CHAPTER I

Introduction

1.1 Statement and Significance of the Problem

Malaria remains one of the most important infectious diseases in the world, and despite some progress in control has re-emerged in tropical regions that have experienced rapid population growth. *Anopheles* mosquitoes are the exclusive vectors of human malaria. Salivary glands are of interest in the *Anopheles* mosquito because malaria sporozoites must invade the mosquito salivary glands in order to be transmitted to a human host. When *Plasmodium* sporozoites invade the salivary glands, the mosquito becomes infectious. It is known that sporozoites only invade the distal portion of the lateral and medial lobes and that recombinant circumsporozoite (CS) protein binds specifically to *Anopheles stephensi* salivary glands, particularly to the medial and distal-lateral lobes of the gland. The salivary glands contain a variety of physiologically and biochemically active molecules that involve food ingestion and digestion. Mosquito saliva is vital for successful blood feeding because it contains anti-coagulant, anti-inflammation, and immunosuppressive factors. In addition, saliva proteins are antigenic and immunogenic, involving immunoglobulin E, immunoglobulin G and T-lymphocyte mediated hyposensitivity response in vertebrate hosts.

Recently, *Anopheles campestris*-like (Chiang Mai strain), a member of *Anopheles barbirostris* complex in Thailand, was infected experimentally with *Plasmodium vivax* and *Plasmodium falciparum*. The results revealed that *An. campestris*-like was a high-potential vector for *P. vivax*, with 66.67% sporozoite rates compared to 90% sporozoite rates recovered from *Anopheles cracens*, it was a refractory vector for *P. falciparum*. Preliminary analysis of female salivary gland proteins of *An. barbirostris* complexes was performed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and nano-liquid chromatography-mass spectrometry (NanoLC-MS). For *An.*

campestris-like, at least eight major and several minor protein bands were detected in the glands. NanoLC-MS analysis revealed that a major protein band matched with the protein, gSG6 involved in blood feeding of *Anopheles gambiae* and *Anopheles freeborni*. No more proteins were reported, as not all of them could be identified by this technique mentioned because some protein bands might consist of several polypeptides that have the same molecular mass but differ in isoelectric points (pI). Therefore, advanced techniques are required to analyze those proteins. Two-dimensional gel electrophoresis is a powerful and high throughput tool for describing the changes in protein expression and modification, which involve separation of cellular proteins according to their isoelectric points and relative molecular masses. The separation of proteins in a sample using 2-DE, and their subsequent identification by biological mass spectrometry (MS), are key elements of classical proteomics, which enable investigation of gene expression at the protein level. These techniques were used to study the salivary gland proteomes of several arthropods, for example, *An. gambiae*, *Aedes aegypti*, *Culicoides nubeculosus* and *Rhipicephalus haemaphysaloides*.

For the purpose of analyzing the salivary gland proteome of *An. campestris*-like, SDS-PAGE, 2-DE coupled with NanoLC-MS will be applied in this study. Proteome profiles of the salivary glands of sugar-fed and blood-fed mosquitoes will be compared to identify proteins secreted during blood feeding and development. Also, proteins differentially expressed in the salivary glands of male and each salivary gland lobe of female mosquitoes will be identified. The new data could contribute to understanding the physiological processes that appear during the blood meal and pathogen transmission to vertebrate hosts. In addition, its salivary gland transcriptome will be analyzed. Complimentary DNAs (cDNAs) encoding abundant salivary gland proteins of *An. campestris*-like will be isolated and identified. The information obtained from this study would help to predict and understand the role of salivary proteins in the mosquito. It also would be an initial step for further identification and characterization of anti- parasite-effector molecules, and promoters that may be useful in the development of genetically-transformed *Plasmodium*-refractory mosquitoes.

1.2 Literature Review

1.2.1 Malaria situation

Malaria is an entirely preventable and treatable mosquito borne illness. In 2014, the transmission of malaria occurs in six WHO regions, 97 countries had ongoing malaria transmission. An estimated 3.2 billion people are at risk of malaria, of which 1.2 billion are at high risk (Figure 1.1). In 2013, there were an estimated 198 million cases of malaria global (range 124–283 million), and malaria disease led to 584,000 deaths (range 367,000–755,000). In the WHO African region, 90 % of all malaria deaths occur and 78 % of all children under 5 years deaths. In 2000 to 2013, an expansion of malaria interferences helped to decrease malaria incidence and mortality rates of 30 % globally.

