-PA | D7 related-1 protein precursor, anti-inflammatory |
| | AGAP008283-PA | D7 related-2 protein precursor, anti-inflammatory |
| | AGAP008281-PA | D7 related-3 protein precursor, anti-inflammatory |
| | AGAP008280-PA | D7 related-4 protein precursor, anti-inflammatory |
| | GI:4127305 | D7 related-5 protein precursor |
| | AGAP004334-PA | GSG3 |
| | AGAP000150-PA | GSG5 precursor |
| | AGAP008216-PA | GSG6, implied in blood-feeding |
| | AGAP008307-PA | GSG7 |
| | AGAP008306-PA | Guanine nucleotide releasing factor |
| | AGAP006494-PA | putative gVAG protein precursor, implied in defense |
| | AGAP003168-PA | Hypothetical 10 kDa protein |
| | AGAP006821-PA | Hypothetical 10.2 kDa protein |
| | AGAP001903-PA | Hypothetical 8.8 kDa protein |
| | AGAP006340-PA | Lysozyme precursor |
| | AGAP004038-PA | Maltase, carbohydrate digestion |
| | AGAP000607-PA | Putative 5′ nucleotidase precursor, anti-platelet |
| | AGAP006507-PA | putative Salivary protein SG |
| | AGAP000175-PA | Salivary D3 protein |
| | AGAP012335-PA | Salivary gland 1-like 3 protein |
| | | SG3 |

Table 3. List of *Anopheles gambiae* salivary proteins possessing a signal peptide and detected by proteomic approaches ([30; 32; 33]

The D7 short proteins bind serotonin with high affinity, as well as histamine and norepinephrine, thus antagonizing the vasoconstrictor, platelet-aggregating, and pain-inducing level of these factors [38]. gVAG is a defense-related protein [39]. These observations suggest an important role of these proteins in the interaction between *A. gambiae*, *Plasmodium* and the mammalian host and a putative modulation of the feeding behaviour of the parasitized mosquito.

### 2.4 *Anopheles gambiae* head proteome

Many of the most dangerous pathogens have been shown to manipulate the behaviour of their vectors, such as feeding behaviour, in ways that enhance the contact with the vertebrate host and consequently favour pathogen transmission {40]. Several studies with different systems support the idea that parasites indeed increase the probing and feeding rate of their vectors by a variety of mechanisms [40].

In the *Plasmodium-Anopheles* interaction, facts showing that *Plasmodium* spp. influence the behaviour of their mosquito vectors, *Anopheles* spp. have accumulated [41; 42; 43; 44; 45; 46]. In this system, an altered behaviour of the vector has been shown when sporozoites have invaded the salivary glands [43; 45; 46]. For instance, malaria parasites were shown to manipulate their mosquito vectors in two different ways and in a stage-dependent mode: when the sporozoites are ready to be transmitted to the vertebrate hosts, the parasite increases the biting rate of its vector [45; 46]. In contrast, at the oocyst stage and yet not transmissible to the vertebrate host, the parasite decreases the contact between vector and vertebrate host by decreasing the natural host-seeking behaviour of the insect [46]. Moreover, it was recently shown that the presence of the parasite extends the oviposition cycle of the mosquito, thereby enhancing malaria transmission [47]. Interestingly, few studies have been undertaken to demonstrate the existence of a general manipulative mechanism by which the parasite manipulate the host central nervous system. A 2-D DIGE coupled with MS was used to analyse and compare the head proteome of *A. gambiae* infected with *Plasmodium berghei* with that of uninfected mosquitoes [48]. This approach allowed detecting modulations of 12 protein spots in the head of mosquitoes infected with sporozoites. After their identification by MS, these proteins were functionally classified as belonging to metabolic, synaptic, molecular chaperone, signalling and cytoskeletal groups. These results indicate an altered energy metabolism in the head of sporozoite-infected mosquitoes. Some of the up-/down-regulated proteins identified, such as synapse-associated protein, 14-3-3 protein and calmodulin, have previously been shown to play critical roles in the CNS of both invertebrates and vertebrates. Interestingly, two proteins revealed in the study have been demonstrated to be involved in behavioural modifications in other host–parasite systems. Tropomyosin has been shown to be involved in the behavioural manipulation of crustacean gammarids by acanthocephalans [49], while phosphoglycerate mutase was involved in cricket behavioural manipulation induced by hairworms [50]. Furthermore, a heat shock response (HSP 20) and a variation of cytoarchitecture (tropomyosins) have been shown. Discovery of these proteins demonstrates potential molecular mechanisms that trigger behavioural modifications and offers new insights into the study of close interactions between *Plasmodium* and its *Anopheles* vector.

