CHAPTER IV

Discussion

Salivary glands of male and female *Anopheles* mosquitoes are morphologically different. Salivary glands of male mosquitoes consist of a single small lobe whereas female mosquitoes are composed of two lateral lobes with distinct proximal and distal regions and a median lobe (Moreira-Ferro et al. 1999; Jariyapan et al. 2007). Their feeding success is related to salivary proteins. Male mosquitoes feed only on sugar while females feed on both sugar and blood. Proximal-lateral lobes produce enzymes involved in sugar feeding. Molecular molecules related to blood feeding are synthesized by the distal-lateral and the medial lobes (James 2003). Determination of the *An. campestris*-like salivary gland extracts showed that the male glands contained approximately ten times less protein than the female ones. These values are consistent with the morphological differences observed between the salivary glands of males and females. Also, these morphological and protein content differences have been observed in other mosquito species and are related with the different feeding habits of males and females (Moreira-Ferro et al. 1999; Nascimento et al. 2000; Siriyasatien et al. 2005; Jariyapan et al. 2007; Phumee et al. 2011).

Previous study of mosquito salivary glands using SDS-PAGE in *An. stephensi* (Suwan et al. 2002), *An. cracens* (formerly *Anopheles dirus* B) (Jariyapan et al. 2007), *An. albimanus* (Cázares-Raga et al. 2007) and *An. barbirostris* species A2 (Jariyapan et al. 2012) demonstrated that there are approximately 12–15 major and several minor proteins in the females. In this study, at least 12 major proteins were found in the female salivary glands and each morphological region are contained different major proteins in salivary gland of *An. campestris*-like. Previous study in *An. albimanus* using 2-DE revealed that the salivary gland protein profile of the male was similar to the female proximal-lateral lobes (Cázares-Raga et al. 2007), suggesting that these lobes are responsible for sugar feeding. In our study, as only major proteins were selected for identification, no protein involved in the sugar digestion was identified. An explanation

is that proteins involved in sugar feeding such as alpha-glucosidase may be produced with a very small amount in the glands and collected in the crop, a sac-like compartment for the digestion of sucrose (James et al. 1989; Marinotti and James 1990; Marinotti et al. 1996; Moreira-Ferro et al. 1999). Various studies of alpha-glucosidase activities have been detected in salivary glands of *Ae. albopictus* (Marinotti et al. 1996), *An. darlingi* (Moreira-Ferro et al. 1999), and *An. cracens* (Jariyapan et al. 2007); whereas, no activity has been detected in the crop of *Anopheles aquasalis* but in the midgut (Souza-Neto et al. 2007). Several studies in *Phlebotomus* sandflies *P. langeroni* (Dillon and el-Kordy 1997), *Lutzomia longipalpis* (Gontijo et al. 1998), and *P. papatasi* (Jacobson and Schlein 2001) show that alpha-glucosidase activities have also been detected in midgut but not in the crop suggesting that sugar digestion is carried out in the midgut. Although, salivary alpha-glucosidase may be used for assisting solubilization of sugars (Eliason 1963) and for intra cellular metabolism (Dillon and el-Kordy 1997). Characterization of alpha-glucosidase activity in the *An. campestris*-like would help to clarify this issue.

Several techniques including SDS-PAGE, 2-DE, and liquid chromatography tandem mass spectrometry (LCMS/MS) are combined and used to describe *An. gambiae* salivary gland and saliva contents (Kalume et al. 2005; Choumet et al. 2007). The studies identified 5 saliva proteins and 122 more proteins from the salivary glands, including the first proteomic description for 89 of these salivary gland proteins. Proteomic analyses of the salivary glands of *An. campestris*-like lead to the discovery of proteins that promote blood feeding consist of putative 5′-nucleotidase/apyrase, anti-platelet protein, long-form D7 salivary protein, D7-related 1 protein, and gSG6. They are secreted proteins and synthesized and accumulated in the distal-lateral lobes and medial lobe of the glands. This result is consistent with previous studies on salivary gland proteome profiles of *An. gambiae* (Kalume et al. 2005; Choumet et al. 2007) and *An. barbirostris* species A2, a closely related species in the *An. barbirostris* complex (Jariyapan et al. 2012) and in situ hybridization results of genes involved with blood feeding in *Ae. aegypti* (Juhn et al. 2011).