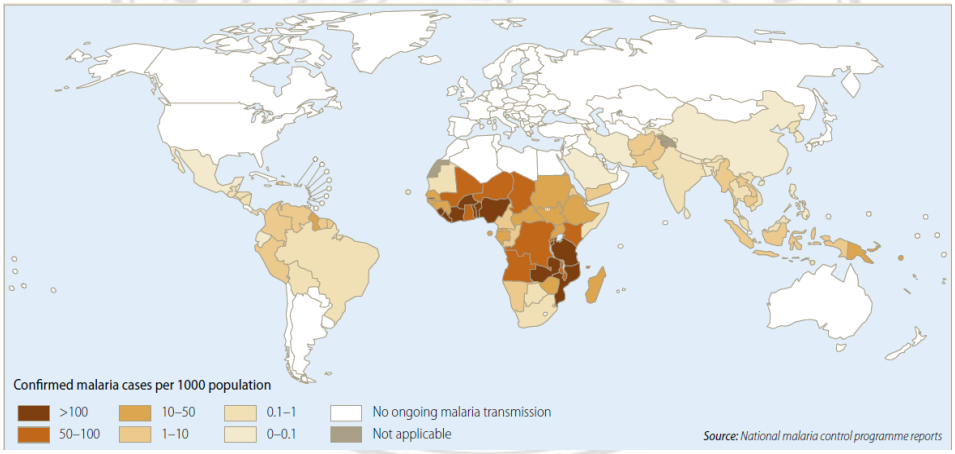

Figure 1.1 Countries with ongoing transmission of malaria (WHO 2014)

Malaria is caused by five species of the parasite belonging to the genus *Plasmodium*. Four of these – *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale* – are human malaria species, which are spread from one person to another by female *Anopheles* mosquitoes. There are about 400 different species, but only 30 of *Anopheles* mosquitoes are major vectors. In recent years human cases of malaria have also been recorded due to *P. knowlesi* – a species that causes malaria among monkeys, and occurs in certain forested areas of South-East Asia.

In the African continent, *P. falciparum* is most frequently and responsible for most deaths from malaria. *P. vivax* has a wider geographic distribution than *P. falciparum* because it can develop at lower temperatures in the Anopheles mosquito vector, and can survive in cooler climates and at higher altitudes. It also has a dormant liver stage (known as a hypnozoite) that enables it to survive for long periods as a potential reservoir of infection. The hypnozoites can activate months later to cause a relapse. Although *P. vivax* can occur throughout Africa, the risk of infection with this species is quite low, because of the absence in many African populations of the Duffy gene, which produces a protein necessary for *P. vivax* to invade red blood cells. In many areas outside Africa, infections due to *P. vivax* are more common than those due to *P. falciparum*.

1.2.2 Sporogonic cycle and salivary gland invasion of *Plasmodium* species in mosquito vectors

1) Sporogonic cycle of *Plasmodium* species in mosquitoes.

Gametocytes are ingested by a female Anopheline mosquito with the blood meal. The time require for *Plasmodium* development in the mosquito rages from 10-21 days (depending on species and temperature) and involves the transformation of gametocytes into gametes (Figure 1.2). The motile male gametes merge into female ones, produce zygotes within 1 hour that differentiate into motile ookinetes. To reach the hemocoel (the mosquito cavity), ookinetes must cross peritrophic matrix (PM) and the midgut epithelium in 24 hours. The ookinete also lodged between the epithelium and the basal lamina and transforms into an oocyst. The mature oocyst ruptures and releases thousands of sporozoites that make contact with many tissues and cell types, but it specifically recognizes and invades only the distal-lateral and medial lobes of the salivary glands (Sterling et al. 1973; Ghosh and Jacobs-Lorena 2009).

Figure 1.2 Life cycle of the *Plasmodium* parasite in its mosquito vector. Female (1) and male (2) gametocytes differentiate into gametes (3 and 4). After completion of meiosis, the male gametocyte generates eight gametes (4) in a process known as 'exflagellation'. A male gamete fertilizes a female gamete (5) to generate a zygote (6), which in turn differentiates into a motile ookinete (7). About 24 hr later, the mature ookinete first traverses the peritrophic matrix (orange line) and then the midgut epithelium (8), after which it differentiates into an oocyst (9). During the next 10 days the oocyst grows (10 and 11) and when mature, it releases sporozoites into the open haemolymph circulation (12). The circulating sporozoites recognize and invade the salivary glands (13) where they are stored until released at the time when the mosquito bites the next individual (Ghosh and Jacobs-Lorena 2009).

2) Salivary gland invasion

Initially, sporozoites also attach to the filamentous basal lamina by their anterior tip and along their entire length. As the parasite penetrates the basal lamina, it loses its thick coat, and the sporozoite anterior end is found pointing towards the epithelial cell. No information is available as to how the sporozoites traverse the basal lamina. Invasion begins with invagination of the epithelial cell membrane that

adopts the shape of the parasite, leading to the formation of a parasitophorous vacuole. The parasitophorous vacuole disintegrates during invasion and free parasites accumulate within the cell (Rodriguez and Hernández-Hernádez 2004). Next, the sporozoite invades the apical membrane of its host's epithelial cell, resulting in sporozoite release into the central secretary cavity of the gland. The ultrastructural studies details of sporozoite invasion of the salivary gland were conducted with *Aedes aegypti* and *Plasmodium. gallinaceum* (Sterling et al. 1973; Pimenta et al. 1994) (Figure 1.3). During early stages of infection, sporozoites are found mostly inside the cytoplasm of the secretary cell, while very few are seen inside the central secretary cavity. At later stages, sporozoites are found mostly inside the secretary cavity, where they aggregate into large bundles (Pimenta et al. 1994).

