SPATIO-TEMPORAL CHARACTERIZATION OF THE INSULIN SIGNALING CASCADE AND ITS ROLE IN REGULATING HEMOCYTE PROLIFERATION IN *AEDES AEGYPTI*

By

JULIO CESAR CASTILLO

(Under the Direction of Michael R. Strand)

ABSTRACT

Hemocytes are central for cell based immunity. In this study, I compared an improved technique (this study) to collect hemocytes from mosquitoes to other welle stablished methods. Collection method greatly affected the number of hemocytes and contaminants obtained from adult females of each species. Using a collection method called high injection/recovery I was able to show that hemolymph from *An. gambiae* and *Ae. aegypti* adult females contains three hemocyte types (granulocytes, oenocytoids and prohemocytes) that were distinguished from one another by a combination of morphological and functional markers. Granulocytes were the most abundant cell type in both species while oenocytoids and prohemocytes comprised less than 10% of the total hemocyte population. The number of hemocytes recovered from sugar fed females declined with age but blood feeding transiently increased hemocyte abundance. In order to understand the nature of the increase in blood cells after a blood meal, the role of insulin signaling as key regulator of cell proliferation was investigated. Several brain-specific ILPs (-3, -4, -7, and -8) were found to be expressed in hemocytes, and their expression pattern differ between blood fed and non-blood fed female mosquitoes. Experiments showed that decapitated females exhibited no increase in hemocyte abundance. ILP-3 injected into blood fed/decapitated females rescued the observed cell increase phenotype, and hemocyte increase was restored. BrdU labeling of hemocytes showed that blood fed females had higher

numbers of BrdU positive cells when compared to non blood fed controls, indicating that blood feeding stimulates cell proliferation. RNAi knock down using dsRNA targeting the mosquito insulin receptor (MIR) inhibited hemocyte increases. Additionally, the total hemocyte number in dsRNA-MIR treated blood fed and non-blood fed females was considerably lower, suggesting that ILPs may also serve as a survival signal. Lastly I found that the increase in hemocyte numbers observed in blood fed animals confered resistance to bacterial infection.

INDEX WORDS: insulin-like peptides, ILP, hemocytes, MIR, insulin receptor, mosquito, *Aedes aegypti*, *Anopheles gambiae*, *Drosophila melanogaster*, cell proliferation.

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DEDICATION

To my beloved family, whose unconditional constant support and encouragement made it possible for me to complete this important part of my life.

A mi familia, quienes con su apoyo incondicional y motivacion hicieron possible que yo cilminara esta importante etapa de mi vida.

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Chapter 1 corresponds to "Characterization of hemocytes from the mosquitoes *Anopheles gambiae* and *Aedes aegypti*" that appears in the journal Insect Biochemistry and Molecular Biology by Castillo et al. (2006), and includes work made by Anne Robertson under the supervision of Michael R. Strand.

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CHAPTER 1

LITERATURE REVIEW

Insulin signaling regulates many processes in metazoan physiology. Insulin is not only responsible for regulating important metabolic functions, such as carbohydrate and energy metabolism, but also activities of the immune system (1). Insect hemocytes are an important group of cells responsible for cell-based immunity in insects and other invertebrates. Key hemocyte functions include phagocytosis, encapsulation and melanization, tissue repair, cell signaling and wound healing. Hemocytes represent the "armed forces" of the immune system. Mosquitoes are an important group of insect vectors that are responsible for the transmission of many different disease-causing pathogens including malaria, dengue, yellow fever, West Nile virus, and other diseases. *Aedes aegypti* is a very vector species, responsible for transmitting dengue and yellow fever in tropical parts of the world. A unique aspect of mosquito physiology is the need to blood feed on a vertebrate hosts in order to produce eggs. In the following sections I will be discussing the importance of insulin signaling from mammals to insects, how it interacts with the immune system and hemocytes in particular and the rationale behind our study.

1.1 Role of the Insulin superfamily of related polypeptides in vertebrates and other taxa

Introduction

The discovery of insulin in 1921 by Frederick Banting and Charles Best (2), was a significant biomedical discovery that shaped the understanding one of an important public health problem of

modern society, diabetes. Its discovery and intensive study by numerous scientists contributed in advancing many scientific fronts, from protein chemistry, biophysics, and structural biology to immunoassays, cellular biochemistry and molecular engineering. The insulin/IGF/relaxin family consists of family of functionally diverse peptides that affect different biological processes such as sugar balance and metabolism, and cell division. In humans this protein family includes insulin, insulin-like growth factors (IGF) I and II and seven members of the relaxin-like peptide family (3-5). Insulins and relaxins contain two peptide chains (A and B) as the result of post-translational processing (6-8), whereas IGFs are single-chain polypeptides containing A- and B-domains, an intervening connecting C domain, and C-terminal D-domain (9, 10). Genomic data and phylogenetic analysis have led to the identification of novel insulin superfamily members in other species such as apes, mouse, frogs and fish (11).

Insulin and Insulin-like peptides (INS and INSL)

Insulin and/or insulin-like peptides have been described in unicellular eukaryotes as well as in metazoan invertebrates such as insects, tunicates, annelids, and molluscans (12). Insulin molecules are expressed as pro-hormones consisting of four regions considered to be "Insulin's signature" features: (1) an N-terminal signal peptide for secretion, (2) a conserved B chain, (3) a non-conserved C peptide, and (4) a C-terminal A chain (12). The positioning and pattern of highly conserved (cysteine motifs) disulfide bridges as well as the position of the B and A chain are relatively invariant between the different hormones of the family and between their variants in different species for a specific hormone. These cysteine motifs characterize the family; specifically, the motif present in the A peptide has been termed the insulin signature (12). All members of the insulin family of hormones are synthesized as pre pro-hormones with the primary peptide undergoing posttranslational modification to generate a disulfide bond-linked heterodimer of the B and A peptides that functions as the active hormone.

In mammals, Insulin is synthesized in the pancreas within the beta cells (β-cells) of the islets of Langerhans. In the beta cells, insulin is synthesized from its precursor (pro-insulin) by the action of proteolytic enzymes, known as pro-hormone convertases (PC1 and PC2), as well as the exo-protease and carboxypeptidase E (13). This series of modifications in proinsulin have proven critical for removal of the center portion of the molecule (C-peptide) , from the C- and N- terminal regions of proinsulin (13). The resulting polypeptide of approximately 51 amino acids corresponds to B- and A- chains bound by disulphide bonds, with the rest of the peptide (C-peptide) removed. Since insulin chains were identified based on mass, and because the C peptide was identified after the other two, the primary sequence of proinsulin goes in the order "B-C-A" (14). The essential endocrine role of insulin is controlling metabolic responses to nutritional state has been established for decades. However, it was only until recently that novel functions have been assigned to other insulin-like factors (15).

Insulin is the main hormone controlling intermediary metabolism. Its most obvious acute effect is to lower blood glucose. The main factor controlling the synthesis and secretion of insulin is the blood glucose concentration (Beardsall et al., 2008). Other stimuli necessary for insulin release include fatty acids, amino acids (particularly arginine and leucine) and gastrointestinal hormones. In vertebrates, insulin inhibits the production and release of glucose from the liver by blocking gluconeogenesis and glycogenolysis. This occurs through a direct effect on the liver, as well as by influencing substrate availability (16). Similar to what happens with carbohydrate metabolism, insulin also promotes the synthesis of lipids, and inhibits their degradation (17). Insulin decreases blood glucose by increasing glucose uptake into muscle and fat cells via GLUT-4 (an insulin-responsive glucose transporter expressed in muscle and fat cells), increasing glycogen and fatty acids synthesis, DNA replication and protein synthesis, decreasing proteolysis, lipolysis, gluconeogenesis and glycogen breakdown (Beardsall et al., 2008).

Insulin Growth Factors (IGFs)

Insulin-like growth factors (IGF-I and IGF-II in humans) are single-chain polypeptides with structural homology to pro-insulin (18). They regulate proliferation and differentiation of different cell types and are capable of inducing insulin-like metabolic effects. Unlike insulin, they are produced by most tissues of the body and are abundant in circulation. They have the potential to act through endocrine as well as autocrine and/or paracrine mechanisms (19). Regulation of inflammation and immunity requires a vast network of interacting cells and cytokines (20). Insulin-like growth factors (IGFs) have a broad range of physiological functions and the complexity of their function regulates processes from embryogenesis to senescence. IGFs are known to regulate innate immunity and proliferation of myeloid lineage cells (20) as well as anti-apoptotic mechanisms(21). This combined effect of proliferation coupled with anti-apoptosis is thought to be responsible for the modulation of hematopoiesis in vertebrates. Insulin growth factors exert their effects at the cellular level by interacting with the Type-I IGF receptor (IGF-I receptor). They also bind to the Type II 1mannose- 6-phosphate receptor (IGF-II receptor) and insulin receptors, as well as high affinity binding proteins (IGFBPs).

Relaxins

In humans, relaxins consist of 7 peptides that share structural homology but low sequence similarity: relaxin genes (H1), (H2), and (H3), insulin-like peptide 3 (INSL3, also called Leydig insulin-like peptide and relaxin-like factor (RLF)), insulin-like peptide 4 (INSL4, also known as placentin and early placenta insulin-like (EPIL)), insulin-like peptide 5 (INSL5), insulin-like peptide 6 (INSL6) in humans. All of these molecules share high structural similarity to insulin due to the presence of six cysteine residues forming two inter-chain and one intra-chain disulfide bonds. Structurally, relaxin is a heterodimer of two peptide chains of 24 and 29 amino acids that are linked by disulfide bridges. Relaxin is synthesized

as a prohormone, by splitting off one additional peptide chain (22). Studies from Wilkinson and collaborators (11) concluded that relaxin and insulin had derived from a common ancestral gene and were therefore grouped within the insulin superfamily, which later included other members such as insulin-like growth factors I and II (IGF-1 and -2). To conclude, insulin, insulin-like peptides and relaxins have different biological properties that make them regulators of human physiology.

Insulin Receptor Structure and Insulin signaling cascade

The integration of multiple trans-membrane signals is especially important during development and maintenance of the nervous system, communication between cells of the immune system (proliferation, migration, homing, and population expansion), evolution of transformed cells (normal vs cancer), metabolic control and many other processes.

The insulin receptor (IR) is a receptor tyrosine kinase (RTK) that, once phosphorylated upon binding to its ligand insulin or insulin-like peptides, transduces the signal downstream by activating various signaling cascades. These pathways are regulated by complex networks of signaling inputs (23). The balancing of signals that transit the pathways stimulated by insulin provide the specific cell response to insulin signaling (24). Insulin signaling is initiatiated by sequential downstream cascades of phosphorylation and dephosphorylation, guanine nucleotide exchange changes and spatial addition of signaling factors, scaffolding, and adaptor molecules. Insulin receptor substrate (IRS) family members, SHC (Src homology domain containing protein), PI3K and GRB10 (insulin receptor binding protein) interact directly with the insulin receptor and are responsible for transducing the insulin stimuli to the appropriate responsive pathway (17).

The insulin receptor gene has a modular organization that comprises 22 exons and 21 introns. The receptor is synthesized as a single chain pre-pro-receptor polypeptide with a 30-residue signal peptide cleaved co-translationally. The IR-precursor encodes an α- and β-subunits that are glycosylated, folded and dimerized before they are transported to the Golgi apparatus, where they are processed to yield the mature α2β2- holo-hetero dimer receptor (25, 26). Ligand binding specificity is critical and occurs at specific cysteine rich regions in the α-subunits whereas the β-subunits contain the tyrosine kinase activity domain responsible for downstream activation of responsive genes (26).

Extensive studies show that the ability of the receptor to autophosphorylate and phosphorylate intracellular substrates is crucial for the mediation of the complex cellular responses to insulin (27, 28). Structural biology studies reveal that the two α subunits jointly participate in insulin binding and that the kinase domains in the two β subunits juxtapose one another allowing autophosphorylation of tyrosine residues, the first step of insulin receptor activation (29). Binding of insulin to its receptor induces auto phosphorylation at a number of tyrosine residues located in the intracellular portion of the receptor. Certain residues (tyrosine) are recognized by the insulin receptor substrate family of proteins (IRS), which are recruited to near the receptor at the cell membrane. There are at least nine intracellular substrates (IRS) of the insulin/IGF-I receptor kinases that have been identified.

The different IRS proteins are responsible for regulating different tasks at the cellular level, as evidenced by their differential tissue distribution, subcellular localization, intrinsic activity of the proteins and the nature of the response required. Four of these belong to the family of insulinreceptor substrate (IRS) proteins *per se* (30). Other substrates include Gab-1 (GRB2-associated binding protein), $p60^{dok}$ (GAP-associated protein, Cbl (Casitas B-lineage Lymphoma-prrotooncogene), APS (SH2B adaptor protein 2) and isoforms of Shc (src homology C domain-containing protein) (31). The phosphorylated

tyrosines present in these substrates act as 'docking sites' for proteins that contain SH2 (Src-homology-2) domains. Many of these SH2 proteins are adaptor molecules, such as the p85 regulatory subunit of PI(3)K and Grb2, or CrkII, which activate small G-proteins by binding to nucleotide exchange factors. Others substrates act as enzymes as well, including the phosphotyrosine phosphatase SHP2 and the cytoplasmic tyrosine kinase Fyn. Substrate binding to these SH2 proteins can regulate their activities, or in some cases their subcellular location (31).

There are three main downstream pathways that transmit signals generated through the insulin receptor: the IRS/phosphatidylinositol-3 kinase (PI3k) pathway; the retrovirus- associated DNA sequences (RAS)/mitogen-activated protein kinase (MAPK) pathway; and the Cbl-associated protein (CAP)/Cbl pathway.