### 2.5 Proteome of the eggshell

Insect eggshells offer the embryo protection from physical and biological damages and assure their survival. Most current knowledge of insect eggshell morphology and composition are based on studies of *Drosophila melanogaster* [51; 52]. Mosquito eggshells show notable diversity in physical properties and structure, presumably resulting from adaptation to the large variety of environments exploited by these insects.

In contrast to *Aedes* mosquitoes, which are highly resistant to desiccation allowing embryos to survive for months in dry conditions [53], eggshells of the human malaria vector, *A. gambiae* are more permeable, restricting their survival and development to humid environments [54]. Greater knowledge of the proteins that comprise eggshells is required to understand these differences and how they contribute to successful mosquito reproductive strategies. A mass spectrometry/proteomics approach was used to identify 44 proteins as putative components of the eggshell (Table 4) [55]. Among the identified molecules are two vitelline membrane proteins and a group of seven putative chorion proteins. Enzymes with

peroxidase, laccase and phenoloxidase activities, expected to be involved in cross-linking reactions that stabilize the eggshell structure, were also identified. Seven odorant binding proteins were found in association with the mosquito eggshell, although their role has yet to be demonstrated.

| **Eggshell** | Protein/gene ID | Domain/motifs/Known/putative function/comments |
|---|---|---|
| | Odorant binding proteins | |
| | AGAP000641 | OBP34/37 |
| | AGAP000642 | OBP35 |
| | AGAP000643 | OBP36 |
| | AGAP002025 | OBP11 |
| | AGAP011647 | OBP1 |
| | AGAP010648 | OBP44 |
| | AGAP002189 | OBP13 |
| | Enzymes | |
| | AGAP004038 | chorion peroxidase |
| | AGAP006176 | Laccase 2 |
| | AGAP005959 | Yellow-g2-dopachrome conversion enzyme |
| | AGAP004978 | Prophenoloxidase 9 |
| | AGAP003233 | Peroxidase |
| | AGAP007020 | Thioredoxin |
| | Vitelline membrane components | |
| | AGAP002134 | Vitelline membrane component |
| | AGAP008696 | Vitelline membrane component |
| | Putative chorion components | |
| | AGAP00655 | Putative chorion protein |
| | AGAP006555 | Putative chorion protein |
| | AGAP006553 | Putative chorion protein |
| | AGAP006554 | Putative chorion protein |
| | AGAP006549 | Putative chorion protein |
| | AGAP006556 | Putative chorion protein |
| | AGAP006550 | Putative chorion protein |
| | Others | |
| | AGAP006524 | Unknown |
| | AGAP006563 | Unknown |
| | AGAP003149 | Unknown |
| | AGAP010147 | Unknown |
| | AGAP004182 | Unknown |
| | AGAP006527 | Unknown |
| | AGAP003047 | Schistosoma mansoni egg protein |
| | AGAP004969 | Ionotropic glutamate receptor |

| | | |
|---|---|---|
| | AGAP010252 | Ribosomal protein L14 |
| | AGAP002306 | Ribosomal protein L4 |
| | AGAP002830 | c-1-Tetrahydrofolate synthase |
| | AGAP012261 | Unknown |
| | AGAP005802 | Unknown |
| | AGAP005061 | Unknown |
| | AGAP000547 | Unknown |
| | AGAP006584 | Unknown |
| | AGAP005338 | Unknown |
| | AGAP003911 | Unknown |
| | AGAP004887 | Ribosomal protein S17 |
| | AGAP000672 | Unknown |
| | AGAP007758 | Unknown |
| | AGAP006805 | Unknown |