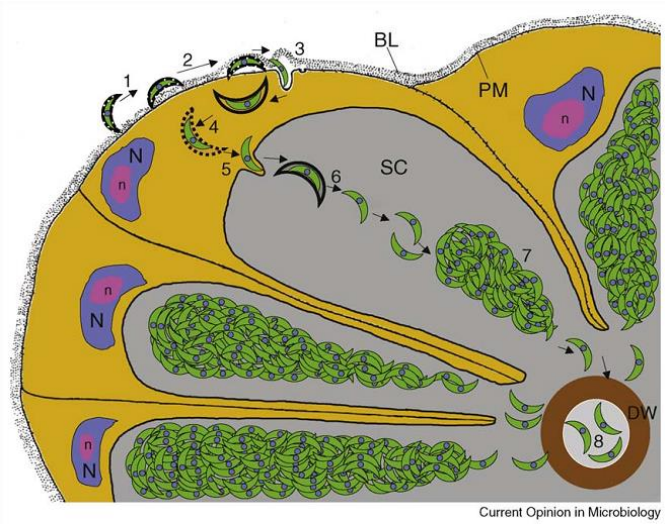

Figure 1.3 Progression of sporozoite invasion of the salivary gland (1) The sporozoite attaches to the basal lamina; (2) sporozoite passage to the space between the basal lamina and basal epithelial cell plasma membrane, a process associated with loss of the sporozoite's thick coat; (3) penetration of the basal plasma membrane; the sporozoite resides within a vesicle; (4) release of the sporozoite from the surrounding membrane by an unknown mechanism; (5) invasion of the apical membrane and entry into the secretory cavity; (6) sporozoites are released from the surrounding membrane by an unknown mechanism; (7) sporozoites assemble into bundles within the secretory cavity; and (8) a small number of sporozoites enter the secretory duct by an unknown mechanism. BL: basal lamina; DW: duct wall; N: nucleus; n: nucleolus; PM: plasma membrane; SC: secretory cavity (Pimenta et al. 1994).

2.1) Salivary gland receptors for sporozoite invasion:

Several previous studies, identify candidate receptors for sporozoite invasion have been reported. By using purified monoclonal antibodies that specifically bind to median and distal lateral-lobes, which one the regions preferentially invaded by sporozoites (Sterling et al. 1973), a new salivary gland surface (SGS) protein family was identified in *Ae. aegypti*. Each SGS gene is encoded by a ~ 10 kb open reading frame and all SGS proteins possess predicted multipass transmembrane domains near their C-terminal ends. Antibody raised against the aaSGS1 protein inhibits sporozoite invasion (Sterling et al. 1973). Four SGS orthologues, agSGS2, agSGS3, agSGS4 and agSGS5, are also found in *An. gambiae* (Korochkina et al. 2006).

Two additional proteins, which are only expressed in the distal-lateral lobes of female salivary glands, have been shown to serve as sporozoite receptors (Brennan et al. 2000; Korochkina et al. 2006). One of these proteins, called Saglin, is a secreted protein that contains several putative glycosylation sites (Okulate et al. 2007) and its expression is induced by blood feeding (Korochkina et al. 2006). Furthermore, Saglin can bind to the A domain of TRAP *in vitro* (Ghosh et al. 2009), which is the same domain as that previously required for parasite invasion (Matuschewski et al. 2002). It is likely that the binding of TRAP to Saglin is required also *in vivo*, as this interaction can be inhibited *in vivo* by a synthetic peptide, called SM1, which binds to Saglin and abolishes the parasite's ability to invade salivary glands (Ghosh et al. 2001).

2.2) Sporozoite ligands for salivary gland invasion:

To date, no data formally show rhoptry, dense granule or microneme secretion that is required for sporozoite invasion of the mosquito salivary gland. However, in salivary gland invasion important micronemal proteins have been present: the circumsporozoite protein (CSP); the thrombospondin-related anonymous protein (TRAP); the apical membrane antigen/erythrocyte binding-like protein (MAEBL); the up-regulated-in-oocysts sporozoite protein 3 (UOS3) (Mikolajczak et al. 2008), also called S6/TREP (Combe et al. 2009; Steinbuechel and Matuschewski 2009); and cysteine repeat modular proteins (PCRMPs) (Thompson et al. 2007).

2.2.1) Circumsporozoite protein (CSP): CSP is a parasite surface molecule essential for sporozoite development within oocysts and invasion of salivary glands (Menard et al. 1997; Sidjanski et al. 1997). In addition to oocyst development and sporozoite differentiation, CSP also seems to play a role in sporozoite recognition of the mosquito salivary glands. An anti-CSP monoclonal antibody completely suppresses invasion of *P. gallinaceum* sporozoites (Warburg et al. 1992). While this finding could be interpreted simply as antibodies causing steric hindrance for sporozoite access to the salivary gland surface, additional evidence suggests that CSP may play an active role. The recombinant protein binds to salivary glands in preference to other mosquito organs and this binding is inhibited by a highly conserved 15-amino acid motif that includes the 5-amino acid sequence known as region I (Dame et al. 1984; Lal et al. 1987; McCutchan et al. 1996; Sidjanski et al. 1997). Further experiments demonstrated that the 15-amino