Phosphatidylinositol 3-kinases (PI(3)-kinases or PI3-Ks) are a family of related enzymes involved in regulating cell growth, proliferation, differentiation, motility, survival and intracellular trafficking. The PI3-kinase pathway is key in signaling downstream upon insulin stimulation, and it is the branch of the insulin cascade responsible for the metabolic and mitogenic activity of insulin. The importance of this family of kinases relies on its unique ability to activate protein kinase B, also known as Akt. Akt possesses a pleckstrin homology that allows it to to bind to the phosphatidyl inositols tri/di phosphate (PtdIns (3,4,5)P3 and PtdIns (3,4)P2) produced by PI3K. The interaction between Akt and PtdIns occurs after Akt translocates into the membrane (32). PDK1 (phosphoinositide-dependent protein kinase 1) which also has a pleckstrin homology domain binds directly to PtdIns(3,4,5)P3 and PtdIns(3,4)P2, causing it to translocate to the plasma membrane upon activation of PI3-kinase. PDK1, then phosphorylates (Thr 308) and partially activates Akt. For Akt to be fully activated it needs to be phosphorylated at Ser 473 by the TORC2 complex of the mTOR protein kinase (33). mTOR integrates the signals from upstream

pathways, including insulin, growth factors (IGF-1 and IGF-2), and mitogens The RAS/MAPK cascade is largely involved in gene regulatory responses in insulin-sensitive tissues and does not play a role in the acute regulation of glucose transport. RAS proteins are also critical effector molecules previously associated with cell proliferation in flies (34).

The CAP/Cbl pathway, a PI3-kinase-independent pathway participates in insulin-mediated glucose transport through activation of TC10, a member of the Rho family of small guanosine triphosphate (GTP) -binding proteins (35). This second pathway involves tyrosine phosphorylation of the protooncogene Cbl by its direct association with the adapter protein CAP protooncogene (36, 37). CAP belongs to a family of adapter proteins with similar organization containing three SH3 domains and a region referred to as a sorbin homology (SoHo) domain. Upon phosphorylation, the Cbl–CAP complex translocates to lipid raft domains in the plasma membrane. Translocation of phosphorylated Cbl recruits the adapter protein CrkII, which forms a complex with the guanyl nucleotide-exchange protein C3G. Once translocated into lipid rafts, C3G comes into proximity and interact with the G protein TC10, catalysing the exchange of GTP for GDP, resulting in the activation of the protein. Once activated, TC10 provides a second signal to the GLUT4 protein that functions in parallel with the activation of the PI3K pathway (38).

Role of insulin in regulating cell proliferation in vertebrates

In humans, insulin and insulin growth factors (IGFs) are important molecules responsible for regulating cell proliferation, cell survival and immunomodulation. In vertebrates, blood cells are produced from four main tissues that function as hemopoietic organs such as, lymph nodes, spleen, bone marrow and the thymus. Each of these centers is influenced by many different humoral factors that regulate cell proliferation, survival and differentiation. In humans for instance, IGFs are involved in

regulation of cell differentiation, stimulation and survival of lymphocytes, antigen presenting cells and macrophages in lymph nodes (39). Similarly IGF-1 expression in macrophages has been shown to affect motility and chemotaxis (40). Additionally, IGF-1 has been shown to work as a survival signal and stimulated the production and release of regulatory cytokines (41). Macrophages are not only able to respond to IGF-1 but also to release it into neighboring tissues or internal milieu (42). In summary, insulin-like peptides and IGFs in particular have regulatory roles responsible for affecting cell proliferation and cell survival.

Much like other growth factors in vertebrates, insulin/IGFs stimulates the mitogen-activated protein (MAP) kinase extracellular signal-regulated kinase (ERK). This pathway involves the tyrosine phosphorylation of IRS proteins and/or Shc, which in turn interact with the adapter protein Grb2, recruiting the Son-of-sevenless (SOS) exchange protein to the plasma membrane for activation of Ras. The activation of Ras also requires stimulation of the tyrosine phosphatase SHP2, through its interaction with receptor substrates such as Gab-1 or IRS1/2. Once activated, Ras operates as a molecular switch, stimulating a serine kinase cascade through the stepwise activation of Raf, MEK and ERK. Activated ERK translocates into the nucleus, where it catalyses the phosphorylation of transcription factors such as $p62^{TCF}$, initiating a transcriptional program that leads to cellular proliferation or differentiation (43).

The molecular events initiated by insulin to modulate cell proliferation and survival have been the subject of intense investigations. Several lines of evidence sugest that the PI(3) Kinase branch of the insulin signaling cascade is responsible for regulating these two processes. Indeed, blocking the PI(3) Kinase by either chemical inhibitors or overexpression of dominant negative mutants in mammalian cell lines inhibited both the mitogenic (44, 45) and antiapoptotic (23) effects of insulin in different cell types.

The PI(3)K pathway has also been shown to interact with the Ras signaling pathway to initiate the proliferative response induced by insulin (46, 47). The way that PI3K interacts to controlling cell proliferation is believed to be through its effect on mRNA translation. The PI3K branch of the insulin signaling cascade (through mTOR) regulates many translation initiation factors, including eIF4E, eIF4B, and eIF4G, phosphoproteins whose phosphorylation states are directly proportional to the translation and cell growth and this is achieved by selectively targeting gene targets directly involved in cell proliferation, such as FGF (fibroblast growth factor), and VEGF (vascular endothelial growth factor) (32).

To mediate the protective effect exerted by insulin against apoptosis, PI 3-K has been demonstrated to activate Akt/PKB (48), a serine/threonine kinase that phosphorylates and thereby inactivates components of the cell death machinery, such as the Bcl-2 family member Bad (49), the glycogen synthase kinase-3 (50), the protease caspase 9 (51), and the transcription factor FKHR (52).

C-Jun N-terminal kinases (JNKs) are serine/threonine kinases that belong to the mitogen-activated protein kinase (MAPK) family. JNKs are regulated by an upstream kinase cascade and are directly phosphorylated by the dual specificity MAPK kinases (SEK1/MKK4 and MKK7) (53, 54). The activation of JNKs was initially shown to be induced by stress- and inflammation-related stimuli and to be associated with cell growth arrest and apoptosis. In addition, some studies revealed that growth factors and oncogenes induced a sustained activation of JNKs to mediate their effects on proliferation and transformation (55-59). The implication of the insulin signaling cascade in vertebrates together with the effects of RAS proteins in regulating cell proliferation and its regulation though the MAP kinase pathway are indicative of their interaction in regulating cell proliferation and population expansion.

1.2 The Insulin signaling pathway in Insects-Overview

Introduction

The presence of an insulin-like hormone in insects was proposed in the 1970s (60, 61). Since then, genetic and molecular analysis has demonstrated the presence and function of a well conserved insulin signaling pathway. Herein, I will summarize what we have learned about insulin signaling in insects, and its role in immunity.

Insulin-Like peptides in Arthropods-Overview

Insulin-like peptides have been discovered in a variety of different invertebrates: sponges (Porifera), *C. elegans*, mollusks (Molluscan Insulin Peptides or MIPs) and insects (bombyxins in *Bombyx mori* and insulin-like peptides (ILPs) in *Locusta migratoria* (Orthoptera), *Drosophila* and Aedes aegypti and anopheles gambiae mosquitoes (Diptera)), just to name a few (62-68).

The typical members of this family consist of heterodimers of A and B chains of about 20 and 30 amino acids respectively (64). Cysteine residues are located at the same sites as in their vertebrate cousins and disulphide bridges contribute to the folding of the mature structure, which is very well conserved between members. Also other structural features such as a hydrophobic core, α-helices and sharp turns are preserved (69). Insect ILPs are expressed mainly in protocerebral neurosecretory cells (62, 66, 70). By contrast, one of the *Drosophila* dilp transcripts (dilp-7) previously found in third instar larvae was also present in neurons of the abdominal ganglia, as well as in salivary glands and imaginal discs (62). The production of ILPs by median neurosecretory cells is supported by older studies of a variety of insects that utilized antisera to mammalian insulin (71-73). Bombyxin was the first insulin-like molecule discovered in the silkmoth *Bombyx mori*. Discovered in 1984, this molecule acted as a brain peptide, exerting prothoracicotropic activity in the moth species, *Samia Cynthia ricinii* (74-76)

stimulating prothoracic glands to synthesize and release the molting hormone, ecdysone. This peptide did not show such activity in *Bombyx mori*. On the contrary, another peptide named prothoracicotropic hormone or PTTH, was shown to be the endogenous ecdysteroidogenic hormone in *Bombyx mori* (77). The structure of bombyxin resembled that of Insulin-like molecules, a heterodimer of Insulin-like A and B chains (66, 69, 75). Based on these data, bombyxin was considered to be the first member of the insulin-like peptide superfamily in insects. The A and B chains appeared to be linked by means of disulfide bonds in exactly the same way as in its mammalian counterpart. Nevertheless, the gene organization of bombyxin is different from the vertebrate gene in two ways, there is an expanded number of bombyxin-related genes (30 genes) per genome and bombyxin is completely intronless (78). Four pairs of median neurosecretory cells are the major site of bombyxin gene expression in the moth's brain. Besides bombyxins in *B. mori*, other related peptides have been characterized from the saturniid moth *Samia Cynthia ricinii* (79) and the hornworm *Agruis convolvuli* (80).

Another insulin-like peptide was later identified in Orthoptera, more specifically in *Locusta migratoria* by Hietter et al, (81). Cloning and sequencing of a cDNA fragment isolated from the corpora cardiaca (CCs) led to the discovery of a 5 KDa peptide, the Locust insulin-related peptide (LIRP) (82). This small peptide did not display any amino acid sequence conservation to other insulin precursors. Instead, it corresponded to the C-chain of the LIRP precursor. The organization of the *L. migratoria* LIRP gene was different from that of *Bombyx* and *Samia cynthia ricinii* ILP genes. Indeed, while ILP genes from *Bombyx mori* and *Samia cynthia ricinii* are intronless, the organization of the LIRP gene is very similar to the vertebrate insulin gene, containing three exons separated by two introns.

There is limited evidence for ILPs in other insect groups, but there have been reports on the identification of these peptides in Hemiptera (*Rhodnius prolixus)* (83), Coleoptera (*Tenebrio molitor*) (84) and more recently in *Apis mellifera* (Hymenoptera) (84-88). Other invertebrates also express a large

number of insulin-like peptides (ILPs); for example, 38 putative ILP genes were found in the genome of *Caenorhabditis elegans* which may have different functions by virtue of their differential spatio-temporal expression patterns. In addition, while insulins are canonical activators of Receptor Tyrosine Kinases (RTK), some ILP ligands in *C. elegans* are hypothesized to work as agonists and antagonists of Insulin/IGF signaling (IIS) (89).

Insulin-Like Peptides in Drosophila

Seecof and Dewhurst (60) provided the first evidence of the presence of an insulin-like molecule in fruit flies (60). Years later, a family of seven ILPs was identified in flies (Dilp-1–7) (62). The seven *Drosophila* ILPs (Dilps) resemble pre-proinsulin at the structural level, and are therefore considered orthologous to mammalian insulin. *Drosophila's* insulin-like peptides (Dilp-1–5) were predicted to be most closely related to mammalian insulin, while DILP6 and DILP7 were predicted to be more similar to IGF-1 and relaxin, respectively, based on amino acid sequences, position of cysteine residues and consensus cleavage sites (62). The genes encoding the *Drosophila* insulin-like peptides are independently transcriptionally regulated in response to nutrition, as well as in a tissue- and stage-specific manner during development (90, 91).

Five of the ILPs (Dilp-1–5) are clustered on chromosome 3 (Dilp-5 is separated from Dilp-4 by one intervening gene), whereas Dilp6 and Dilp-7 are located on the X chromosome at two distinct loci. In addition, A- and B chains display highly conserved cysteine residues which are typical of all known members of the insulin superfamily (91, 92). Dilp6 has a different structural variation consisting of, a decapeptide attached to the N terminus of the B chain, and a truncated C peptide (KRRKR) -that resembles the structure of IGFs, with conserved processing sites (93). The direct interaction between Dilps and the *Drosophila* insulin receptor (DInR) has been demonstrated in DInR overexpression experiments and Dilp-1–5 deletion mutants, clearly showing that overexpressed DInr is dependent on

DILPs for its activity (89) . Mutations in chico, the *Drosophila* homolog of the insulin receptor substrate, caused growh defects in adult flies by making them smaller than normal control flies (94). Moreover, Dilp-2 interacts with the DInR to stimulate cell division and cell growth that translate to bigger flies. Mutational changes in the *Drosophila* insulin receptor (DInR) suppressed Dilp-2-induced formation of large flies.

The expression of different ILPs in *Drosophila* has been characterized using *in situ* hybridization and immunocytochemistry-based approaches. Dilp-1, -2, -3 and 5 transcripts are found in the same paired cluster of medial neurosecretory cells (MNCs) (62). The remaining Dilps are expressed in midgut tissue (Dilp-4, -5 and -6), imaginal discs (Dilp-2), ventral nerve cord (Dilp-7), and salivary glands (Dilp-2) of larvae, but not in the fat body. In adult females, Dilp-5 transcripts were detected in the follicle cells surrounding oocytes (95). Immunocytochemistry using an anti-Dilp-2 antibody showed reactivity in the brain's medial neurosecretory cells (MNCs) with axons enervating the corpora cardiaca (CC), the primary release site of ILPs in larvae and females. Moreover, no significant immunoreactivity was observed in other tissues (96, 97).

Insulin-Like Peptides in Mosquitoes

Insulin-like peptides also have been also found in different mosquito species (Diptera: Culicidae), including *Aedes aegypti* and *Anopheles gambiae*. Although, there have been some studies reporting the existence of genes involved in the insulin signaling pathway in *Anopheles stephensii* (98) and *Culex pipiens* (99), these genes have been characterized in more detail in *Aedes aeg*ypti and *Anopheles gambiae* mosquitoes. Seven ILPs have been identified in *Anopheles gambiae* and eight in *Aedes aegypti*. In *Anopheles gambiae*, ILP gene organization contrasts to that of *Drosophila*, despite the fact that the A and B chains share distinct similarities (100). In *Anopheles gambiae*, the location of certain ILPs is similar to *Drosophila's,* ILP-1-4 are arrayed on chromosome 3, but a pair of genes, ILP3/1 are

duplicated as ILP6/7 approximately 23 kb away (100). Transcripts corresponding to ILP-2 and ILP-5 were detected in heads, thoraces and abdomens of all life stages by RT-PCR, possibly indicating a role as a growth factor function. ILP1/7, 3/6, and ILP-4 were detected only in heads and thoraces of all life stages, and this distribution was the same as previously observed in brain MNC cells by immuno-staining, indicating a putative neurohormonal role.