Table 4. Proteome of *Anopheles gambiae* eggshell

### 2.6 Functional genomics approaches

Genomics, transcriptomics and proteomics studies have been complemented by studies using RNAi for gene silencing. RNAi allows characterization of genes *in vivo* which can later be targeted for transmission blocking studies. Boisson et al. [56] demonstrated the role of the gene AgApy, which encodes an apyrase, in the probing behaviour of *A. gambiae*. An RNAi-mediated gene silencing approach has also been used to assess the potential involvement of 10 selected *A. gambiae* salivary gland genes in regulating mosquito blood-feeding capacity [36]. Silencing of several salivary gland transcripts; D7L2, anophelin, peroxidase, 5'nucleotidase and SG2 precursor, produced a significantly lowered blood-feeding phenotype and increased probing time, confirming that these genes could play an important role in blood-feeding.

## 3. Conclusion

In a recent publication, the results of an extensive qualitative proteomic analysis of *Anopheles gambiae* to better understand gene structures and their functions were presented [57]. In their manuscript, the authors reported validation of existing genes, correction of existing gene models, identification of novel genes, identification of novel splice variants, confirmation of splice sites and assignment of translational start sites based on high-resolution mass spectrometry-derived data. A total of 2,682 peptides were identified that could not be mapped onto existing VectorBase annotations. This study emphasizes on the interest of proteomic tools to complement other approaches for genome annotation.

Dissecting the molecular basis of the interplay between vector and pathogen is essential for vector-borne disease transmission. Deciphering the proteome of the main mosquito barriers for parasite development and transmission may pave the way to novel disease control mechanisms.

## 4. References

[1] Crompton P.D., Pierce S.K. & Miller L.H. (2010) Advances and challenges in malaria vaccine development. *J Clin Invest* 120, 12 (Dec 2010), pp. 4168-4178, 1558-8238.

[2] Lavazec C. & Bourgouin C. (2008). Mosquito-based transmission blocking vaccines for interrupting Plasmodium development. *Microbes Infect* 10, 8 (Jul 2008), pp. 845-849, 1286-4579.

[3] Vlachou D., Schlegelmilch T., Christophides G.K. & Kafatos F.C. (2005) Functional genomic analysis of midgut epithelial responses in Anopheles during *Plasmodium* invasion. *Curr Biol* 15, 13 (Jul 2005), pp. 1185-1195, 0960-9822.

[4] Aly A.S., Vaughan A.M. & Kappe S.H. (2009) Malaria parasite development in the mosquito and infection of the mammalian host. *Annu Rev Microbiol* 63, pp. 195-221, 1545-3251.

[5] Killeen G.F., McKenzie F.E., Foy B.D., Schieffelin C., Billingsley P.F. & Beier J.C. (2000) A simplified model for predicting malaria entomologic inoculation rates based on entomologic and parasitologic parameters relevant to control. *Am J Trop Med Hyg*. 62, 5 (May 2000), pp. 535-44, 0002-9637.

[6] Zdobnov E.M., von Mering C., Letunic I., Torrents D., Suyama M., Copley R.R., et al. (2002) Comparative genome and proteome analysis of *Anopheles gambiae* and *Drosophila melanogaster*. *Science* 298, 5591 (Oct 2002), pp 149-59, 1095-9203.

[7] Holt R.A., Subramanian G.M., Halpern A., Sutton G.G., Charlab R., Nusskern D.R., et al. (2002). The genome sequence of the malaria mosquito *Anopheles gambiae*. *Science*. 298, 5591 (Oct 2002):129-49, 0036-8075.

[8] Adams M.D., Celniker S.E., Holt R.A., Evans C.A., Evans C.A, Gocayne J.D, et al. (2000) *The genome sequence of Drosophila melanogaster*. *Science*. 287, 5461 (Mar 2000), pp 2185-2195, 0036-8075.