acid peptide, as well as the whole CSP protein, inhibited sporozoite invasion (Myung et al. 2004). However, the 5-amino acid region I peptide by itself had no inhibitory activity (Sidjanski et al. 1997). In agreement with these findings, recombinant parasites carrying a deletion of region I showed no impairment of motility or infectivity of the mosquito (Tewari et al. 2002). A second conserved CSP motif, region II, bears a striking homology to a cell adhesion motif of thrombospondin. Sporozoites carrying a CSP gene with a deleted region II had no motility and were unable to invade the salivary glands (Tewari et al. 2002). A subsequent study determined that deletion of region II allowed development of a normal number of sporozoites, but in contrast to previous study, these sporozoites were unable to exit the oocysts (Rogers et al. 1992). The discrepancy may be explained partly by the fact that former studies were conducted with hybrid *Plasmodium berghei* parasites that expressed the *P. falciparum* CSP protein. These sporozoites had 10-fold lower salivary gland infectivity.

2.2.2) Thrombospondin-related anonymous protein (TRAP): TRAP is expressed during sporozoite differentiation in the oocyst and stored in micronemes. It is essential for sporozoite gliding and cell invasion (Rogers et al. 1992; Sultan et al. 1997). Upon contact with a target cell, TRAP is released from the micronemes onto the sporozoite's anterior tip (Gantt et al. 2000). It is released also on the substrate during gliding locomotion (Kappe et al. 1999; Entzeroth et al. 1992). After invasion of the salivary gland, TRAP is found to coves the whole surface of *P. berghei* sporozoites, and in other apicomplexans, the orthologous protein is found over the entire parasite surface. TRAP contains two adhesive modules in its extracellular portion A-domain of the von Willebrand factor (Gantt et al. 2000) and a thrombospondin type I repeat (Wengelnik et al. 1999). Transgenic *P. berghei* sporozoites with a mutated TRAP A-domain are impaired in salivary gland invasion, but not in gliding motility (Wengelnik et al. 1999; Matuschewski et al. 2002). However, a very small number of *Plasmodium yoelii* TRAP knockout sporozoites were able to bind and invade salivary glands (Mota et al. 2001). A detailed mutational analysis revealed that two specific mutations in the A-domain-T126A and D157A-abrogated the sporozoite's ability to invade the salivary glands (Matuschewski et al. 2002).

2.2.3) Apical membrane antigen/erythrocyte binding-like protein (MAEBL): MAEBL is a micronemal protein of about 200 kDa, which was identified in asexual stages of parasite development. MAEBL has a single transmembrane domain and is related structurally to members of the *Plasmodium* Duffy binding-like (DBL) family (Kappe et al. 2001). While MAEBL was characterized initially in asexually reproducing parasites, its expression was described more recently in sporogonic stages in the mosquito (Kariu et al. 2002; Srinivasan et al. 2004). Targeted disruption of MAEBL revealed that the gene is essential for salivary gland invasion. Gliding motility and infectivity to the vertebrate host were unaffected by MAEBL disruption, but mutant sporozoites showed a 20-fold reduction in attachment to the salivary gland surface (Kariu et al. 2002). The putative interacting salivary gland molecules have not been identified.

2.2.4) Up-regulated-in-oocysts sporozoite protein 3 (UOS3 and TREP/S6): *P. yoelii* USO3 (upregulated in oocyst sporozoites) was first identified by the Kappe laboratory (Mikolajczak et al. 2008). The related TREP protein, first identified via subtractive hybridization experiments (Kaiser et al. 2004), has been named alternatively TREP (Combe et al. 2009) or S6 (Steinbuechel et al. 2009). The two proteins have related structural features in that they are surface proteins (they possess a transmembrane domain) and have a thrombospondin repeat that presumably functions in protein–protein interactions. Structurally, USO3 differs from TREP/S6 by the lack of an adhesion domain A in the latter protein. USO3 is localized to the apical end of oocyst sporozoites, while TREP/S6 is localized to the plasma membrane. Surface localization of both proteins and expression before salivary gland invasion are consistent with a role in invasion. Indeed, USO3 knockout of *P. yoelii* parasites leads to a complete inhibition of salivary gland invasion, while TREP/S6 knockout in *P. berghei* parasites partially inhibits salivary gland invasion. Knockout of both genes leads to partial loss of motility. It is not known whether either of the proteins directly interacts with mosquito salivary gland proteins, as shown for TRAP.

2.2.5) Cysteine repeat modular proteins (PCRMPs): The cysteine repeat modular proteins 1 and 2 are encoded by a small gene family conserved in malaria and other Apicomplexan parasites. *P. berghei* PCRMP1 is transcribed in

developing oocysts and its abundance increases in sporulating oocysts, while PCRMP2 is transcribed in sporulating oocysts and salivary gland sporozoites. Both proteins localize on the sporozoite surface. PCRMP1 and PCRMP2 knockout sporozoites are unable to invade salivary glands, suggesting a role in salivary gland invasion (Thompson et al. 2007).