In *Aedes aegypti,* Riehle and collaborators (101) conducted a thorough characterization of ILPs by using an *in silico* approach and the aid of an *Aedes* genome database. From these studies they confirmed the existence of 5 ILPs with shared homology to other dipteran ILPs. Three *Aedes* ILPs seemed to be unique, ILP-4, -7, and -8, whereas ILP8 is not related to any of the dipteran ILPs (101). The other two remaining ILPS, ILP4 and -7 did not show homology to any dipteran orthologues either. In *Aedes aegypti*, ILP transcripts showed three different expression patterns, ILP-2 and -6 are expressed in all body regions and at all life stages. ILP-6 resembles a putative IGF, and its widespread expression pattern suggests that it is likely that it behaves like one. The second pattern is for ILP-5, predominantly expressed in the abdomen of different life stages, which is consistent with the expression of its ortholog in *Anopheles gambiae* (100). The third pattern is shared by the remaining ILP genes, which are predominantly expressed in the head of different life stages, possibly suggesting a neuroendocrine function. Some other ILPs such as ILP-1, 3 are also found expressed in thoraces of larvae and ILP-4 which is also found expressed in abdomens of female mosquitoes only, making it a female specific ILP (totally absent in males) (101).

Many possible explanations have been suggested regarding the different number of ILP variants in different species. Recently, Wen and collaborators (102) compared the binding affinity of two mosquito ILPs from *Aedes aegypti* (ILP-3 and ILP-4) and found that ILP-3 strongly binds to the insulin

receptor whereas ILP-4 does not, suggesting that different ILP variants may be able to interact with the insulin receptor in different ways. Another possible reason for having several ILPs is that some ILPs could bind to other receptors, more specifically G-protein coupled receptors (GPCRs), of which there are many in the fly and mosquito genomes; and lastly, that each ILP is released on a time/tissue-specific fashion, so that only one ILP can interact with the insulin receptor at a given time and place.

Insulin receptor in Insects

The insect version of the insulin receptor has been identified in many important model insects such as, *Drosophila melanogaster*, mosquitoes (*Aedes aegypti*, *Anopheles gambiae* and *Culex pipiens*) (99, 103, 104), *Bombyx mori* (105, 106), *Apis mellifera* (107), and *Manduca sexta* (108). The *Drosophila* insulin receptor (DInR) displays similarities to its mammalian ortholog. It is a receptor tyrosine kinase (RTK) molecule of about 400 kDa that includes approximately 300 additional amino acid residues at the $^{-}NH_2$ terminus and at the $^{-}$COOH terminus (109). DInR binds human insulin with less affinity, but its signal capacity is similar to that of the human insulin receptor, and so its the function of the β-subunits, which is well conserved from man to insects (109). One important difference between the human insulin receptor and the DIR is the presence of three YXXM motifs (Tyr^{1941}, Tyr^{1957} and Tyr^{1978}) at the $^{-}$COOH-terminal tail that match phosphorylation sites in mammalian IRS1 (Insulin Receptor Substrate1) (110). Because of these motifs, the DIR can bind directly to the phosphatidylinositol-3-OH kinase (PI3k) and activates it in the absence of the Drosophila insulin receptor substrate (DIRS).

Upon binding of insulin to its receptor, the IR β-subunits undergo autophosphorylation, due to activation of their intrinsic tyrosine kinase activity. Unlike other RTKs, the insulin receptor uses insulin receptor substrates (IRS), which are scaffolding proteins necessary to initiate the signaling program (111). Interaction of IRS with the tyrosine-phosphorylated cytoplasmic tail of IR is facilitated by the

cytoskeleton, resulting in phosphorylation of specific tyrosine-residues of IRS (112). Numerous tyrosine phosphorylation sites on IRS act as docking sites for Src-homology (SH2) domain containing molecules such as, Grb2 (growth factor receptor bound protein-2) (113) and PI3K (phosphatidylinositol-3-OH kinase) (114). Each of these proteins can initiate a specific pathway: the Mitogen-Activated Protein Kinase (MAPK) and the PI3K/PKB (protein kinase B) pathway, respectively (113, 115).

The insulin receptor has been also characterized in two mosquito species, *Aedes aegypti* and *Anopheles gambiae* (116). The mosquito insulin receptor (MIR) was first discovered in *Aedes aegypti* from an mRNA transcript extracted from ovaries. This sequence seemed to correspond to a pre-receptor that is likely cleaved proteolytically into α- and β-subunits at a processing site composed of four amino acid residues. Besides being expressed in the ovaries, transcripts were also found in the head and body of female mosquitoes (117). The MIR is different from the *Drosophila*'s DIR in terms of size since it lacks the 300 amino acid extension at the carboxy terminus of the β-subunit (118).

More Recently, Brown and collaborators (119) found that the MIR is fully functional and capable of interacting with chemically synthesized ILP-3 and therefore stimulated the ovaries to produce yolk. Moreover ILP-3 is also able to stimulate the synthesis and release of ecdysteroids from ovaries. Ecdysteroids are also responsible for promoting cell proliferation in imaginal discs, optics lobes and epidermis (120). This is the first report of an insect ILP capable of binding and signaling through the mosquito insulin receptor. In hemocytes ecdysteroids have been found expressed in hemocytes of the crayfish *Procambarus clarkia.* In crayfish hemocytes, ecdysteroids are able to induce immune activation and participate in wound healing (121).

1.3 Biological Function of the Insulin pathway in Insects

In vertebrates, hormones and growth factors play a key role in controlling overall growth because they coordinate cell division, survival and growth. Insulin is well known for its role in the regulation of metabolic activity: it induces glucogenesis (formation of glycogen from glucose), inhibits gluconeogenesis, and shows an indirect anabolic effect on lipid and protein metabolism, and other roles in immunity. In insects, carbohydrate levels present in the hemolymph are also controlled by hormones. Insulin plays many functions in regulating the physiology of insects, by controlling their metabolic activity, reproductive physiology, regulation of growth and size, control of life span, diapause (99) and immunity (122). Insulin signaling plays a very important role in regulating reproductive functions in the mosquito, *Aedes aegypti.* The mosquito insulin receptor (MIR) is localized in follicular cells surrounding developing oocytes. In adult female insects, follicle cells are the primary source of ecdysteroids.

Interestingly, bovine insulin applied to cultured ovaries showed a direct stimulatory effect on mosquito ovarian ecdysteroid production and protein synthesis in a dose-dependent manner (123). Similarly, in *Drosophila*, an intact insulin pathway is required to regulate the rate of egg production in response to nutritional changes, such as a protein-rich diet, and to enter vitellogenesis [17]. Animals with decreased insulin signaling not only display reduced fertility, but also display changes in energy homeostasis.

One of the biggest effects of insulin signaling is the regulation of life span. This phenomenon was first observed in *Caenorhaditis elegans* by noticing that the lack of signaling through the insulin receptor DAF-2 lead to larval development arrest and lifespan extension. This state or arrest in dauer larvae is characterized by the lack of feeding and a metabolic shift to energy storage mode. This insulin-

based effect in longevity is not limited to worms and mammals but it also occurs in *Drosophila* and probably in other insects as well (124).

In *Aedes aegypti*, insulin signaling stimulates ovarian ecdysterogenesis and protein synthesis, leading to yolk deposition, and egg maturation. Moreover, insulin-like peptides, more specifically ILP-3 was shown to reduce sugar levels in hemolymph and to elevate carbohydrate and lipid store in the fat body. Similarly ILP-3 was also able to stimulate yolk deposition, as evidenced by RNAi and injection of chemically-synthesized ILP-3. RNAi knockdown of the mosquito insulin receptor was able to block yolk deposition whereas ILP-3 injection in decapitated females completely restored normal yolk (119).

More limited data also suggest that insulin-like peptides have mitogenic properties (125) (126). For example, molecular genetic studies using *Drosophila* have shown that insulin signaling controls organ growth and body size in insects (127, 128). Bombyxin (an insulin-related lepidopteran peptide) was able to stimulate cell growth of wing imaginal disc of the butterfly *Precis coenia* acting as a growth factor (129) (130). It has also been reported that bombyxin promotes mitotic division of circulating granulocytes in *Bombyx mori* (131). In silkworm larvae, the number of cells in hematopoietic organs increases during feeding periods (132-134), suggesting that hematopoiesis in the HPO is linked to nutritional status.

More recently, Kim and collaborators (125) reported that dPI3K and dAKT -two important kinases and part of the insulin cascade- are involved in insulin-induced ERK pathway activation leading to proliferation in *Drosophila* S2 (Schneider) cells. S2 (Schneider) cells treated with insulin showed increase activities of dPI3K and dAKT with activation of the dERK pathway components dMEK and dERK. When dPTEN and AKT-specific inhibitors were used dERK and dAKT activation was blocked, indicating involvement of dPI3K and dAKT in the insulin-induced dERK and dAKT activations. This series of

experiments were the first ones to indicate an involvement of the insulin signaling cascade in regulating proliferation as well as dERK pathway activation by both dPI3K and dAKT in *Drosophila* S2 (Schneider) cells, which is not activated the same way in mammals (125).

1.4 Insect Hemocytes: Role in immunity

Overview

Vertebrates protect themselves from infectious agents by using two defense systems, the innate and acquired immune system. The first one relies on factors determined genetically and expressed upon pathogen entry and specific recognition by cognate receptors. On the other hand, the acquired immune system does his part by means of complex gene rearrangements in order to produce a diversity of recognition molecules suitable for detection of intruders. These mechanisms also lead to the development of memory and establishment of populations of sentinel cells. Insects lack an acquired immune system but they have developed a very efficient innate mechanisms that are divided into two categories: humoral and cellular immunity (135). The humoral defense mechanisms are various and include the production of antimicrobial peptides (136), reactive species of oxygen and nitric oxide (137, 138), and oxidative molecules involved in melanization (139). The cellular defense system is basically the use of immune/blood cells or hemocytes to phagocytose or encapsulate pathogens or foreign objects (140).

In vertebrates, all blood cells derive from hematopoietic stem cells that differentiate into different lineages under control of transcription factors like GATA 1–3 and AML1 (141). In flies, hemocytes first develop during embryogenesis from cell layers on the head mesoderm, a so called first wave (142-144). Later during larval or nymphal stages hemocytes are produced via division of stem cells in hematopoietic organs (derived from mesodermal tissue) (lymph glands) and/or by continued division of hemocytes already in circulation (132, 143, 145, 146). In *Drosophila*, the GATA homolog Serpent

(Srp) is expressed in hematopoietic stem cells (147). Two additional transcription factors, Glial Cell Missing (Gcm) and the AML1-like protein Lozenge (Lz), function downstream of Srp and are required for plasmatocyte and crystal cell development, respectively (135). Serpent is considered a master regulator for hemocyte differentiation. Other genes implicated in proliferation and differentiation of hemocytes include the Janus kinase (JAK)/signal transducer and activator transcription (STAT) (148, 149) Toll signaling pathways (150) and the Vegf/PVR signaling pathway, involved in cell proliferation and migration in flies (151, 152).

Classification

Insects produce several types of hemocytes that have been identified using morphological characteristics, histochemical staining and functional markers (153). The most common types of hemocytes reported in the literature are prohemocytes, granular cells (granulocytes), plasmatocytes, spherule cells (spherulocytes), and oenocytoids. These hemocyte types have been described from species in diverse orders including Lepidoptera, Diptera, Orthoptera, Blattaria, Coleoptera, Hymenoptera, Hemiptera, and Collembola (154-163).

In lepidopteran larvae, granular cells and plasmatocytes are the only types of hemocytes capable of adhering to foreign surfaces, and together usually comprise more than 50% of the total number of hemocytes in circulation (164-166). The other hemocytes described from Lepidoptera are non-adhesive spherule cells, oenocytoids and prohemocytes. Spherule cells have been suggested to transport cuticular components (167), while oenocytoids contain cytoplasmic phenoloxidase precursors that likely play a role in melanization of hemolymph (168-170). Prohemocytes are hypothesized to be stem (progenitor) cells that differentiate into one or more of the aforementioned hemocyte types. The nomenclature used for *Drosophila* hemocytes is somewhat different from those of most other insects,

including lower dipterans such as culicids and Simuliids (171). During embryogenesis, the only hemocyte type present is called macrophages or plasmatocytes. In larvae, plasmatocytes remain the most abundant hemocyte type in circulation but two other cell types, lamellocytes and crystal cells, are also present. Lamellocytes are adherent whereas crystal cells have hyaline inclusions and regulate melanization.

In anophiline mosquitoes, hemocytes are known to be phagocytic and have also been observed in proximity to melanotic capsules (161, 172, 173). Although there are several studies (174) illustrating the different types of hemocytes produced by mosquitoes, many of them only used morphological criteria (175, 176). My work in characterizing hemocytes from *Aedes aegypti* and *Anopheles gambiae* is presented in chapter 2.