However, Calvo et al. (2006b) compared approximately 1,000 randomly sequenced clones of an adult male salivary gland cDNA library of *An. gambiae* with a

previous data set of the female salivary gland cDNAs (Arca et al. 2005). Results show that female transcribed genes codes for proteins, D7L1, D7r1, D7r2, D7r3, and D7r4 which are implicated in anticlotting and anti-bradykinin production as well as biogenic binding activities (Calvo et al. 2006a; Isawa et al. 2007), anti-platelet aggregation proteins, 5′-nucleotidase (5p_nuc), and apyrase (Ribeiro and Francischetti 2003; Sun et al. 2006); antithrombin protein, cE5, homologous to *An. albimanus* anopheline (Francischetti et al. 1999; Valenzuela et al., 1999); and proteins with unknown function, SG1-like 3 long, trio, gSG1b, gSG7-2, gSG7, hyp17, 30_kDa, and hyp15 are not found in the *An. gambiae* male salivary gland cDNA library. In contrast to *An. gambiae*, the blood-feeding proteins, apyrase, putative 5′-nucleotidase/apyrase, anti-platelet protein, D7, and short form D7r1, were found in 2-DE gels with very small amount in the male salivary glands of *An. campestris*-like. Study on differentially expressed genes in the salivary glands of female and male *An. campestris*-like using Switching Mechanism At RNA Termini Polymerase Chain Reaction (SMART-PCR) followed by suppression subtractive hybridization (SSH-PCR) techniques (Ghorbel and Murphy 2011) would help to explain this issue. Information from the study may be helpful in finding additional peptides and proteins with a function in blood or sugar feeding in mosquitoes as studies in *Culex pipiens pallens* (Chen et al. 2007) and *Anopheles anthropophagus* (Geng et al. 2009).

In our study, *An. campestris*-like female salivary glands were found at least 14 glycoproteins. However, few proteins in the saliva of *Anopheles* mosquitoes studied so far have been described as glycoproteins, i.e., 5′-nucleotidase/apyrase, anti-platelet protein (30 kDa allergen/GE-rich), and D7 protein. Secretory proteins are often glycosylated or modified by phosphorylation as they pass through the Golgi apparatus (Alberts et al. 2002). Glycoproteins contain oligosaccharide chains covalently attached to polypeptide side chains. These glycoproteins are involved in a wide range of biological functions such as receptor binding, cell signaling, immune recognition, inflammation, and pathogenicity. Glycoproteins contain three major types of oligosaccharides (glycans): N-linked, O-linked, and glycosylphosphatidylinositol (GPI) lipid anchors. Most of the proteins in saliva are glycosylated. Carbohydrates have many hydroxyl (−OH) groups that bind to water molecules, and thus increase stability. Therefore the glycoproteins of saliva lean to lubricate the food chewed, in part to allow

easier swallowing of food and its passage through the esophagus. For examples, mucins, which are found extensively in the sialotranscriptomes of insects, contain many short O-linked glycans (Calvo et al. 2007; Alves-Silva et al. 2010). These glycoproteins increase the viscosity of the fluids in which they are dissolved. Therefore, they are postulated to help maintain the insect mouthparts, in addition to other possible functions (Alves-Silva et al. 2010). Most 5′-nucleotidases are typically extracellular proteins bound to the membrane by glycosylphosphatidylinositol (GPI) anchors attached to their carboxyterminal domain.