1.2.3 Mosquito salivary glands

The salivary glands of adult mosquitoes are sexually dimorphic and it is clear that the structural and functional differences between the male and female organs reflect the ability of the latter to engage successfully in hematophagy (James 1994; Stark and James 1996). The salivary glands of females are much larger and paired structures than males. Each gland consists of three lobes that are attached to a common salivary duct. The duct in anopheline mosquitoes extends only partway along the lobe. Each lobe comprises a secretory epithelium surrounding a duct into which saliva is released. The cells in each lobe are organized into a single layer epithelium with characteristic basal and apical surfaces. The basal ends of the epithelial cells from the outside surface of the glands and are in contact with a basement membrane that provides the cohesiveness of the glands.

Female salivary glands are included two laterals and one medial lobe. The proximal-lateral lobes in females are involved in sugar feeding, and overlap the male salivary glands functionally (James 1994; Arca et al. 1999). In contrast, the medial lobe and distal-lateral lobes are involved in hematophagy (Champagne et al. 1995; Smartt et al. 1995; Beerntsen et al. 1999; Stark and James 1998; Arca et al. 1999). Furthermore, their gene expression characteristics, the surface properties of the different salivary gland lobes are also variable. A number of studies, with lectins and monoclonal antibodies raised to whole salivary glands, show differential binding of these agents to the lobes of the female glands (Perrone et al. 1986; Barreau et al. 1995, 1999). Some of these reagents specifically recognize the distal-lateral and/or medial lobes, indicating differentiation among the regions of the glands. These differences are particularly important because results of a number of studies have interpreted that sporozoites preferentially invade the distal-lateral and medial lobes of the female glands (Sterling et al. 1973; Rossignol et al. 1984; Golenda et al. 1990; Pimenta et al. 1994). In a striking

demonstration of this specificity, the peptide, SM1, binds to the distal-lateral and medial lobes of the female glands of *An. gambiae* and *An. stephensi*, and slightly blocks more than 90% of *P. berghei* sporozoite invasion in the latter species (Ghosh et al. 2001).

Some of the most exciting work being done in vector physiology is the discovery and characterization of a large number of proteins and their corresponding genes that are involved in facilitating hematophagy. Classes of proteins which appear common to all blood-feeding arthropods, include polyphyletic groups of enzymes that prevent coagulation, cause vasodilation and prevent platelet aggregation (Stark and James 1996). Furthermore, proteomic approaches have provided comprehensive lists of individual gene products in the 'sialomes' of various mosquito vectors, including *Ae. aegypti*, *An. gambiae*, *An. stephensi*, *An. darlingi* and *Cx. pipiens quinquefasciatus* (Valenzuela et al. 2002b, 2003; Francischetti et al. 2002; Calvo et al. 2004; Ribeiro et al. 2004). These studies have revealed an amazing diversity in the recruitment of gene family members to roles in hematophagy as well as a remarkable amount of apparent redundancy in each recognized functional class. For example, proteins that function as vasodilatory agents include the small peptides, sialokinins, from *Ae. aegypti* (Champagne et al. 1995), and the larger enzymes, catechol oxidases/peroxidases, in *An. albimanus* (Ribeiro and Nussenzveig 1993). Furthermore, the products of different genes function as anti-coagulants in individual mosquito species (Stark and James 1998; Valenzuela et al. 2002b, 2003; Francischetti et al. 2002).

1.2.4 Examples of mosquito salivary gland proteins

1) Esterases and other hydrolases: In arthropods, Esterases and other hydrolases are ubiquitous enzymes that are involved in various functions such as digestion, pheromone recognition, insecticide resistance, juvenile hormone catabolism and reproduction. Transcripts coding for hydrolases, in particular serine proteases have been found regularly in sialotranscriptomes of blood-feeding arthropods (BFA). These enzymes could play a role in degrading fibrin, or activating plasminogen, a vertebrate protein, which is proteolytically activated to an enzyme that has high specificity for fibrin, and is part of the self-regulation of the clotting process. Tabfiglysin from *Tabanus yao* is one such blood-feeding insects (BFI) salivary enzyme shown to degrade fibrinogen, and possibly fibrin (Xu et al. 2008), with an activity similar to that of tick

metalloproteases (Francischetti et al. 2003). Bioinformatic analysis of many mosquito and *Triatoma* salivary serine proteases revealed enzymes similar to chymotrypsin, suggesting that they could play a role as an elastase, or in degrading other components of the extracellular matrix (ECM). Support for a salivary function associated with ECM degradation is found also in the form of hyaluronidases that have been described in *Cx. quinquefasciatus* mosquitoes, sand flies, black flies, *Culicoides* and tabanids but absent in *Aedes* or *Anopheles* mosquitoes (Charlab et al. 1999; Ribeiro et al. 2000; Cerna et al. 2002; Campbell et al. 2005; Volfova et al. 2008; Wilson et al. 2008; Xu et al. 2008). Except for *Culex* a possible vessel feeder, all other insects are strict pool feeders, indicating that telmophagy may promote evolution of enzymes which digest ECM constituents, with the benefit of creating an enlarged feeding cavity. Hyaluronidase decreases the viscosity of the ECM, thus favouring the spread or diffusion of salivary agonists in the host skin and, for example, preferring diffusion of vasodilators from the surface of the skin to the deeper precapillary sphincter in the arteriolar plexus. *Cx. quinquefasciatus* also possess a salivary endonuclease that was functionally characterized (Calvo and Ribeiro 2006), and found to be abundantly secreted in saliva. Endonucleases may help to reduce skin viscosity caused by DNA released from broken cells, and may additionally produce pharmacologically active DNA products. Transcripts coding for endonucleases also have been found in sand fly and tsetse sialotranscriptomes, but their recombinant forms have not been characterized yet (Ribeiro and Arca 2009).