1.5 General Summary

There has been considerable progress over the past few years in unravelling the mechanisms of insulin action, and the molecular defects that give rise to pathology in humans. Recent advances dissecting the signaling pathways in insects, with sophisticated genetic analysis have yielded quantum leaps in our understanding on how proteins and tissues interact to control glucose and lipid metabolism. Furthermore, novel roles involving these molecules are appearing to be the widespread. Taken together, the observations on the role of the insulin/IGF/ILP signaling pathways in vertebrates, and invertebrates (insects and nematodes, etc) revealed the existence of an evolutionarily conserved signaling pathway which regulates a variety of interrelated biological processes, such as the control of metabolism, growth, reproduction and life-span (1). It is known that insulin-peptides can induce hemocyte proliferation in lepidopteran insects. More recent molecular geneticic studies using *Drosophila* have shown that it also controls organ growth and body size in insects. Bombyxin, an insulin-

related peptide functions as a growth factor in Lepidoptera, as well as stimulating mitotic division in granulocytes in *Bombyx mori*. Also, the number of cells in the hematopoietic organ seems to also increase. The fact that ILPs have immunomodulatory effects (hemocyte proliferation/hemopoiesis and increased PPO activity) lead us to believe that immune activation does occur after a blood meal and therefore hemocytes might play a role in defending the mosquito from pathogens present in the blood meal.

Previous precedent in the literature has highlighted the importance of brain produced factors in the regulation of biological processes in mosquitoes. Factors such as ILPs, ecsdysteroids and ovarian ecdysterogenic hormone have been shown to affect the reproductive biology of mosquitoes by regulating yolk deposition. Given the importance of the insulin signaling pathway, MIR and ILPs in mosquito physiology and the importance of hemocytes as the armed branch of the immune system, we wanted to investigate the role that mosquito insulin plays in influencing the immune system of mosquitoes, more specifically their effects on hemocytes. I hypothesize that the same signaling pathway involved in regulating reproductive physiology after a blood meal is responsible for affecting hemocyte physiology. Knowing about their types, function, abundance and dynamics, is key to our understanding of the intricacies of mosquito cell based immunity. We propose the following specific objectives:

1. Characterize the hemocyte types produced by *Aedes aegypti* and their abundance during larval, pupal and adult stages. I hypothesize that hemocytes produced by mosquitoes belong to a few classes with different functions and their abundance might be conditioned to conditions such as life stage, time, and blood feeding. Blood feeding is known for stimulating the start of a developmental program that leads to egg production, and that affects mosquito physiology as a whole.
2. Characterize the expression of the Mosquito insulin receptor (MIR), and its cognate ligands (Insulin-like peptides or ILPs) in mosquito hemocytes. The expression of components of the insulin signaling

cascade in blood/immune cells is not new and their functions are usually associated with cell proliferation survival and immunity. I hypothesize that the different ILPs and the MIR are expressed in mosquito hemocytes.

3. Characterize the effects of the MIR and insulin-like peptides in maintenance of hemocyte populations following blood feeding. The localization of transcripts for the different ILPs in human lymphocytes as well as their role in regulating lymphocyte function and dynamics led me to hypothesize that the insulin cascade is responsible for affecting hemocyte population by inducing cell proliferation and or survival.

CHAPTER 2

CHARACTERIZATION OF HEMOCYTES FROM THE MOSQUITOES *ANOPHELES GAMBIAE* AND *AEDES AEGYPTI*

2.1 Introduction

The ability to isolate and identify hemocytes is essential for studies in insect cellular immunity. Hemocyte classification schemes based on morphology or a combination of morphological and functional characters have been developed for the model dipteran *Drosophila melanogaster* and selected lepidoptera species (153, 164, 165, 177-182). Much less is known about the types of hemocytes produced by other insects including numerous species of economic importance. Key challenges include the small size of many insects which makes collection and identification of hemocytes difficult due to the limited amount of hemolymph and cells present in circulation.

The hemocyte types insects produce and the names they are given also sometimes differs between taxa such that classification schemes and criteria used to identify hemocytes in one group of insects may not be fully applicable to another (135). The difficulty of collecting and classifying insect hemocytes is especially apparent in vector arthropods like mosquitoes. In vivo studies indicate that hemocytes comprise an essential branch of the mosquito immune system required for phagocytosis and encapsulation of foreign targets (161, 173, 183-189). Mosquito hemocytes are also important sources of signaling and effector molecules released into hemolymph (188, 190, 191). However, current understanding of the types of hemocytes mosquitoes produce, their relative abundance, and

their functions is limited. Hemocytes from adult *Aedes aegypti* were recently classified into granulocytes, oenocytoids, adipohemocytes, and thrombocytoids on the basis of morphology, binding of selected lectins, and enzymatic activity (192). Using strictly morphological criteria, other investigators have classified hemocytes from *Aedes aegypti* and *Culex quinquefasciatus* into plasmatocytes and oenocytoids (175, 193) or have recognized multiple cell types including putative stem cells named prohemocytes (194, 195).

Far less is known about the hemocytes produced by other mosquitoes including *Anopheles gambiae*, a major vector of human malaria. Hemocytes from anophiline mosquitoes are known to be phagocytic and have also been observed in proximity to melanotic capsules (161, 173, 189, 196). The types of hemocytes involved and their function in mediating these responses, however, are unclear. Identifying the hemocyte types mosquitoes produce and understanding their functions in immunity would benefit from increased uniformity in methods for collecting cells and in the criteria used for classifying and naming different hemocyte types. It would also be valuable if comparative data collected using similar methodology were available to determine if important vector species produce similar or different hemocyte types.

Toward this end, we conducted a comparative study with *Anopheles gambiae* and *Aedes aegypti.* We first examined how different collection methods affected the number and types of hemocytes obtained from mosquitoes. We then used a combination of morphological and functional markers to classify the hemocytes present in different life stages. We conclude that both species produce three types of hemocytes that are identifiable using similar criteria.

2.2 Materials and methods

Insects

An. gambiae (G3 strain) and *Ae. aegypti* (UGAL strain) were reared in a dedicated insectary in the Department of Entomology at the University of Georgia at ~27 °C with a 16 h light:8 h dark cycle. After hatching, larvae were reared in deionized water in shallow aluminum pans (200-250 larvae/~400 ml/tray) and fed a defined daily regimen of finely ground mixture of TetraMin Rich Mix fish food. Under these conditions, development to pupae is highly synchronous. Adults have access to 8% fructose (*Anopheles gambiae*) or 5% sucrose (*Aedes aegypti*) solution, and prior to blood feeding, caged mosquitoes were starved and kept in total darkness for at least an hour.

Hemocyte collection and primary culture

Previous approaches used for collecting mosquito hemocytes include clipping the proboscis of cold anesthetized adult females (197-199) and displacement perfusion (200). We compared these approaches to two other approaches we developed and named low and high injection/ recovery. In the low injection/recovery method, adult *An. gambiae* and *Ae. aegypti* were cold anesthetized on ice for 15 min followed by injection of 8-10 ul of 60% Schneider's medium (Sigma), 10% fetal bovine serum (FBS) (Hyclone) and 30% citrate buffer **(**=anticoagulant) (98 mM NaOH, 186mM NaCl, 1.7mM EDTA and 41 mM citric acid, buffer pH 4.5) (vol/vol) between the last two abdominal schlerites using a glass needle mounted on a micromanipulator. Diluted hemolymph was then collected by capillary action using a clean, empty glass needle placed next to the injection site in the abdomen. In the high injection/ recovery method, adults were again injected with 10-12 ul of Schneider's:FBS:anticoagulant (60:10:30) and placed on ice for 20 min. We then injected 25 ul of Schneider's:FBS:anticoagulant (60:10:30) into the lateral wall of the mesothorax and collected the diluted hemolymph by capillary action from the original injection site in the abdomen using a second hand-held glass needle. The diluted hemolymph

from both approaches was collected in a microfuge tube on ice or placed in Teflon-lined wells on glass slides (see below). After allowing cells to settle, diluted hemolymph was removed and replaced with fresh Schneider's medium plus 10% FBS. Hemocytes were collected from 3-day old pupae and third instar larvae using the high injection/recovery method. Other media including Grace's insect medium, TC-I00, L-15, and HyQ (Sigma, Hyclone) with or without FBS were also tested as alternatives for collecting and maintaining mosquito hemocytes in primary culture. However, none of these media improved collection or viability of mosquito hemocytes compared to Schneider's medium plus 10% FBS (data not presented).

Functional assays and immunocytochemical procedures

Fluorescent probes tested as potential markers for mosquito hemocytes are listed in Table 2.1. Staining conditions (buffer, pH, temperature, incubation time) were first optimized by titrating with monochlorobimane (MCB)(201). Cells were incubated with probes diluted in Schneider's medium for 30 min at room temperature in the dark at the concentrations indicated in Table 2.1. Cells were washed once in medium and then held at 4°C or at room temperature before examination. Phagocytosis assays were conducted using fluorescein isothiocyanate (FITC)-conjugated *Escherichia coli* prepared as previously described (202). About 1 x 10^3 bacteria were injected into cold anesthetized mosquitoes. After 1 h at room temperature, hemocytes were collected using the high injection/ recovery method as described above. Alternatively, hemocytes were collected from individual mosquitoes and overlayed with 1 x 10^3 bacteria for 1 h. We then scored the percentage of each hemocyte type that had ingested particles by counting 100 hemocytes per sample using the fluorescent quenching method (202).

The capacity of mosquitoes to encapsulate a foreign target was tested by inserting the tip of a glass needle between the intersegmental membrane of two abdominal schlerites. The fiber was then

examined for binding of hemocytes 24-48 h later. Six antibodies generated against specific *An. gambiae* immune proteins provided by collaborators were also tested (Table 2.2). These were antisera to: serpin6 (SRPN6), serpin10 (SRPN10), the chitin binding serine protease SP22D, and prophenoloxidase6 (PP06) (K. Michel and F.E. Kafatos, Imperial College); lysozyme c-1 (LYS c-1) (S. Paskewitz, University of Wisconsin); and a subunit of the 26S proteasome (PSMD3) previously named diphenol oxidase A2 (DOXA2) (P. Romans, University of Toronto; see (203). An anti-histone HI antibody (Santa Cruz) was also used as a marker for cell nuclei in selected experiments. Hemocytes from non-immune challenged mosquitoes and mosquitoes injected 3 h earlier with *E. coli* were fixed in 4% paraformaldehyde in PBS (13.7 mM NaCI, 0.27mM KCI, 0.43mM Na2HP04, 0.14mM KH_2P0_4, pH 7.3) for 15min, rinsed with PBS and then permeabilized for 15 min in PBT (PBS plus 0.1 % Triton X-100). After blocking for 1 h with 1 % bovine serum albumin (BSA; fraction V, Boehringer Mannheim) in PBS (blocking solution), cells were incubated with primary antibody at the dilution indicated in Table 1. After rinsing 4 x in PBT, hemocytes were incubated with FITC, Texas Red (TR)-conjugated secondary antibodies (1:1000) (Jackson Labs) diluted in blocking solution. Staining for phenoloxidase activity was performed by fixing hemocytes in 4% paraformaldehyde in PBS for 15 min, rinsing with PBS, and permeabilizing in 50% methanol. After rinsing in PBS, cells were incubated with 2 mg/ml L-dopamine in PBS for 3 h (204). For detection of acid phosphatase activity, samples were incubated for 30min in IOrng/ml lead nitrate and 3% f3-glycero-phosphate in 0.05 M acetate-acetic acid, pH 5.0. Cells were then rinsed 3 x in water followed by incubation for 1 min in 1.0% ammonium sulfide in water (205). Peroxidase activity was detected by incubating fixed cells in 500 flg/ml diaminobenzidine (DAB) in PBS followed by addition of 0.01 % hydrogen peroxide.

Microscopy, image processing, and statistical analysis

Samples were examined using a Leica TCS confocal microscope fitted with differential interference contrast (DIC) optics. Some DIC and epifluorescent images were directly captured using a digital camera (Q Capture) while others were obtained by confocal microscopy using Leica software. All captured images were exported to Adobe Photoshop as .tif files for assembly of figures. Each treatment was tested against hemocytes collected independently from five or more mosquitoes of specific age or stage. Proportional data were arcsin transformed prior to analysis. The data were then analyzed by *t-test* or one way analysis of variance (ANOVA) using JMP 3.0 software (SAS Institute, Cary, NC, USA) (206).

2.3. Results

Collection method affects the number and types of cells recovered from adult mosquitoes

Established methods for collecting mosquito hemocytes include bleeding from a cut proboscis (197-199) and perfusion whereby saline or medium is injected between the head and thorax, and diluted hemolymph is collected from an incision made in the abdomen (200). Mosquito hemocytes have also been obser-ved in close proximity to trachea and other tissues in the hemocoel (187, 189). This suggests that some hemocytes are sedentary or adhere to tissues in contact with hemo-lymph which could also affect collection. In insects like Lepidoptera, the adhesive properties of hemo-cytes have been reduced during collection by using low pH, anticoagulant buffers (204, 207).

We therefore assessed whether use of an anticoagulant could facilitate collection of mosquito hemocytes by comparing a method we called injection/ recovery to the proboscis and perfusion methods. Four day old sugar fed *An. gambiae* and *Ae. aegypti* adult females were used for these comparisons by assessing the total number of hemocytes and contaminants (cuticle, scales, and internal

tissues like the fat body) collected. Live hemocytes were discriminated from contaminants by adding the vital dye propidium iodide (PI) and MCB to each sample. MCB enters living cells and interacts with the antioxidant glutathione to produce fluorescent glutathioneS-bimane. MCB is also reported to preferentially label mammalian and insect immune cells because of their elevated glutathione levels (201). The high injection/recovery method yielded the most hemocytes per mosquito while cutting the proboscis yielded the fewest (Fig. 2.1A).