However, 5′-nucleotidase/apyrases in several insects include *Ae. aegypti*, *Ae. albopictus*, *Culex pipiens quinquefasciatus*, *Lu. longipalpis*, and *Glossina morsitans morsitans* lack the GPI anchor attachment domain, either through mutation or truncation, thus deduce that these proteins are secreted (Champagne et al. 1995; Charlab et al., 1999; Ribeiro et al. 2004; Ribeiro et al. 2007; Alves-Silva et al. 2010; Dong et al. 2012). An acidic glycoprotein of 35 kDa (GP35 ANOAL) from female salivary glands of *An. albimanus* contains several potential post-translational modifications predicted in its amino acids sequence. Among them, two potential N-glycosylation and nine potential Oglycosylation sites have been identified (Cázares-Raga et al. 2007). Putative N-glycosylation and O-glycosylation sites occur in all identified members of the 30-kDa allergen family (Xu et al. 1998; Ribeiro and Francischetti 2003; Valenzuela et al. 2003; Calvo et al. 2004; Jariyapan et al. 2006; Cázares-Raga et al. 2007; Yoshida et al. 2008). Proteins that contain N-glycosylation sites may play important roles in the induction of allergic responses (Wal 2001; Malandain 2005). Several study of D7 family proteins, only D7 long forms of *An. gambiae*, *An. stephensi*, *Anopheles arabiensis*, *Anopheles funestus*, and *An. darlingi* contain glycosylation sites (Francischetti et al. 2002; Suwan et al. 2002; Valenzuela et al. 2002; Calvo et al. 2007; Calvo et al. 2009). Glycoproteins in the salivary glands of mosquitoes should be studied for their structure and specific biological functions in receptor binding, cell signaling, and immune recognition that may be involved in pathogen transmission.

Three major protein spots of *An. campestris*-like salivary glands related to housekeeping proteins from other arthropod species consist of serine/threonine-protein kinase rio3 [*Culex quinquefasciatus*], sil1, putative [*Ixodes scapularis*], and cyclophilin

A, putative [I. *scapularis*]. Interestingly, SN6 Protein that matched the serine/threonine-protein kinase rio3 of *C. quinquefasciatus* (Arensburger et al. 2010) was a protein expressed abundantly in the medial lobe of female salivary glands but found with a small amount in both regions of the lateral lobes and the male salivary glands. The RIO family of atypical serine protein kinases has been first characterized in *Saccharomyces cerevisiae* (Angermayr and Bandlow 1997). It included enzymes that contain a unique domain with a characteristic kinase sequence motif and usually some additional domains. At least two RIO proteins, Rio1 and Rio2, are present in organisms varying from Archaea to humans, with a third Rio3 subfamily present only in multicellular eukaryotes (Manning et al. 2002). Human Rio3 is characterized at the DNA level only, in two splice variants, and is identified as a protein upregulated in the core of malignant melanomas (Roesch et al. 2003). To date, no report of the purification of mosquito kinase Rio3 is available. Moreover, SN 10 matched sil1, putative [*I. scapularis*]. It was predominantly expressed in both the medial and distal-lateral lobes. SIL1 in *Homo sapiens* is a native endoplasmic reticulum (ER), N-linked glycoprotein with an N-terminal ER targeting sequence, 2 putative N-glycosylation sites, and a C-terminal ER retention signal. This protein is required for protein translocation and folding in the endoplasmic reticulum (ER). It functions as a nucleotide exchange factor for the heat-shock protein 70 (HSP70) chaperone HSPA5 (Anttonen et al. 2005). Furthermore, SN 14 matched cyclophilin A, putative [*I. scapularis*]. It was expressed specifically in the distal-lateral lobe. Cyclophilin A also known as peptidylprolyl isomerase A is the most abundant member of the CyP subfamily of immunophilins and has a variety of intracellular functions, including intracellular signaling, protein trafficking, and the regulation of other proteins activity. Cyclophilin A exists in many organisms including bacteria, fungi, plants, helminthes, and mammals (Galat 1999; Wu et al. 2011; Han et al. 2012). In humans, cyclophilin A has been studied as a multifunctional protein that is up-regulated in a variety of inflammatory conditions, such as rheumatoid arthritis, autoimmune disease, and cancer. Besides its intracellular functions, CyPA is a secreted molecule that has a physiological and pathological role in cardiovascular diseases (Satoh et al. 2010). Functional analysis of sil1 and cyclophilin A in *An. campestris*-like mosquito should be carried out as they were major proteins in the female salivary glands.

In this study, SN 5 and 17 were found only in the medial lobe with unknown function whereas SN15 and 16 proteins were expressed only in the distal-lateral lobes with no significant match with protein sequences in the database. These proteins should be identified and characterized as they may be involved in blood feeding and/or pathogen transmission.