2) D7-related proteins/D7 proteins: In *Ae. aegypti* salivary glands, D7 proteins were first described and expressed specifically in the medial and distal-lateral lobes of the female adult glands (James et al. 1991). Moreover, they are a unique protein family distantly related to odorant-binding proteins (Hekmat-Scafe et al. 2000; Calvo et al. 2002). Similar proteins have been shown to present in salivary glands of sand flies and in other mosquitoes (Arca et al. 1999; Valenzuela et al. 2002a). The D7 family separate in two sizes, long and short, and *An. gambiae* has five genes coding for short D7 proteins and three genes coding for long ones (Arca et al. 2005). The first two proteins of the long family are expressed at relatively low levels, but the third hardly so. Recombinant forms of the abundant short D7 proteins of *An. gambiae* were shown to bind the biogenic amines, H, 5-HT and NE (Calvo et al. 2006). Although most proteins

can bind all amines with high affinity, each has its preference for a particular agonist, with a typical process of function divergence of function following gene duplication. Interestingly, in *Aedes* the expression pattern of the family is opposite to that seen in *Anopheles*; where the long and short D7 proteins are expressed highly and poorly, respectively (Ribeiro et al. 2007). The carboxy domain of the long D7 protein of *Aedes* was shown to bind biogenic amines similarly to the short D7 proteins of *Anopheles* (Calvo et al. 2006). The crystal structures of one long D7 protein of *Aedes* (Calvo et al. 2009) and one short protein of *Anopheles* (Mans et al. 2007) have been obtained, showing that the D7 domain has two additional helices as compared to the OBP family. Solution of the crystal structure and its binding pockets revealed that the amino-terminal domain of the long D7 protein of *Aedes* could bind a hydrophobic compound. After testing several potential agonists using isothermal calorimetry, it was discovered that the N-terminal domain of the long *Aedes* D7 binds cysteinyl leukotrienes, making this protein look feasible in targeting mast-cell agonists (Calvo et al. 2009). In *Anopheles*, both domains of the long D7 proteins appeared to be also suitable for high affinity interaction with hydrophobic ligands. These discoveries also explain the relative abundance of proteins in the salivary glands: H and 5-HT accumulate and saturate their receptors at one order of magnitude above the levels required for TXA2 and LTs. According to the brute force needed with the kratagonist design, one needs 10 times less force, or protein mass, to be effective with TXA2 or LTs as compared to biogenic amines., the long D7 of *Aedes* are in high concentrations Possibly for this reason, and the short ones of *Anopheles* are in higher concentrations, the D7 that bind 5-HT and H. The ligands of the short D7 prortein of *Aedes*, are still not known, but they are probably lipidic ligands, possibly; TXA2 (Ribeiro and Arca 2009).

3) Antigen (AG5) Family Member: All of the BFA, sialotranscriptomes, analysed so far, including those from ticks, indicate the expression of one or more members of the antigen 5 protein family, which was described originally in the venom of ants and wasps (King and Spangfort 2000). Insect genomes encode several divergent members of this widely spread protein family that is part of the larger superfamily of cysteine-rich extracellular proteins, found ubiquitously in animals and plants (Schreiber et al. 1997; Megraw et al. 1998). The *Drosophila melanogaster* genome encodes 25 different Ag5 genes (Kovalick and Griffin 2005) and 19 family members sharing 32-

51% amino acid identity that can be seen in the *An. gambiae* genome. At least one member of the Ag5 family was recruited by haematophagous insects to play an essential blood-feeding role as suggested by the abundant and tissue-enriched expression in its salivary glands (Arca et al. 2005, 2007; Ribeiro et al. 2007). Members of this family in snake venoms are associated with ion channel inhibitors and toxins (Yamazaki et al. 2003; Yamazaki and Morita 2004). In *Conus* snails, an Ag5 protein was found to have a specific protease activity (Milne et al. 2003), and as seen above, a protein of this family acquired a RGD domain and acted as a salivary inhibitor of platelet aggregation (Xu et al. 2008). Recently, a recombinant Ag5 protein expressed in the salivary glands of the stable fly, *Stomoxys calcitrans*, was found to bind immunoglobulins strongly, particularly their Fc fragment (Ameri et al. 2008). The action of this protein on activation of the classical pathway of complement was not described. Besides the *Tabanus* and *Stomoxys* proteins, no other salivary Ag5 member from BFA has been characterized functionally so far. It is difficult to predict the function of these proteins in the saliva of BFA because it is a very diverse member of this protein family (Ribeiro and Arca 2009).