[9] Nene V., Wortman J.R., Lawson D., Haas B., Kodira C., Tu ZJ., et al. (2007) Genome sequence of Aedes aegypti, a major arbovirus vector. *Science.* 316, 5832 (Jun 2007), pp. 1718-23, 1095-9203.

[10] Riehle M.M., Markianos K., Niaré O., Xu J., Li J., Touré A.M., Podiougou B., Oduol F., Diawara S., Diallo M., Coulibaly B., Ouatara A., Kruglyak L., Traoré S.F. & Vernick K.D. (2006) Natural malaria infection in Anopheles gambiae is regulated by a single genomic control region. *Science*. 312, 5773 (Apr 2006), pp. 577-579,1095-9203.

[11] Kwon H.W., Lu T., Rutzler M. & Zwiebel L.J. (2006) Olfactory responses in a gustatory organ of the malaria vector mosquito Anopheles gambiae. *Proc Natl Acad Sci* U S A 103, 6 (Sep 2006), pp. 13526-13531, 0027-8424.

[12] Gingras, A. C., Aebersold, R. & Raught, B. (2005) Advances in protein complex analysis using mass spectrometry. *J Physiol* 563 Pt1 (Dec 2004), 11-21, 0022-3751.

[13] Mayer D., Baginsky S. & Schwemmle M. (2005) Isolation of viral ribonucleoprotein complexes from infected cells by tandem affinity purification. *Proteomics* 5, 17 (Nov 2005), pp. 4483-4487. 1615-9853.

[14] Jorba N., Juarez S., Torreira E., Gastaminza P., Zamarreno N., Albar J.P. & Ortin J. (2008) Analysis of the interaction of influenza virus polymerase complex with human cell factors (2008) *Proteomics* 8, 10 (May 2008), p. 2077-2088, 1615-9861.

[15] Gygi S.P., Rist B., Gerber S.A., Turecek F., Gelb M.H. & Aebersold R. (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags (1999) *Nat. Biotechnol.* 17, 10 (Oct 1999), pp. 994-999, 1087-0156.

[16] Guaragna A., Amoresano A., Pinto V., Monti G., Mastrobuoni G., Marino G. & Palumbo G. (2008) Synthesis and proteomic activity evaluation of a new isotope-coded affinity tagging (ICAT) reagent. *Bioconjug. Chem.* 19, 5 (May 2008), pp. 1095-2104, 1520-4812.

[17] Ross P.L., Huang Y.L.N., Marchese J.N., Williamson B., Parker K., Hattan S., et al. (2004) Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. *Mol. Cell. Proteomics* 3, 12 (Dec 2004), pp. 1154-1169, 1535-9476.

[18] Ong S.E., Blagoev B., Kratchmarova I., Kristensen D.B., Steen H., Pandey A. & Mann M. (2002) Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol. Cell. Proteomics* 1, 5 (May 2002), p. 376-386, 1535-9476.

[19] Mann M. (2006) Functional and quantitative proteomics using SILAC *Nat. Rev. Mol. Cell Biol.* 7, 12 (Dec 2006), pp. 952-958, 1471-0072.

[20] Tellam R.L., Wijffels G. & Willadsen P. (1999) Peritrophic matrix proteins. *Insect Biochem Mol Biol* 29, 2 (Feb 1999), pp. 87-101, 0965-1748.

[21] Billingsley P.F. & Rudin W. (1992) The role of the mosquito peritrophic membrane in bloodmeal digestion and infectivity of Plasmodium species. *J Parasitol* 78, 3 (Jun 1992), pp. 430-440, 0022-3395.

[22] Huber M., Cabib E. & Miller L.H. (1991) Malaria parasite chitinase and penetration of the mosquito peritrophic membrane. *Proc Natl Acad Sci* U S A 88, 7 (Apr 1991), pp. 2807-2810, 0027-8424.