Several studies have demonstrated changes in amount of total salivary gland proteins and/or SDS-PAGE gel electrophoretic protein profiles after the first blood meal of various mosquito species, such as, *Ae. aegypti*, *Aedes caspius*, *An. stephensi*, *An. albimanus*, *An. gambiae*, *An. freeborni*, *Armigeres subalbatus*, *Cx. pipiens*, *Cx. quinquefasciatus,* and *Mn. uniformis* (Golenda et al. 1995; Siriyasatien et al. 2005; Phumee et al. 2011; Soliman et al. 1999). However, only salivary gland proteins differentially expressed after the first blood feeding in *Ae. aegypti* have been identified (Wasinpiyamongkol et al. 2010). Up-regulated salivary gland proteins ten days post blood feeding in *Ae. aegypti* include three D7 proteins, salivary apyrase, apyrase precursor, aldehyde dehydrogenase, salivary serpin putative anticoagulant, putative 30 kDa allergen-like protein, adenosine deaminase, 19.6 kDa secreted protein precursor, putative secreted protein, and a putative uncharacterized protein (Wasinpiyamongkol et al. 2010). In addition, microarray transcriptome analyses of *Ae. aegypti* and *An. gambiae* salivary glands response to blood feeding have been performed (Das et al. 2010; Thangamani et al. 2009). Down-regulated salivary gland transcripts of *Ae. aegypti* during the first three hours post-blood feeding have included an odorant binding protein, protease inhibitors, and immune genes (Thangamani et al. 2009). Das et al. (2010) have reported that a small proportion of the salivary gland transcriptome of *An. gambiae* is dynamically changing already at two hours in response to blood feeding. The salivary gland transcripts encoding secretory proteins that displayed a lower abundance after blood feeding have included two OBPs (OBP 10 and OBP 7), two D7 long-form precursors (L1 and L2), two aminopeptidases, a trypsin 6 precursor, a salivary lipase, a 5'-nucleotidase precursor, an apyrase, E1 protein, cecropin 3, defensin 1, and a hypothetical 6.2 precursor (Das et al.2010). It is generally assumed that mRNA levels are a useful surrogate for protein concentration. However, mRNA levels do not necessarily correspond to concentration of proteins due to requirements for

posttranslational modifications of some, and potential regulation of translation. In this study, a proteomic approach, 2-DE coupled with NanoLC-MS, was used.

Our study showed that 19 and 14 major salivary proteins of *An. campestris*-like females decreased in quantity after the first and the second blood meals, respectively. Significantly depleted proteins in the these groups included apyrase, 5'-nucleotidase/apyrase, D7, D7-related 1, short form D7r1, gSG6, anti-platelet protein, serine/threonine-protein kinase rio3, putative sil1, cyclophilin A, hypothetical protein Phum_PHUM512530, AGAP007618-PA, and two non-significant hit proteins, indicating that these polypeptides are introduced into the vertebrate hosts during blood feeding and facilitate the process of blood-feeding and potentially pathogen transmission. Our results, together with those from previous studies in *Ae. aegypti* and *An. gambiae* (Das et al. 2010; Wasinpiyamongkol et al. 2010; Thangamani et al. 2009), indicate that proteins involved in hematophagy and pathogen transmission include salivary apyrase, 5'-nucleotidase/apyrase, D7 proteins, and putative 30 kDa allergen-like protein/anti-plateletprotein.

Apyrase and 5'-nucleotidase proteins are known to facilitate the acquisition of a blood meal by the degradation of adenosine diphosphate (ADP), a mediator of platelet aggregation and inflammation (Ribeiro 1995) and prevent neutrophil activation (Sun et al. 2006). Two genes of the 5'-nucleotidase family, putative 5'-nucleotidase and salivary apyrase, are expressed in the salivary glands of *An. gambiae* (Arca et al. 2005). The sialotranscriptome of *An. darlingi* also presents evidence for the two orthologues, a full-length orthologue of the salivary '-nucleotidase of *An. gambiae* and a 5'-truncated clone best matching the *An. gambiae* salivary apyrase (Calvo et al. 2009). *An. gambiae* has been shown to require longer probing times during blood-feeding when an apyrase gene has been silenced (Boisson et al. 2006). It has also been reported that apyrase is reduced in *Plasmodium berghei*-infected *An. gambiae* mosquito salivary glands (Choumet et al. 2007). The reduction of apyrase levels in *P. gallinaceum* infected *Ae. aegypti* salivary glands caused an increase in mosquito probing time (Rossignol et al. 1984). These studies indicate a role for apyrase and 5'-nucleotidase in increasing the time for *Plasmodium* transmission to a new vertebrate host. Our results showed that at least two proteins in the salivary glands of *An. campestris*-like mosquitoes matched