4) 30 kDa antigen: This protein was first described in *Ae. aegypti* mosquitoes (Simons and Peng 2001) and later found in the salivary glands of *An. gambiae* (Francischetti et al. 2002). It has a long region of low amino acid complexity, consisting mainly of Gly and Glu residues. The 30 kDa glycoprotein family also is very abundant in the saliva of female mosquitoes, and is stained negatively under standard silver stain conditions (Cazares et al. 1998). In *Anopheles albimanus*, this protein is present only in female saliva. Although the function of this protein has been elucidated, its location, abundance and sex specificity suggest that it could be involved in blood feeding.

5) Apyrase: All mammalian BFI studies so far have found large amounts of salivary apyrase (ATP diphosphohydrolase) activity. The apyrase reaction degrades both ATP and ADP to AMP and orthophosphate, thus inhibiting platelet aggregation. This activity was associated with the first papers describing platelet aggregation inhibitors in the saliva of *Rhodnius* and tsetse (Ribeiro and Garcia 1980; Smith et al. 1980; Mant and Parker 1981) and later several other blood feeding arthropods (Ribeiro et al. 1984, 1985, 1986, 1989, 1990, 1991; Kerlin and Hughes 1992; Cupp et al. 1993, 1995; Marinotti et

al. 1996; Valenzuela et al. 1996; Cheeseman 1998). Interestingly, lizard (Ribeiro et al. 1989) or bird-feeding (Ribeiro 2000) species have very small amounts of enzyme activity, which is relevant to platelets being a mammalian invention that uses ADP as a main agonist. Accordingly, insects selecting mammals to feed from were better off if they found ways of destroying the ADP. Male mosquitoes, which do not blood feed, and mosquito species that have lost their blood-feeding ability also indulge in very little salivary apyrase activity (Ribeiro et al. 1985; Calvo et al. 2008). Mosquitoes and triatomine bugs of the *Triatoma* genus (but probably not *Rhodnius* genus) elect the 5'-nucleotidase family to take care of host ADP and ATP (Champagne et al. 1995b; Faudry et al. 2004; Sun et al. 2006). This also appears to be the case with tabanids, where a collagen-induced platelet aggregation inhibitor, named chrysoptin, is a member of the 5'-nucleotidase family, although a study reported this protein, possibly erroneously, as a specific collagen receptor inhibitor (Reddy et al. 2000). Bed bugs and sand flies (Valenzuela et al. 1998, 2001) selected the *Cimex* type of apyrase, then a novel protein family that was found later to exist ubiquitously in eukaryotes (Failer et al. 2002). Fleas appear to have elected the CD-39 family of nucleotidases (Andersen et al. 2007), although the evidence so far is circumstantial. Salivary apyrase is so abundant in these organisms that, unusually for enzymes, they display as a strong protein band in SDS-PAGE experiments (Francischetti et al. 2002; Valenzuela et al. 2002b, 2004). The presence of different protein families to serve the apyrase function in BFA is an example of convergent evolution that occurred when unrelated BFI had to adapt to the feeding change that happened with the extinction of dinosaurs and irradiation of mammals, ~60 million years ago (Ribeiro and Arca 2009).

6) Maltases and amylases: Adult mosquitoes feed on sugars, mostly in the form of nectars and honeydew. The sugar meal work as an energy source for physical functions, flight and general metabolism, and contributes slightly of female fertility (Foster 1995; Holiday-Hanson et al. 1997). Sugar digestion initiates when saliva mixes with nectar during feeding (Eliason 1963), and continues in the crop (Christophers 1960). Mosquito salivary glands express enzymes consist of maltases or α -glucosidase (Jame et al. 1989; Marinotti et al. 1990) and α -amylase (Grossman and James 1993) that help to digest in sugar-meal. In *Ae. aegypti*, the α-amylase gene is expressed specifically in the salivary glands and its function has been proposed as involved with carbohydrate

metabolism. However, amylase activity is detected at a very low level in *Ae. aegypti* salivary gland extracts (Grossman and James 1993).

7) Lysozyme: Lysozyme is an antibacterial enzyme that was first identified in the salivary glands of both *Ae. aegypti* adult male and female mosquitoes (Rossignol and Lueders 1986). Salivary lysozyme may help to prevent bacterial growth in the sugar meals of mosquitoes that are stored in the crop (Moreira-Ferro et al. 1998; Pimentel and Rossignol 1990). Recently, other immune-related products, for example lectins and antimicrobial peptides, have been found to express in the salivary gland of infected mosquitoes (Dimopoulos et al. 1997), and the trancriptomes of this insect (Valenzuela et al. 2002b, 2003).

8) SG proteins/gSGs: Anopheline salivary gland proteins, described as SG or gSG proteins (Arca et al. 1999; Lanfrancotti et al. 2002), do not yield significant similarities (by BLASTP) to other proteins in the NCBI database, except among its own members. The subfamilies of SG protein family are included SG1, SG2, SG3, SG7 and SG8. However the gSG6 protein was first identified in *An. gambiae*, it has been reported further as being highly conserved among *Anopheles* species. To optimize this biomarker candidate, peptide design has been applied recently using the bioinformatics approach to generate five *Anopheles* species peptides (gSG6-P1 to gSG6-P5). Among them, only the gSG6-P1 peptide was validated as a specific biomarker of exposure to malaria vectors. Indeed, the level of human IgG to gSG6-P1 peptide evaluated that of exposure to *An. gambiae* bites in human populations (Poinsignon et al. 2008). The IgG response to this peptide has been confirmed also as a biomarker for evaluating very low-level exposure to *An. gambiae* (Poinsignon et al. 2009) as well as *An. funestus* (the second major malaria vector in Africa) (Poinsignon et al. 2010).