[23] ] Dinglasan R.R., Devenport M., Florens L., Johnson J.R., McHugh C.A., Donnelly-Doman M., Carucci D.J., Yates J.R. 3rd & Jacobs-Lorena M. (2009) The *Anopheles gambiae* adult midgut peritrophic matrix proteome. *Insect Biochem Mol Biol* 39, 2 (Feb 2009), pp. 125-134, 1879-0240.

[24] Pinto S.B., Lombardo F., Koutsos A.C., Waterhouse R.M., McKay K., An C., Ramakrishnan C., Kafatos F.C. & Michel K. (2009) Discovery of *Plasmodium* modulators by genome-wide analysis of circulating hemocytes in Anopheles gambiae. *Proc Natl Acad Sci* U S A 106, 50 (Dec 2009), pp. 21270-21275, 1091-6490.

[25] Paskewitz S.M. & Shi L. (2005) The hemolymph proteome of Anopheles gambiae. *Insect Biochem Mol Biol* 35, 8 (Aug 2005), pp. 815-824, 0965-1748.

[26] Kumari A., Gakhar S.K. & Hooda V. (2009) Antibodies raised against hemolymph of *Anopheles culicifacies* reduce the fecundity and malaria parasite development. *J Vector Borne Dis* 46, 4 (Dec 2009), pp. 255-260,0972-9062.

[27] Kappe S.H., Gardner M.J., Brown SM, Ross J, Matuschewski K., Ribeiro J.M., Adams J.H., Quackenbush J., Cho J., Carucci D.J., Hoffman S.L. & Nussenzweig V. (2001) Exploring the transcriptome of the malaria sporozoite stage. *Proc Natl Acad Sci* U S A 98, 17 (Aug 2001), pp. 9895-9900, 0027-8424.

[28] Kappe S.H., Buscaglia C.A. & Nussenzweig V. (2004) *Plasmodium* sporozoite molecular cell biology. *Annu Rev Cell Dev Biol* 20, pp. 29-59, 1081-0706.

[29] Ribeiro JM, Francischetti IM. (2003) Role of arthropod saliva in blood feeding: sialome and post-sialome perspectives. Annu Rev Entomol 48 (Jun 2003), pp.73-88, 0066-4170.

[30] Francischetti, I. M., Valenzuela, J. G., Pham, V. M. , Garfield, M. K., Ribeiro, J. M. (2002) Toward a catalog for the transcripts and proteins (sialome) from the salivary gland of the malaria vector *Anopheles gambiae*. *J Exp Biol* 205 Pt16 (Jul 2002), 2429-2451, 0022-0949.

[31] Valenzuela, J. G., Charlab, R., Gonzalez, E. C., de Miranda-Santos, I. K., Marinotti, O., Francischetti, I. M., Ribeiro, J. M. (2002) The D7 family of salivary proteins in blood sucking diptera. *Insect Mol Biol* 11, 2 (Apr 2002), 149-155, 0962-1075

[32] Kalume D.E., Okulate M., Zhong J., Reddy R., Suresh S., Deshpande N., Kumar N. & Pandey A. (2005) A proteomic analysis of salivary glands of female *Anopheles gambiae* mosquito. *Proteomics* 5, 14 (Sep 2005), pp. 3765-3777, 1615-9853

[33] Choumet V., Carmi-Leroy A., Laurent C., Lenormand P., Rousselle J.C., Namane A., Roth C. & Brey P.T. (2007) The salivary glands and saliva of *Anopheles gambiae* as an essential step in the *Plasmodium* life cycle: a global proteomic study. *Proteomics* 7, 18 (Sep 2007), pp.3384-94, 1615-9853.

[34] Champagne D.E. (2005) Antihemostatic molecules from saliva of blood-feeding arthropods. *Pathophysiol Haemost Thromb* 34, 4-5, pp. 221-227, 1424-8832

[35] Lombardo F., Ronca R., Rizzo C, Mestres-Simòn M., Lanfrancotti A., Currà C., Fiorentino G., Bourgouin C., Ribeiro J.M., Petrarca V., Ponzi M., Coluzzi M. & Arcà B. (2009) The *Anopheles gambiae* salivary protein gSG6: an anopheline-specific protein with a blood-feeding role. I*nsect Biochem Mol Biol* 39, 7 (Jul 2009), pp. 457-466, 1879-0240.