Table 2.1

Non-antibody probes tested as markers for mosquito hemocyte

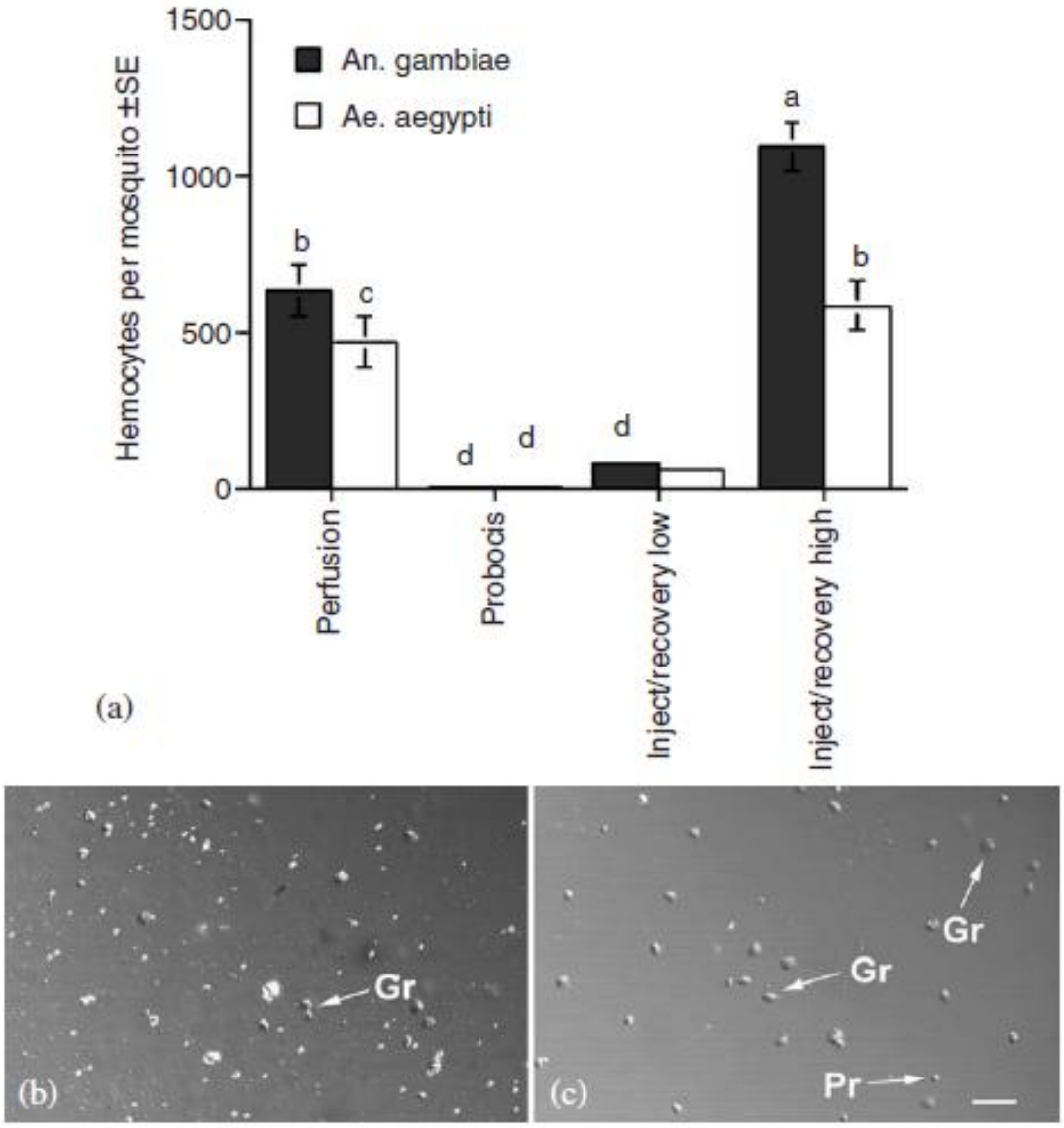
Reagent	Source	Concentration	Reactivity
Monochlorobimane	Molecular probes	10µM	Intracellular glutathione
Diaminofluorescein diacetate	Sigma	10µM	Intracellular nitric oxide and Peroxynitrite
Rhodamine 123	Sigma	20 ng/ml	Intracellular reactive oxygen
Fluo3-FF-AM	Sigma	0.3 µM	Intracellular calcium
Propidium iodide	Santa Cruz Biotech.	2.0 µg/ml	Cell-impermeable nucleic acid dye (vital dye)
Alexa 488 phalloidin	Molecular Probes	1:200 dilution	F-actin
3,4 Dihydroxy—phenylamine	Sigma	2 mg/ml	Intracellular Phenyloxidase activity
Glycerol 2-phosphate	Sigma	1 mM	Intracellular acid phosphatase
FITC-conjugated Escherichia coli	Lab stock	1 x 10^3 cells mosquito	Phagocytosis
FITC-conjugated peanut lectin	Sigma	10µg/ml	fl-gal(1–3) Galactosamine
FITC-conjugated soybean lectin	Sigma	10 µg/ml	Surface a/fl-N-acetyl Galactosamine and galactopyranoside
FITC-conjugated wheat germ lectin	Sigma	10 µg/ml	Surface N-acetyl Glucosamine and N-acetyl neuramine acid

Table 2.2

Antibody probes tested as markers for mosquito hemocytes

Antisera	Dilution	Antigen	Source
Anti-H1 histone	1:200	Cell nuclei	Santa Cruz Biotech.
Anti-SRPN6	1:1000	Serpin6 An. gambiae	Abraham et al. (2005) (199)
Anti-SRPN10	1:100	Serpin10 An. gambiae	Danielli et al. (2003) (208)
Anti-PPO6	1:500	Phenoloxidase 6 An. gambiae	Muller et al. (1999) (209)
Anti-SP22D	1:500	Chitin binding serine protease SP22D An. gambiae	Danielli et al. (2000) (210)
Anti-PSMD3	1:800	Regulatory subunit 26S proteosome An. gambiae	Romans, unpubl.
Anti-LYS c-1	1:200	Lysozyme-c1 An. gambiae	Li and Paskewitz (2006) (211)

We also noted that more hemocytes were recovered on average from *An. gambiae* than *Ae. aegypti* using each collection method despite their smaller size (Fig. 2.1A). Visual inspection of the samples revealed that the perfusion method consistently produced the largest amount of contaminants which included a mixture of cells and tissue fragments from other organs (fat body, Malpighian tubules), unidentifiable subcellular debris, and scales from the abdomen (Fig. 2.1B). The proboscis and low injection recovery methods produced the least contamination while the high injection/recovery method produced slightly more contamination that consisted primarily of subcellular debris (Fig. 2.1C). This level of contamination, however, was much lower than the perfusion samples and was also more easily removed by gently washing the collected hemocytes with fresh Schneider's medium. Hemocyte viability from both species using the high injection/recovery method was greater than 90% as evidenced by PI staining (data not presented). Increasing the amount of anticoagulant or reducing the amount of FBS in the solution used to collect hemocytes significantly elevated mortality and did not increase the number of hemocytes recovered (data not presented). As previously noted, Hillyer and Christensen in 2002 classified hemocytes from *Ae. aegypti* into granulocytes, oenocytoids, adipo-hemocytes, and thrombocytoids (192). Granulocytes were described as being approximately 9 µm diameter with numerous granules in the cytoplasm. Granulocytes were also the most abundant hemocyte type, were phagocytic and bound to foreign surfaces like glass slides. Adipohemocytes were reported to be the second most abundant hemocyte type, averaging 40 µm in diameter with large nuclei and prominent lipid droplets in the cytoplasm. Oenocytoids were described as being approximately 9 µm in diameter with a homogeneous cytoplasm. Oenocytoids also contained phenoloxidase activity and were non-phagocytic. Thrombocytoids were reported to be rarest hemocyte type and were characterized as elongate cells (30 µm in length) with a homogeneous cytoplasm that were non-adhesive in vitro.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500
0
Hemocytes per mosquito ±SE
An. gambiae
Ae. aegypti
a
b
b
c
d
d
d
Perfusion
Probocis
Inject/recovery low
Inject/recovery high
(a)
Gr
(b)
Gr
Gr
Pr
(c)

Figure 2.1. Collection method affects hemocyte number and the level of contaminants recovered from *An. gambiae* and *Ae. aegypti* adult females. (A) Total hemocyte counts±SE recovered per mosquito by perfusion, clipping the proboscis, low injection/recovery, and high injection/recovery. A minimum of 10 individuals were bled per treatment ($F_{7, 77} = 34.7$; $p<0.0001$). Means with the same letter do not significantly differ from one another (Tukey–Kramer HSD multiple comparison procedure a = 0.05). Low magnification/differential interference contrast (DIC) micrographs of samples collected by perfusion **(B)** or high injection/recovery **(C)**. The perfusion sample contains large amounts of contaminants along with hemocytes while the injection/recovery sample contains primarily hemocytes with low levels of contamination (see text). Representative granulocytes (Gr) and prohemocytes (Pr) are indicated. Scale bar = 40 pm.

Using this classification scheme as a reference point, we determined that perfusion samples from *An. gambiae* and *Ae. aegypti* contained hemocytes matching the above descriptions for granulocytes, adipohemocytes and oenocytoids but we did not observe any cells that could unambiguously be identified as thrombocytoids (Fig. 2.2A). A small percentage of cells recovered from both species also did not morphologically correspond to any of the hemocyte types described above. These cells were 4-6 μm in diameter, spherical, non-adhesive, and had a high nuclear to cytoplasmic ratio as visualized by phalloidin and anti-histone staining (see below). We classified these cells as prohemocytes, because of their resemblance to hemocytes named prohemocytes in other insects (135, 212). Samples from the high injection/ recovery method consisted primarily of granulocytes and much smaller percentages of prohemocytes and oenocytoids (Fig. 2.2B). However, almost no adipohemocytes were collected. The size and morphology of prohemocytes, granulocytes and oenocytoids collected from *An. gambiae* and *Ae. aegypti* using the high injection/recovery method were very similar to one another (Fig. 2.2C-H).

The percentage, morphology and size of each hemocyte type in the proboscis and low injection/recovery samples was also very similar to the high injection/recovery samples (data not presented). Given the near absence of adipohemocytes in the proboscis and injection/recovery samples and their identical morphology to fat body cells in the hemocoel, we concluded that adipohemocytes were not hemocytes but rather contaminating fat body cells that were most frequently collected using the perfusion method.

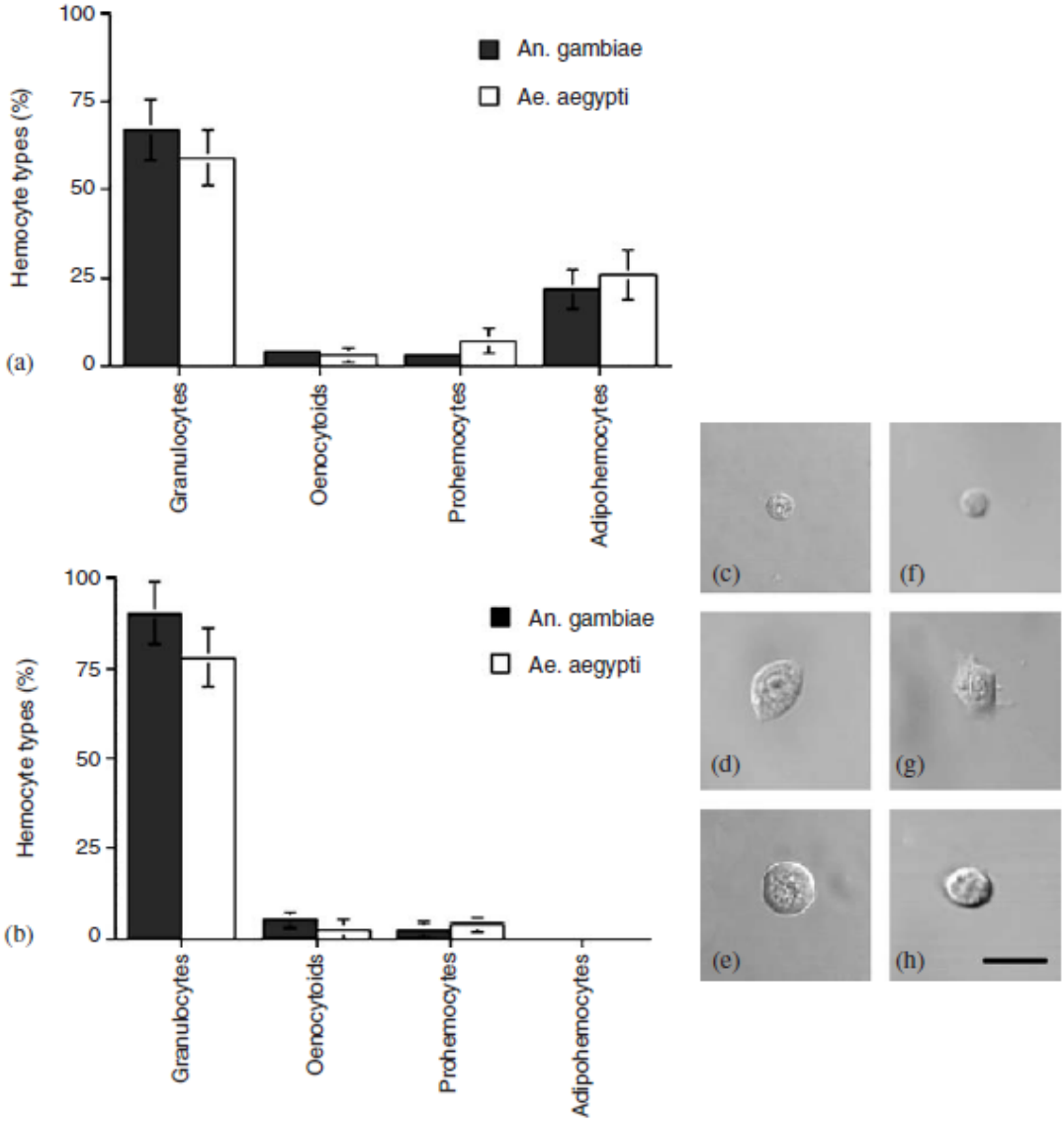
100
75
50
25
0
Hemocyte types (%)
(a)
An. gambiae
Ae. aegypti
Granulocytes
Oenocytoids
Prohemocytes
Adipohemocytes
(b)
(c)
(f)
(d)
(g)
(e)
(h)

Figure 2.2. Differential hemocyte counts for perfusion (A) and high injection/recovery (B) samples from *An. gambiae* and *Ae. aegypti*. Counts were made on the same samples used to produce the total hemocyte counts presented in Fig. 2.1. Hemocytes were classified as granulocytes, oenocytoids, prohemocytes, and adipohemocytes on the basis of morphology and staining with different markers (see text). DIC images of a representitive prohemocyte **(C)**, granulocyte **(D)** and oenocytoid **(E)** from an An. gambiae adult female. A prohemocyte **(F)**, granulocyte **(G)**, and oenocytoid **(H)** from an *Ae. aegypti* adult female are also shown for comparison. Note the close similarity in morphology and size of each hemocyte type from the two species. Scale bar ¼ 25 mm.