1.2.5 *Anopheles campestris*-like Form E (Chiang Mai strain)

An. campestris-like is one of the member of *Anopeles (Anopheles) barbirostris* complex in Thailand. It has at least three karyotypic forms, i.e, Form B (X2, Y2), Form E (X1, X2, X3, Y5) and Form F (X2, X3, Y6) (Saeung et al. 2007; Thongsahuan et al. 2009) Form B has been found only in Chaing Mai and Kamphaeng Phet populations, while Forms E and F are widely distributed throughout the species

range. Genetic crosses between the 12 isolines, which were arbitrarily selected as representatives of *An. campestris*-like Forms B, E and F, revealed genetic compatibility that provided viable progeny through F2 generations, suggesting a conspecific nature of these karyotypic forms. These results are supported by the very low intraspecies variation (genetic distance < 0.005) of ITS2, COI and COII from genomic DNA of the three karyotypic forms (Thongsahuan et al. 2009).

Recently, *An. campestris*-like (Chiang Mai strain), a member of *An. barbirostris* complex in Thailand, was infected experimentally with *P. vivax* and *P. falciparum*. The results revealed that *An. campestris*-like was a high-potential vector for *P. vivax*, with 66.67% sporozoite rates compared to 90 % sporozoite rates recovered from *An. cracens*, it was a refractory vector for *P. falciparum* (Thongsahuan et al. 2011). Preliminary analysis of female salivary gland proteins of *An. barbirostris* complexes was performed using sodium dodecyl sulphate polyacrylamide gel electrophoresis and nano-liquid chromatography-mass spectrometry (Jariyapan et al. 2010). For *An. campestris*-like, at least eight major and several minor protein bands were detected in the glands. NanoLC-MS analysis revealed that a major protein band matched with the protein, gSG6 involved in blood feeding of *An. gambiae* and *An. freeborni*. No more proteins were reported; as not all of them could be identified by this technique mentioned (Jariyapan et al. 2010) because some protein bands might consist of several polypeptides that have the same molecular mass but differ in isoelectric points (pI). Therefore, advanced techniques are required to analyze those proteins. Two-dimensional gel electrophoresis is a powerful and high throughput tool for describing the changes in protein expression and modification, which involve separation of cellular proteins according to their isoelectric points and relative molecular masses. The separation of proteins in a sample using 2-DE, and their subsequent identification by biological mass spectrometry (MS), are key elements of classical proteomics, which enable investigation of gene expression at the protein level (Nabby-Hansen et al. 2001). These techniques were used to study the salivary gland proteomes of several arthropods, for example, *An. gambiae* (Choumet et al. 2007), *Ae. aegypti* (Wasinpiyamongkol et al. 2010), *C. nubeculosus* (Langner et al. 2007, Wilson et al. 2008, Russell et al. 2009) and *Rh. haemaphysaloides* (Xiang et al. 2009).

For the purpose of analyzing the salivary gland proteome of *An. campestris*-like, SDS-PAGE, 2-DE coupled with NanoLC-MS were applied in this study. Proteome profiles of the salivary glands of sugar-fed and blood-fed mosquitoes were compared to identify proteins secreted during blood feeding and development. Also, proteins differentially expressed in the salivary glands of male and each salivary gland lobe of female mosquitoes was identified. The new data could contribute to understanding the physiological processes that appear during the blood meal and pathogen transmission to vertebrate hosts. In addition, its salivary gland transcriptome was analyzed. Complimentary DNAs (cDNAs) encoding abundant salivary gland proteins of *An. campestris*-like was isolated and identified. The information obtained from this study would help to predict and understand the role of salivary proteins in the mosquito. It was an initial step for further identification and characterization of anti parasite-effector molecules, and promoters that may be useful in the development of genetically-transformed *Plasmodium*-refractory mosquitoes.

1.3. Purpose of This Study

1.3.1. To analyse proteomic profiles of the salivary glands of male and female *An. campestris*-like mosquitoes.

1.3.2. To determine and compare proteomic profiles of female salivary glands from *An. campestris*-like mosquitoes obtained at different ages of adult and diet after emerging.

1.3.3. To identify and isolate of cDNA clones encoding abundant secreted proteins in female salivary glands of *An. campestris*-like mosquitoes.

1.4. Usefulness of This Study

The information generated by this sequencing project was represent new challenges in terms of predicting the function of a particular gene (by sequence analysis) and comparing to it the MS data to identify protein expressed specifically and abundantly in the salivary gland of *An. campestris*-like mosquitoes. These results were provide basic knowledge and information for further study of the possible role or testing of the salivary gland protein function in sugar/blood feeding, immunity, and pathogen transmission. As a novel disease control strategy has been proposed, using genetically

engineered mosquitoes resistant to parasite development, the molecule(s) and promoter(s) could be good candidates for developing genetically-transformed mosquitoes resistant to malaria parasites.