[36] Das S., Radtke A., Choi Y. J., Mendes A. M., Valenzuela J. G., Dimopoulos G. (2010) Transcriptomic and functional analysis of the *Anopheles gambiae* salivary gland in relation to blood feeding. BMC Genomics 11 (Oct 2010), 566, 1471-2164.

[37] Rizzo C., Ronca R, Fiorentino G., Mangano V.D., Sirima S.B, Nèbiè I., Petrarca V., Modiano D. & Arcà B. (2011) Wide cross-reactivity between *Anopheles gambiae* and *Anopheles funestus* SG6 salivary proteins supports exploitation of gSG6 as a marker of human exposure to major malaria vectors in tropical Africa (2011) *Malar J* 10, 206 (Jul 2011), pp. 1475-2875, 1475-2875.

[38] Calvo, E., Mans, B. J., Ribeiro, J. M., Andersen, J. F. (2009) Multifunctionality and mechanism of ligand binding in a mosquito antiinflammatory protein. *Proc. Natl. Acad. Sci. USA* 2009, 106, 3728-3733.

[39] Dong Y., Aguilar R., Xi Z., Warr E., Mongin E. & Dimopoulos G. (2006*) Anopheles gambiae* immune responses to human and rodent *Plasmodium* parasite species. *PLoS Pathog* 2, 6 (Jun 2006), pp. e52, 1553-7374.

[40] Hurd H. (2003) Manipulation of medically important insect vectors by their parasites. *Annu Rev Entomol* 48, pp. 141-161. 0066-4170

[41] Rossignol P.A., Ribeiro J.M. & Spielman A. (1984) Increased intradermal probing time in sporozoite-infected mosquitoes. *Am J Trop Med Hyg* 33, 1 (Jan 1984), pp. 17-20, 0002-9637.

[42] Rossignol P.A., Ribeiro J.M. & Spielman A. (1986) Increased biting rate and reduced fertility in sporozoite-infected mosquitoes. *Am J Trop Med Hyg* 35, 2, pp. 277-279. 0002-9637

[43] Wekesa J.W., Copeland R.S. & Mwangi R.W. (1992) Effect of *Plasmodium falciparum* on blood feeding behavior of naturally infected *Anopheles* mosquitoes in western Kenya. *Am J Trop Med Hyg* 47, 4 (Oct 1992), pp. 484-488, 0002-9637.

[44] Koella J.C. & Packer M.J. (1996) Malaria parasites enhance blood-feeding of their naturally infected *vector Anopheles punctulatus*. *Parasitology* 113 ( Pt 2), pp. 105-109, 0031-1820

[45] Koella J.C., Sorensen F.L. & Anderson R.A. (1998) The malaria parasite, *Plasmodium falciparum*, increases the frequency of multiple feeding of its mosquito vector, *Anopheles gambiae*. *Proc Biol Sci* 265, 1398 (May 1998), pp: 763-768, 0962-8452.

[46] Anderson R.A., Koella J.C. & Hurd H. (1999) The effect of Plasmodium yoelii nigeriensis infection on the feeding persistence of *Anopheles stephensi* Liston throughout the sporogonic cycle. *Proc Biol Sci* 266, 1430 (Sep 1999), pp. 1729-1733, 0962-8452.

[47] Charlwood JD, Tomás EV.Do developing malaria parasites manipulate their mosquito host? Evidence from infected *Anopheles funestus* (Giles) from Mozambique (2011) *Trans R Soc Trop Med Hyg* 105, 6 (Jun 2011), pp. 352-354, 1878-3503.

[48] Lefevre T., Thomas F., Schwartz A., Levashina E., Blandin S., Brizard JP, Le Bourligu L., Demettre E., Renaud F & Biron D.G. (2007) Malaria *Plasmodium* agent induces alteration in the head proteome of their *Anopheles* mosquito host. *Proteomics* 7, 11 (Jun 2007), pp: 1908-1915, 1615-9853.