Functional markers further discriminate hemocyte types

We next assessed whether any of the fluorescent or enzymatic markers listed in Table 2.1 discriminated the morphological hemocyte types described above using samples collected from sugar fed (non-immune challenged) 4-day-old adult females. When first collected from mosquitoes, granulocytes were spherical or slightly tear-shaped cells that could not be fully distinguished from oenocytoids or prohemocytes by light microscopy alone. Unlike oenocytoids and prohemocytes, however, granulocytes rapidly bound and spread on the surface of glass slides (Fig. 2.3A and B). Staining with phalloidin and anti-HI histone clearly visualized the filopodia, focal adhesions, and low nuclear/ cytoplasmic ratio typical of granulocytes after spreading on glass slides (Fig. 2.3C). Phagocytosis assays further indicated that granulocytes were the only hemocyte type in *Ae. aegypti* and *An. gambiae* that internalized bacteria (Fig. 2.3D-G). Most granulocytes from both mosquito species spread symmetrically on glass slides but some cells spread asymmetrically with one axis clearly longer than the other (Fig. 2.3A-C). We originally thought this difference in spreading morphology could be functionally significant since other insects produce adhesive hemocyte types that assume different spreading morphologies in vitro which in turn correlate with different immune functions.

In most Lepidoptera, for example, granulocytes spread symmetrically in vitro and are the professional phagocytes, whereas plasmatocytes spread asymmetrically, are non-phagocytic, and function as the main capsule-forming hemocyte type (summarized by Lavine and Strand, 2002; Strand et al., 2006) (135, 213). Analogously, the professional phagocytic hemocytes in *Drosophila* (called plasmatocytes) differ in size and assume a different spread morphology from lamellocytes that form capsules (171, 181).

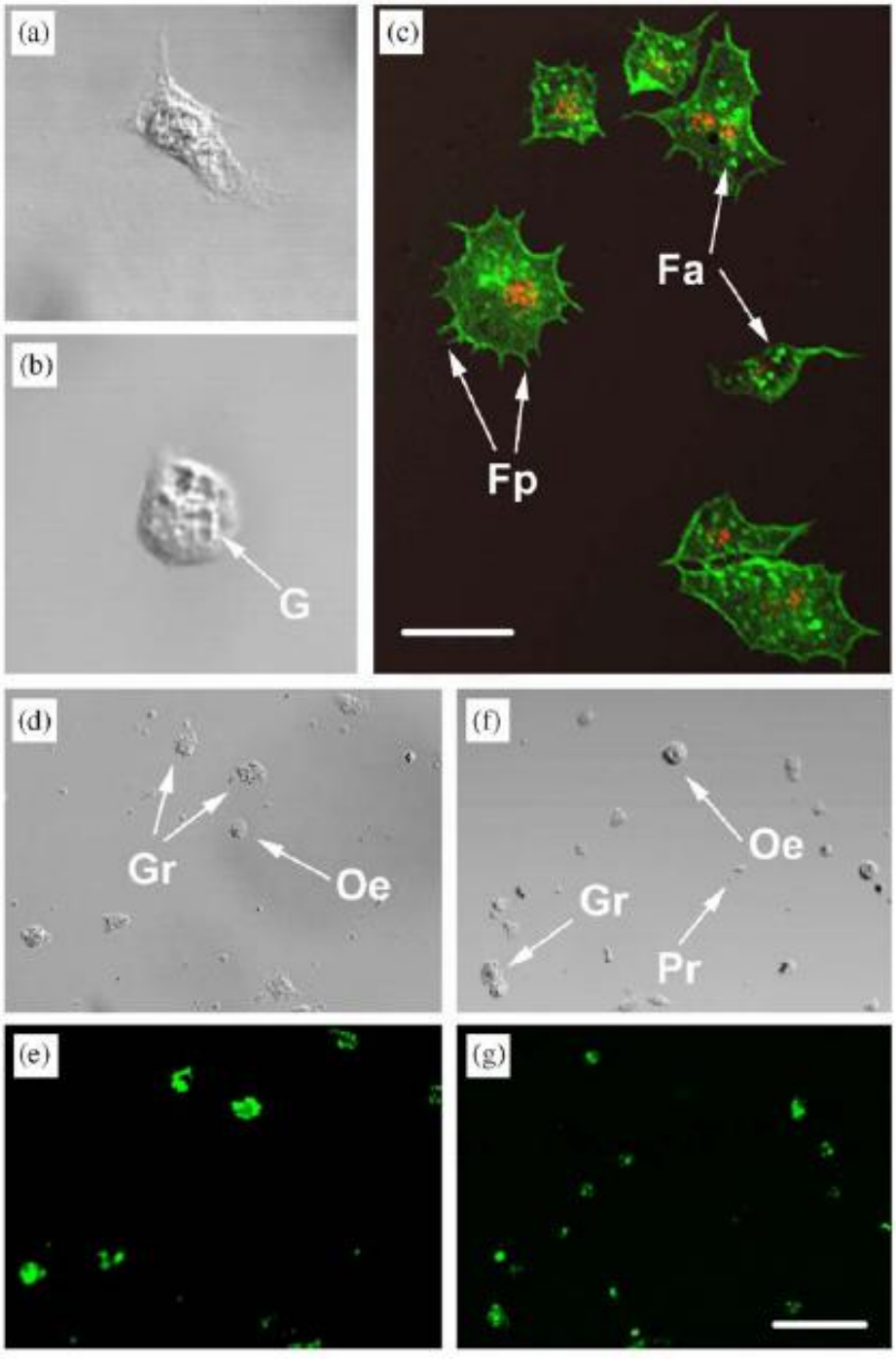
(a)
(b)
G
(c)
Fa
Fp
(d)
Gr
Oe
(f)
Oe
Gr
Pr
(e)
(g)

Figure 2.3. Granulocytes from *An. gambiae* and *Ae. aegypti* adult females. (A) and **(B)** DIC images of granulocytes from An. gambiae 1 h after placement on a glass slide in Schneider's medium plus 10% FBS. One cell **(A)** has spread asymmetrically while the other **(B)** has spread symmetrically. Granules (G) are visible in the granulocyte presented in b. **(C)** Confocal image of *An. gambiae* granulocytes stained with phalloidin that labels Factin (green) and anti-histone H1 that labels nuclei (red). Filopodia (Fp) and focal adhesions (Fa) associated with F-actin and adhesion to the glass slide are clearly visible. The projected composite image was generated from six optical sections that were one-half micron in thickness. Scale bar in C ¼ 10 mm with the same magnification in a and b. Low magnification DIC **(D)** and epifluorescent image **(E)** of hemocytes from *An. gambiae* 1h after incubation with FITC-labeled E. coli. DIC **(F)** and epifluorescent **(G)** images of hemocytes from *Ae. aegypti* 1 h after incubation with FITClabeled E. coli is also presented. All granulocytes (Gr) from both species phagocytized bacteria (green), whereas oenocytoids (Oe) and prohemocytes (Pr) have not. Scale bar in G ¼ 80 mm with the same magnification in d–f.

However, we found that symmetrically and asymmetrically spreading granulocytes from both mosquito species phagocytized bacteria. A small number of hemocytes also attached to glass fibers inserted into the hemocoel but did not form multilayered capsules typically observed in Lepidoptera or *Drosophila.* Inspection of these glass fibers indicated that both symmetric and asymmetrically spreading granulocytes were present. Oenocytoids from *Anopheles gambiae* and *Aedes aegypti* were distinguished from spread granulocytes by their spheroidal shape (6-20 μm diameter) and weak adhesion to glass or plastic (Fig. 2.4A). Oenocytoids were the only hemocyte type that stained positively for phenoloxidase activity and also were consistently labeled more strongly by MCB than granulocytes and prohemocytes (Fig. 2.4B-D). Oenocytoids from both species usually also had a single nucleus. The rounded morphology and lack of adhesion of prohemocytes was similar to oenocytoids. However, prohemocytes were usually smaller (4-6 μm) and had a larger nuclear to cytoplasmic ratio than oenocytoids (Fig. 2.4A). Prohemocytes also lacked phenoloxidase activity and were stained weakly or not at all by MCB. The other fluorescent probes and enzymatic assays we tested did not unambiguously discriminate a single hemocyte type.

Diaminofluorescein diacetate and dihydrorhodamine 123 that detect reactive nitrogen and oxygen species, respectively, stained granulocytes and oenocytoids similarly but stained prohemocytes very weakly or not at all. The intracellular calcium marker Fluo3-FF-AM, wheat germ agglutinin, soybean lectin, and *Helix pomatia* lectin labeled all hemocytes with varying intensity as did assays for acid phosphatase activity. We tested antibodies generated against six immunerelated proteins from *Anopheles gambiae* against hemocytes from sugar fed (non-immune challenged), 4-day-old adult females of both mosquito species.

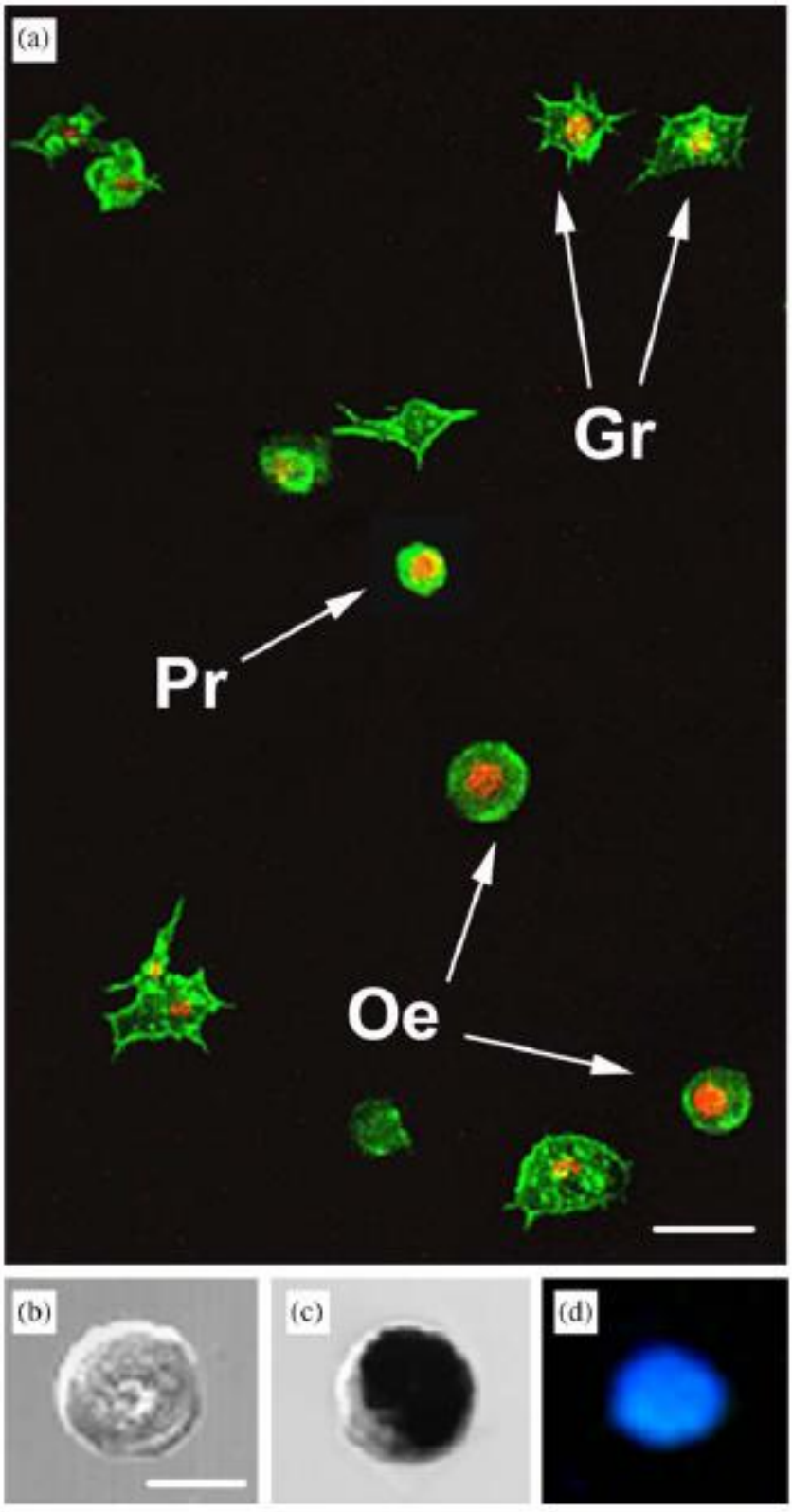
(a)
Gr
Pr
Oe
(b)
(c)
(d)

Figure 2.4. (A) Confocal image of *Ae. aegypti* hemocytes stained with phalloidin (green) and anti-histone-H1 (red). Spread granulocytes (Gr) are distinguished from round, unspread oenocytoids (Oe) and smaller, unspread prohemocytes (Pr). Also note the visibly higher nuclear to cytoplasmic ratio in the prohemocyte compared to the oenocytoids. Scale bar ¼ 20 mm. The projected composite image was generated from six optical sections that were one-half micron in thickness. **(B)** Higher magnification DIC image of an oenocytoid. A DIC image of an oenocytoid following staining for phenoloxidase activity **(C)**, and an epifluorescent image of an oenocytoid following vital staining with monochloro-bimane (MCB) **(D)** are also shown. Staining of *An. gambiae* oenocytoids for phenoloxidase activity and MCB is virtually identical to the images shown for *Ae. aegypti*. Scale bar ¼ 15 mm in B with the same magnification in c and d.