apyrase and 5'-nucleotidase of other *Anopheles* mosquitoes. In addition, the most depleted protein spot of the first and second blood meal groups was SN2 matched with putative 5'-nucleotidase/apyrase [*An. darlingi*] suggesting that platelet aggregation might be the most vital mechanism used for blood feeding in *An. campestris*-like. Further studies on the role in facilitation of pathogen transmission of 5'-nucleotidase/apyrase in *An. campestris*-like should be performed.

gSG6, a small protein with unknown function, was first identified in *An. gambiae* females (Lanfrancotti et al. 2002) and conserved in species members of the *An. gambiae* complex, i.e., *An. gambiae*, *An. melas*, *An. bwambae*, *An. quadriannulatus*, and *An. arabiensis* (Lombardo et al. 2009). These authors demonstrated that silencing of *An. gambiae* salivary gland gene, gSG6, results in increased probing time and reduced blood-feeding ability. Recently, *An. gambiae* gSG6, a reliable marker for exposure to *An. gambiae* bites (Poinsignon et al. 2008; Poinsignon et al. 2009; Rizzo et al. 2011), has been recently reported to be a good indicator for exposure to bites from three main African malaria vectors, i.e., *An. gambiae, An. arabiensis* and *An. funestus* (Poinsignon et al. 2010). In *An. campestris*-like, its gSG6 protein was depleted in both blood feeding groups with approximately 40-60% confirming that a least one of the gSG6 functions is involved in blood feeding. However, further studies are needed to characterize the biological properties of this unknown protein.

The 30 kDa/GE-rich/anti-platelet protein family was been first identified as a salivary antigen in *Ae. aegypti* and called 30-kDa allergen of *Ae. aegypti* (Simons and Peng 2001) . The members of this family have been found in salivary transcriptomes and proteomes of both culicine and anopheline mosquitoes (Valenzuela et al. 2003; Calvo et al. 2004; Jariyapan et al. 2006; Cazares-Raga et al. 2007), where it has been named GE-rich protein. Later, a related unique anti-platelet protein, anopheline anti-platelet protein (AAPP), from the salivary gland of female *An. stephensi* was identified (Yoshida et al. 2008). Several proteomic works have also indicated that the members of 30 kDa/GE-rich/antiplatelet protein family are one of the most abundantly expressed acidic proteins in the female salivary glands of mosquitoes studied so far (Calvo et al. 2009; Valenzuela et al. 2003; Calvo et al. 2004; Jariyapan et al. 2006; Cazares-Raga et al. 2007; Jariyapan et al. 2012), and also found in *An. campestris*-like (Sor-suwan et al.

2013). Recently, members of the 30 kDa/GE-rich/anti-platelet family have been identified as antigens from four *Anopheles* species, i.e., *An. gambiae*, *An. albimanus*, *An. stephensi*, and *An. arabiensis*, and are thus potential candidates to serve as pan-*Anopheles* genus markers of immunological exposure (Jensen et al. 2012). A proteomic study of the salivary glands of *Ae. aegypti* has revealed that putative 30 kDa allergen-like salivary gland protein is up-regulated ten days post feeding (Wasinpiyamongkol et al. 2010). In *An. campestris*-like, the anti-platelet protein was depleted in the blood feeding groups. The result suggested that one of the anti-platelet protein functions is involved in blood feeding. In addition, studying saliva allergens could provide valuable information on immune responses, with a view to developing new diagnostic tests for allergies to mosquito bites.