[49] Biron D.G., Marché L., Ponton F., Loxdale H.D., Galéotti N., Renault L., Joly C., Thomas F. (2005) Behavioural manipulation in a grasshopper harbouring hairworm: a proteomics approach. *Proc Biol Sci* 272, 1577 (Oct 2005), pp. 2117-2126, 0962-8452.

[50] Ponton F., Lefevre T., Lebarbenchon C., Thomas F., Loxdale H.D., Marché L., Renault L., Perrot-Minnot M.J. & Biron DG. (2006) Do distantly related parasites rely on the same proximate factors to alter the behaviour of their hosts? *Proc Biol Sci* 273, 1603 (Nov 2006), pp. 2869-2877, 0962-8452.

[51] Waring G.L. (2000) Morphogenesis of the eggshell in *Drosophila*. *Int Rev Cytol* 198, pp. 67-108. 0074-7696.

[52] Cavaliere V., Bernardi F., Romani P., Duchi S. & Gargiulo G. (2008) Building up the *Drosophila* eggshell: first of all the eggshell genes must be transcribed. *Dev Dyn* 237, 8 (Aug 2008), pp. 2061-2072, 1058-8388.

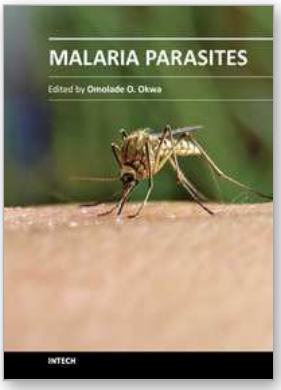
[53] Sota T. & Mogi, M. (1992) Interspecific variation in desiccation survival time of *Aedes* (Stegomyia) mosquito eggs is correlated with habitat and egg size. *Oecologia* 90, pp. 353–358.

[54] Beier J.C., Copeland R., Oyaro C., Masinya A., Odago W.O., Oduor S., Koech D.K. & Roberts C.R. (1990) *Anopheles gambiae* complex egg-stage survival in dry soil from larval development sites in western Kenya. *J Am Mosq Control Assoc* 6, 1 (Mar 1990), pp. 105-109, 8756-971X.

[55] Amenya D.A., Chou W., Li J., Yan G., Gershon P.D., James A.A. & Marinotti O. (2010) Proteomics reveals novel components of the *Anopheles gambiae* eggshell. *J Insect Physiol* 56, 10 (Oct 2010), pp: 1414-1419, 1879-1611.

[56] Boisson B., Jacques J.C., Choumet V., Martin E., Xu J., Vernick K. & Bourgouin C. (2006) Gene silencing in mosquito salivary glands by RNAi. *FEBS Lett* 580, 8 (Apr 2006), pp. 1988-1992, 0014-5793.Calvo E., Mans B.J., Andersen J.F. & Ribeiro J.M. (2006) Function and evolution of a mosquito salivary protein family. *J Biol Chem* 281, 4 (Jan 2006), pp. 1935-1942, 0021-9258.

[57] Chaerkady R., Kelkar D.S., Muthusamy B., Kandasamy K., Dwivedi S.B., Sahasrabuddhe N.A., et al. (2011) A proteogenomic analysis of *Anopheles gambiae* using high-resolution Fourier transform mass spectrometry. Genome Res. 2011 Jul 27.




**Malaria Parasites**

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Malaria is a global disease in the world today but most common in the poorest countries of the world, with 90% of deaths occurring in sub-Saharan Africa. This book provides information on global efforts made by scientist which cuts across the continents of the world. Concerted efforts such as symbiont based malaria control; new applications in avian malaria studies; development of humanized mice to study P.falciparium (the most virulent species of malaria parasite); and current issues in laboratory diagnosis will support the prompt treatment of malaria. Research is ultimately gaining more grounds in the quest to provide vaccine for the prevention of malaria. The book features research aimed to bring a lasting solution to the malaria problem and what we should be doing now to face malaria, which is definitely useful for health policies in the twenty first century.

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