Four of the antibodies (anti-PP06, -SP22D, -SRPN6, -SRPN10) only stained hemocytes from *An. gambiae* while the remaining two (anti-PSMD3 and -LYS c-1) stained hemocytes from *An. gambiae* and *Ae. aegypti.* PP06 is one of nine prophenoloxidases encoded by *An. gambiae* that is thought to be expressed predominantly, if not exclusively, in hemocytes and a subpopulation of cells in the 4a3b cell line that was derived from hemocytes (209, 214). Anti-PP06, however, may cross-react with other PPOs (215). We found that anti-PP06 stained only oenocytoids from non-immune challenged *Anopheles gambiae* (Fig. 2.5A and B) which also were the only hemocytes that stained positively in phenoloxidase assays (see above). Previous in vivo studies detected Sp22D expression in unknown hemocytes present in circulation or bound to tissues like muscle and trachea (216).

We observed that anti-Sp22D strongly stained 20-25% of *An. gambiae* granulocytes but did not stain any oenocytoids or prohemocytes (data not presented). SRPN6 was previously detected in midgut epithelium and hemocytes of unknown type while SRPN10 was detected in the midgut, pericardial cells and hemocytes (199, 217). We found that anti-SRPN6 and SRPN10 labeled the cytoplasm of both granulocytes and oenocytoids in a punctate pattern but did not stain prohemocytes (data not presented). PSMD3 (formerly named DOXA2) is closely linked to a QTL for melanotic encapsulation of malaria ookinetes that is expressed in several tissues including hemocytes (203) and P. Romans (pers. commununication). Anti-PSMD3 weakly stained granulocytes and oenocytoids in *An. gambiae* (Fig. 2.5A and B). The same weak staining pattern was also observed in *Ae. aegypti* (data not presented).

Lysozymes are a family of proteins produced by both invertebrates and vertebrates that are defined by their ability to cleave the glycosidic bond between N-acetylmuramic acid and N-acetyl glucosamine in the peptidoglycan layer of bacterial cell walls. The activity of lysozyme c-1 from *An. gambiae* toward bacterial cell walls has not been determined but recent studies indicate that this protein is

present in hemolymph and surprisingly reduces melanization of foreign targets (211). Similar to anti-PSMD3, anti-LYS c-1 stained the cytoplasm of granulocytes and oenocytoids from *Anopheles gambiae* and *Aedes aegypti* (data not presented).

Immune challenge affects labeling patterns of selected antibody markers

Although PP06 is constitutively expressed in the 4a3b cell line (218), immune challenge is well known to activate the PO cascade in adult mosquitoes (215, 219). In contrast to sugar fed, non-immune challenged females, anti-PP06 stained both oenocytoids and granulocytes collected from *An. gambiae* females immune challenged with dead, FITC-labeled *E. coli.* This is illustrated by comparing the confocal images presented in Fig. 2.5A-D which were obtained using identical settings for gain, aperture, and laser intensity.

Phenoloxidase assays produced the same pattern (data not presented) suggesting that PP06 and possibly other PPOs are constitutively expressed in oenocytoids but are up regulated in granulocytes after exposure to bacteria. Immune challenge with *E. coli* resulted in staining of all granulocytes by anti-SP22D (data not presented). Compared to samples from nonchallenged mosquitoes (Fig. 2.5E and F), we also observed an increase in staining intensity of granulocytes and oenocytoids following immune challenge using anti-PSMD3 (Fig. 2.5G and H). No changes in staining patterns after immune challenge with *E. coli* were observed with antiL YS c-l, -SPRN-6, or –SRPN-IO (data not presented). An overall summary of hemocyte staining properties using the functional assays, fluorescent probes and antibody markers we tested is presented in Table 2.3.

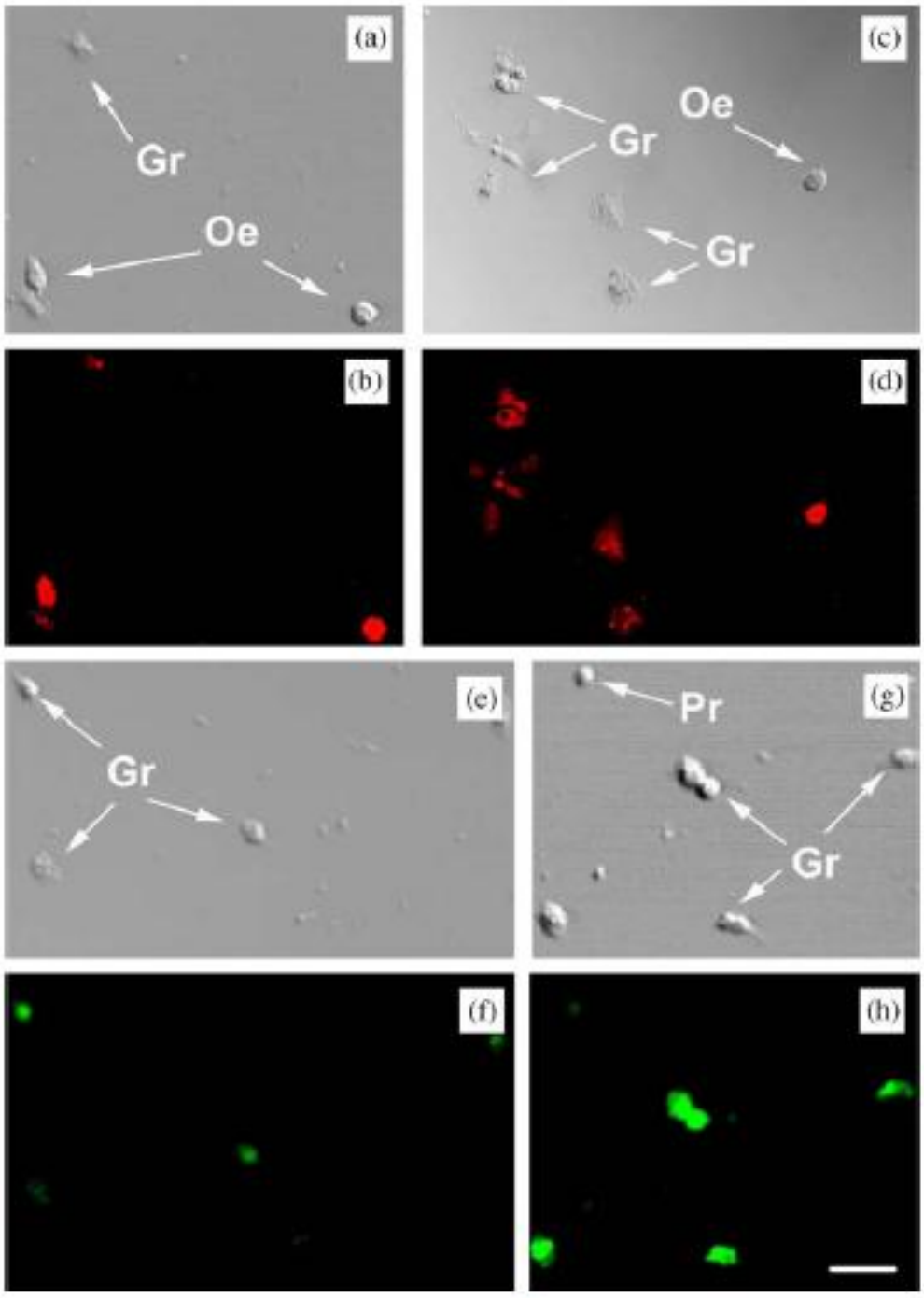
(a)
Gr
Oe
(c)
Oe
Gr
Gr
(b)
(d)
(e)
Gr
(g)
Pr
Gr
(f)
(h)

Figure 2.5. Bacterial challenge alters staining of *An. gambiae* hemocytes using antibodies to PP06 and PSMD3 (formerly Dox-A2). DIC **(A)** and confocal **(B)** micrographs of hemocytes from non-immune challenged mosquitoes stained with anti-PPO6 and a Texas-red conjugated secondary antibody (1:1000). The two oenocytoids in the image are stained strongly whereas the granulocyte is stained weakly. DIC **(C)** and confocal **(D)** micrographs of hemocytes collected 3 h post-injection of FITC-conjugated *E. coli*. Hemocytes were stained with same concentration of PPO6 antiserum and secondary antibody as in A and B. Note that granulocytes are stained more strongly in the challenged sample compared to the non-challenged sample. In contrast, staining intensity of the oenocytoids in the challenged and non-challenged samples are similar. The projected composite images in **B** and **D** were generated from four optical sections that were one-half micron in thickness. Laser intensity, gain, and aperture settings in acquiring the two images were also identical. **(E)** and **(F)** DIC and epifluorescent micrographs of hemocytes from non-immune challenged An. gambiae hemocytes stained with anti-PSMD3 and a FITC-conjugated secondary antibody. All cells in the image are uniformly but weakly labeled. **(G)** and **(H)** DIC and epifluorescent micrographs of hemocytes collected 3 h post-injection of rhodamine-conjugated *E. coli*. Note the stronger staining of granulocytes (Gr) compared to the non-challenged sample. In contrast, PSMD3 staining of the prohemocyte in the image remains weak. Hemocytes were stained with same concentration of anti-PSMD3 and secondary antibody as in e and f. Camera exposure time and gain settings in acquiring the images in f and h were identical. Scale bar in H ¼ 40 mm with the same magnification in e–g.

Table 2.3
Summary of hemocyte staining patterns in *An. gambiae* and *Ae. Aegypti* adult females.

	An. gambiae[a]			Ae. aegypti[a]		
	Gr	Oe	Pr	Gr	Oe	Pr
General markers						
Adhesion to foreign surfaces	+	—	—	+	—	—
Monochlorobimane[b]	+	+	—	+	+	—
Phagocytosis of FITC-labeled E. coli	+	—	—	+	—	—
Phenoloxidase activity[c]	+	+	—	+	+	—
Diaminofluorescein diacetate	+	+	—	+	+	—
Dihydrorhodamine 123	+	+	—	+	+	—
Fluo3-FF-AM	+	+	+	+	+	+
Peanut lectin	+	+	+	+	+	+
Soybean lectin	+	+	+	+	+	+
Wheat germ lectin	+	+	+	+	+	+
Acid phosphatase	+	+	+	+	+	+
Antibody markers						
Anti-SRPN6	+	+	—	—	—	—
Anti-SRPN10	+	+	—	—	—	—
Anti-PPO6[c]	+	+	—	—	—	—
Anti-SP22D[c]	+	—	—	—	—	—
Anti-PSMD3[c]	+	+	—	+	+	—
Anti-LYS c-1	+	+	—	+	+	—

[a] Gr=granulocyte, Oe=Oenocytoid, Pr=Prohemocyte.

[b] Monochlorobimane stains oenocytoids from both mosquito species more strongly than granulocytes while prohemocytes are stained weakly or not at all (see text).

[c] Staining for phenoloxidase activity and PPO6 was restricted almost exclusively to oenocytoids in non-immune challenged mosquitoes but granulocytes also exhibit phenoloxidase activity and PPO6 staining following immune challenge by bacteria (see text). Differences in staining patterns also exist for anti-SP22D and anti-PSMD3 between non challenged and immune challenged mosquitoes (see text).

Adult males, pupae, and larvae contain the same hemocyte types as adult females

Most studies on hemocytes from *Drosophila* and Lepidoptera involve cells collected from larvae, whereas studies with mosquitoes focus on adult females since this is the life stage that vectors medically important pathogens. To assess whether the hemocyte types observed in adult females are present in other life stages, we collected hemocytes from 4-day-old adult males, third stadium larvae, and 2-day-old pupae using the high injection/recovery method. Hemocytes were easily collected from pupae and adult males, whereas the fragile nature of larvae made hemocyte collection more difficult. However, the

use of fine glass needles for injections allowed us with practice to consistently collect large numbers of hemocytes from larvae of both species using the high injection/recovery method. *t-tests* indicated that fewer hemocytes on average were collected from *Anopheles gambiae* adult males compared to adult females *(t* = 4.2; *p<O.OOOl)* but no differences were found in the number of hemocytes recovered from adult male and female *Aedes aegypti (t* = 0.7; *p>O.l)* (see Figs. 2.1A and 2.6A).

Life stage comparisons indicated that significantly more hemocytes were collected from *Anopheles gambiae* pupae and adult females than larvae and adult males *(F* = 5.1; *P* < 0.001). In contrast, no differences in the number of hemocytes recovered were found between life stages in *Ae. aegypti (F* = 0.7; *P* >0.1). Classification of hemocyte types using the morphological and functional markers described above indicated that larvae, pupae and adult males contained similar percentages of granulocytes, oenocytoids and prohemocytes to four day old adult females (Fig. 2.6B-D). No other hemocyte types were observed.