Proteins of the D7 family are distantly related to the OBP (odorant binding proteins) super-family and present in the saliva or salivary glands of several female blood-sucking insects (James et al. 1991; Arca et al. 1999; Valenzula et al. 2002a). The D7 proteins are able to bind host biogenic amines such as serotonin and histamine to antagonize vasoconstrictor, platelet aggregating, and pain-inducing properties (Calvo et al. 2006). The D7 protein exists in two forms: a long form having a molecular mass of 27-30 kDa which is found exclusively in mosquitoes and sand-flies, and the short forms having a molecular mass of 15-30 kDa which are found in mosquitoes, sand-flies and other insects (Valenzuela et al. 2002a; Valenzuela et al. 2002b; Arca et al. 2002). Five D7-related short forms (D7r1, D7r2, D7r3, D7r4, and D7r5) and three D7 long forms have been identified in *An. gambiae* (Arca et al. 2005; Valenzuela et al. 2002). The D7r1, 2, 3, 4 and D7 long forms have been shown to bind to the biogenic amines (Arca et al. 2005; Calvo et al. 2006). Hamadarin, a short D7 protein 1 from *An. stephensi*, has been shown to inhibit the plasma contact system by preventing the activation of kallikrein by Factor XIIa (Isawa et al. 2007). By using a RNAimediated gene silencing method, it was shown that depletion of D7L2 resulted in decreased blood feeding capacity and as well as increased probing time (Das et al. 2010). Also the level of D7 related-1 protein precursor protein is decreased in *P. berghei*-infected salivary glands of *An. gambiae* (Choumet et al. 2007). The decreased production of D7 related-1 protein precursor may induce an increased local inflammatory response to mosquito bites, thus modifying the immune response to the parasite. In our study, a long form D7, matched

best with a D7 protein [*An. stephensi*], and two D7 short forms, matched best with D7-related 1 protein [*An. gambiae*] and short form D7r1 salivary protein [*An. arabiensis*], were depleted significantly after blood-feeding. These results strongly support the involvement of D7 and D7 related-1 proteins in the blood feeding process. Furthermore, a novel gene encoding for a D7 related 1 protein in *An. campestris*-like was isolated for the first time. We observed an abundance of ESTs belonging to the D7 family of proteins. In the sequences of *An. campestris*-like were matched the nucleotide sequence with 66% and 74% shared identity with a mRNA for D7-related 1 protein of *An. stephensi,* respectively This result support that the D7-related 1 protein is expressed abundantly in the salivary glands of female *An. campestris*-like mosquitoes.

A chance of successful blood feeding of female mosquitoes depends on the efficiency of alteration of the host hemostatic response (platelet activation and aggregation, local vasoconstriction, and coagulation), inflammatory, and immune systems (Ribeiro and Francischetti 2003). Results from the proteomic approach used in this study confirmed that *An. campestris*-like used at least anti-platelet aggregation (apyrase and anti-platelet protein), and anti-inflammatory (D7and D7-related) proteins to facilitate blood feeding. Although, analysis in terms of putative functional association networks among the depleted proteins using STRING 9.0 Server (Jensen et al. 2009; Szklarczyk et al. 2011) (http://www.string-db.org) was performed in this study, no predicted confident association was reported (unpublished data). Further investigation on the functions of salivary gland proteins could be performed using transient RNAi gene-silencing assays on the salivary transcribed genes and examining how they influence the mosquito feeding and probing behavior on a vertebrate host and pathogen infected mosquitoes. Also the mechanism that would allow, for example, depletion of approximately 50% of one protein and 75% of another is unknown. However, it is known that individual proteins are not distributed evenly between different parts and lobes of salivary glands, so this may explain differential depletion. More studies on other mosquito species and using different methods of feeding, for example, artificial membrane feeding might provide some clues for explanation of the secretion mechanism in mosquito salivary glands.

Surprisingly, for *An. camprestris*-like, two protein spots (SN4 and 16) disappeared in the unfed females from the control group (17-18 days post emergence) and both unfed and blood fed mosquitoes from the second blood meal groups (17-18 days post emergence and 14 days after the 1st blood meal). The result indicates that both protein spots were absent in old-aged female mosquitoes from at least day 17 post emergence. It suggests that the proteins may not be involved in blood feeding and transmission of malaria sporozoites in *An. camprestris*-like of the same age. However, it is interesting to identify and characterize these proteins in other mosquito species, whether they are involved in blood feeding and transmission of pathogens or not before any conclusions should be drawn.