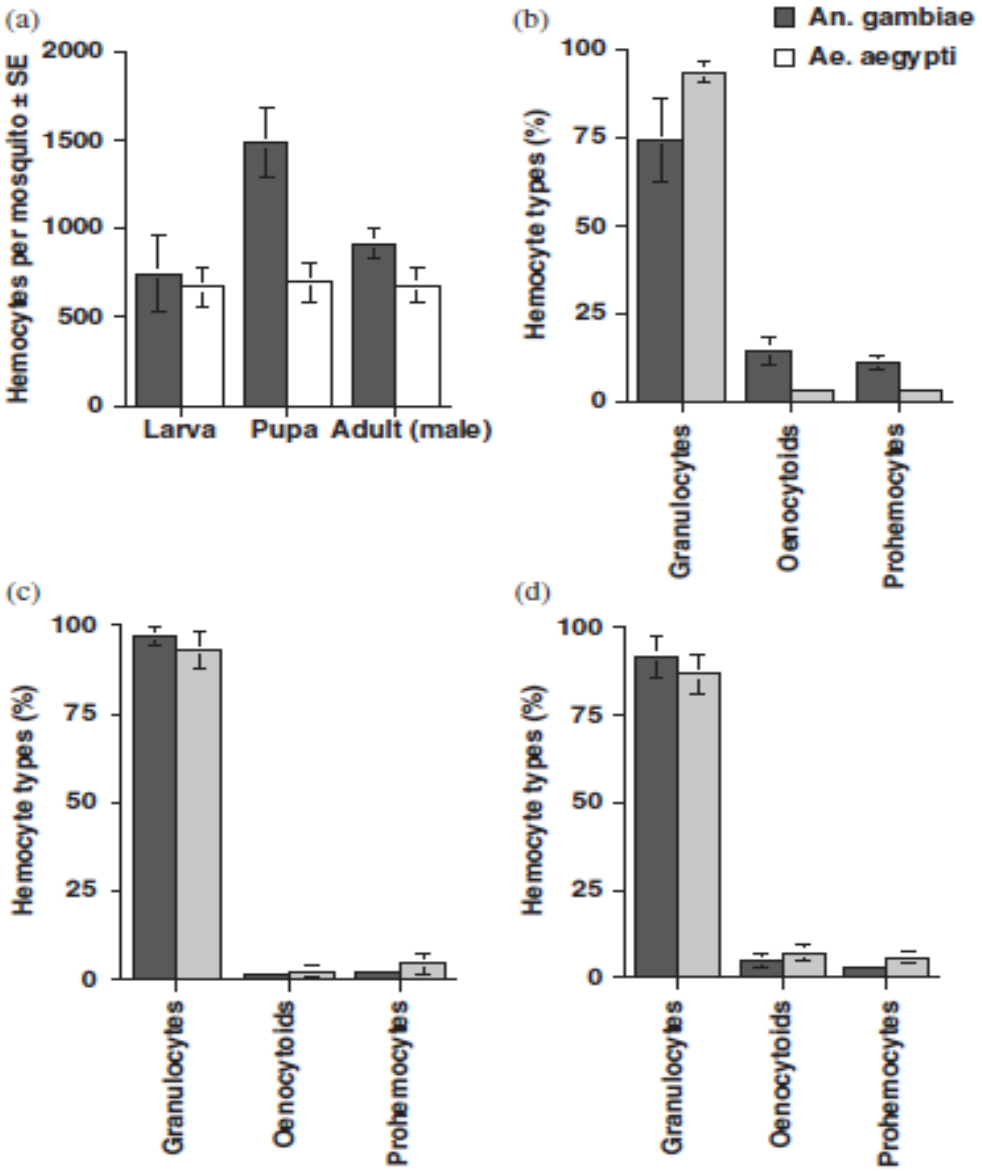
(a)
Hemocytes per mosquito ± SE
2000
1500
1000
500
0
Larva
Pupa
Adult (male)
(b)
An. gambiae
Ae. aegypti
Hemocyte types (%)
100
75
50
25
0
Granulocytes
Oenocytoids
Prohemocytes
(c)
Hemocyte types (%)
100
75
50
25
0
Granulocytes
Oenocytoids
Prohemocytes
(d)
Hemocyte types (%)
100
75
50
25
0
Granulocytes
Oenocytoids
Prohemocytes

Figure 2.6. Hemocyte counts from different life stages of An. gambiae and *Ae. aegypti* larvae. (A) total hemocyte counts from larvae (third instar), pupae (day 4) and adult males (day 4). (**B–D)** Differential hemocyte counts from larvae, pupae, and adult males, respectively. Hemocytes were collected from each stage using the high injection/recovery method. A minimum of 10 individuals were sampled for each life stage. Hemocytes were placed on glass slides in Schneider's medium plus 10% FBS for 1 h and then identified by morphology, monochloro-bimane, and rhodamine 123 staining. See Fig. 2.1A and Fig. 2.2 for total and differential hemocyte counts from adult females.

Hemocyte abundance declines with adult age and transiently increases following a blood meal

The preceding studies focused on a single age class of larvae (third instar), pupae (2-day) and adults (4-day) in order to minimize possible age-related differences in hemocyte abundance when evaluating collection methods and markers for identification. Once these methods were standardized, we next asked whether hemocyte abundance changed in adult females with age or blood feeding since both greatly affect mosquito physiology (220). Hemocyte abundance progressively declined with age in both species when mosquitoes were fed only sugar water (Fig. 2.7A and B).

In contrast, the number of hemocytes collected from the hemocoel transiently increased 24-48 h after a blood meal in comparison to sugar fed females of the same age (Fig. 2.7A and B). Hemocyte abundance then declined to the same levels as sugar-fed females by 72 h post-blood meal. Classification of the hemocyte types present 48 h after blood feeding did not reveal any differences in the percentage of granulocytes, oenocytoids and prohemocytes present compared to sugar-fed females of the same age (data not presented).

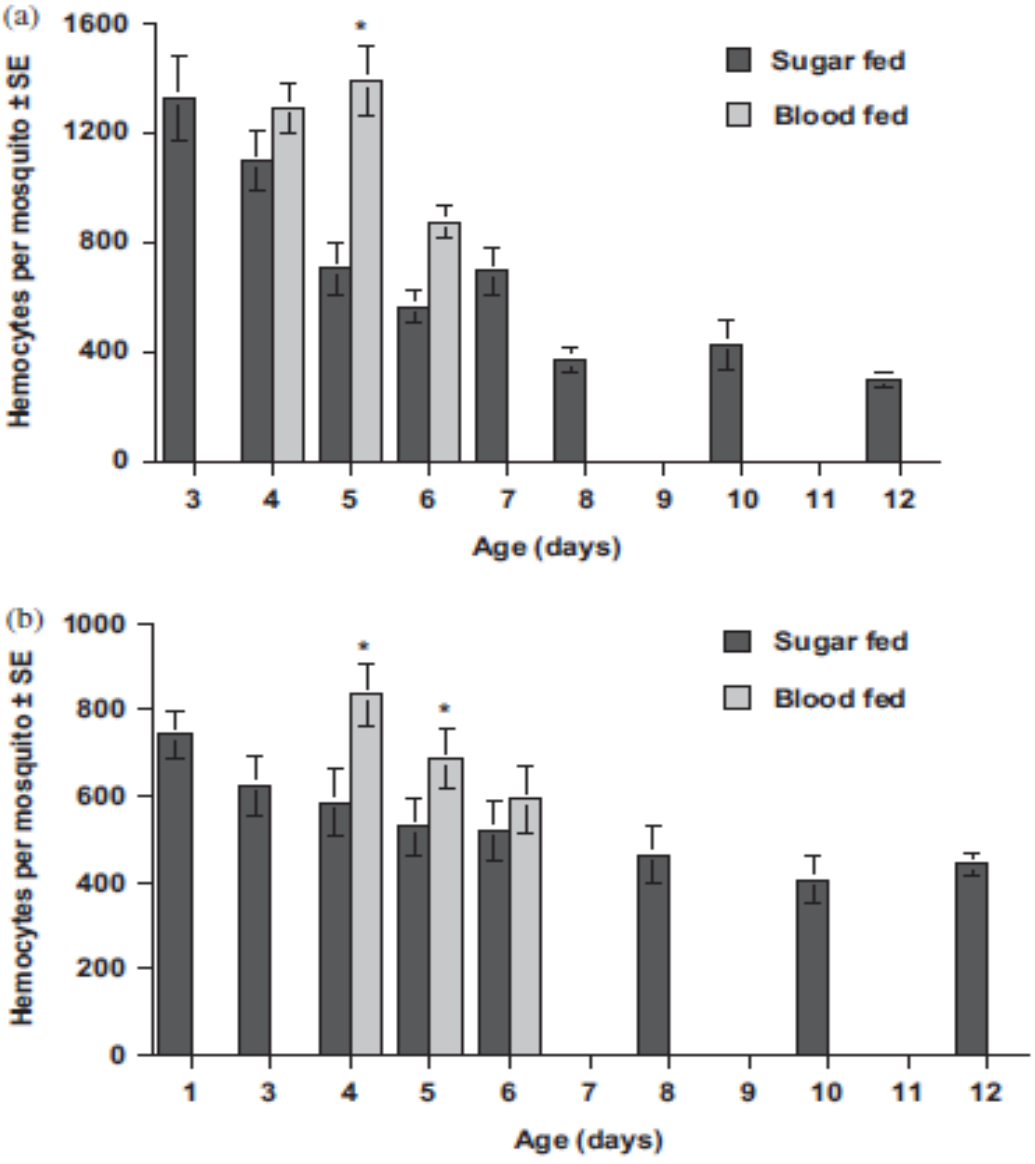
(a)
Hemocytes per mosquito ± SE
1600
1200
800
400
0
*
Sugar fed
Blood fed
3
4
5
6
7
8
9
10
11
12
Age (days)
(b)
Hemocytes per mosquito ± SE
1000
800
600
400
200
0
*
*
Sugar fed
Blood fed
1
3
4
5
6
7
8
9
10
11
12
Age (days)

Figure 2.7. Total hemocyte counts from adult female *An. gambiae* (A) and *Ae. aegypti* (B) of increasing age (days). Cohorts of females fed sugar only were sampled daily using the high injection/recovery method. A second cohort of females from the same starting population was blood fed on day 3 and thereafter sampled on days 4–6. Asterisks above the bar indicate that the total number of hemocytes collected from blood fed females differed significantly from females of the same age that were fed sugar water only (t-test, a ¼ 0.05). A minimum of 10 individuals were bled per time point in each treatment.

2.4. Discussion

Most studies of mosquito hemocytes have relied exclusively on light or electron microscopy for identification (175, 193, 195, 221). Studies in other insects, however, indicate that morphology alone is often inadequate for identification and that functional markers along with uniformity in collection and culture methods are needed to reliably distinguish one hemocyte type from another.

This study, therefore, focused on three needs. First, we compared collection methods and examined different life stages in order to develop a comprehensive data set on the number and types of hemocytes present in mosquitoes. Second, we examined several functional markers to assess whether any could be used in combination with morphology to facilitate hemocyte identication. Some of the markers we tested have been used previously to classify hemocytes from *Ae. Aegypti* (188, 222), whereas others had not. Third, we compared *An. gambiae,* whose hemocytes had not previously been classified, with *Ae. aegypti* to determine if similar criteria can be used in both. We conclude that hemolymph from *An. gambiae* and *Ae. aegypti* contains three hemocyte types that are present in larvae, pupae and adults. Granulocytes are by far the most abundant cell type while oenocytoids and prohemocytes together usually comprise less than 10% of the total hemocyte population. Our results also indicate that collection method greatly affects the number and types of cells obtained when bleeding mosquitoes. The high injection/recovery method yielded the largest number of hemocytes per individual and produced lower levels of contaminates than the perfusion method. This improvement appears to be due to the use of an anticoagulant and a pipette to collect the diluted hemolymph more cleanly. The use of Schneider's medium also improved hemocyte viability in primary culture compared to other methods we tested. The importance of reducing contamination cannot be overemphasized because its presence makes identification and accurate counting of mosquito hemocytes very difficult. For example, adult *Ae. aegypti* were previously reported to contain 2000 hemocytes (223), whereas

subsequent studies suggested this was an overestimate because of fat body and other cellular contaminants in perfusates (192). We too found it difficult to accurately discriminate hemocytes from other contaminants in perfusion samples.

The presence of contaminants also affects hemocyte morphology and spreading behavior which further exacerbates cell identification. In particular, granulocytes phagocytize contaminants which reduces binding of these cells to glass slides and alters spreading morphology. Reducing contamination from other cells and tissues like the fat body is also of obvious importance in any functional genomic study that seeks to identify genes preferentially expressed in hemocytes or a specific hemocyte type (181, 224). The most common types of hemocytes reported in the literature are prohemocytes, granular cells (= granulocytes), plasmatocytes, spherule cells and oenocytoids. These hemocyte types have been described from species in diverse orders including Lepidoptera, Diptera, Orthoptera, Blattaria, Coleoptera, Hymenoptera, Hemiptera, and Collembola (135, 145, 225-227). As noted in the introduction of this paper, these hemocyte names have also been used in prior studies to classify the types of hemocytes observed in mosquitoes. In insects like Lepidoptera, granulocytes are usually the professional phagocytes, plasmatocytes are the main capsule forming cell, oenocytoids are a source of phenoloxidases, prohemocytes are putative stem cells, and spherule cells are potentially a source of cuticular components. In contrast, the most detailed data on hematopoiesis in insects derives from *Drosophila* whose hemocytes, for historic reasons, are named differently from most other insect species. *Drosophila* larvae contain three recognized types of hemocytes in circulation named plasmatocytes, lamellocytes and crystal cells (171). While rare, hemocytes similar to prohemocytes in other insects, are also observed in circulation in *Drosophila,* whereas no hemocytes resembling spherule cells are observed (171). Plasmatocytes are the professional phagocyte, lamellocytes are specialized capsule forming cells, and crystal cells are a primary source of phenoloxidase activity.

Based on morphology and functional activity *Drosophila* plasmatocytes are most analogous to hemocytes named granulocytes in Lepidoptera and other insects, lamellocytes are most similar to plasmatocytes, and crystal cells are analogous to oenocytoids (135, 227). With this background in mind, we carefully considered whether to name the hemocytes observed during the current study using the terminology of previous studies on mosquitoes or to adopt the terminology used for *Drosophila.* In the end, we decided to continue using the nomenclature adopted in previous studies on mosquito hemocytes, because it was less confusing and made it easier to compare our data to earlier results. There clearly is a need though for workers studying hemocytes from mosquitoes, *Drosophila,* Lepidoptera, and other insects to develop more uniform terminology.

Although some authors have named phagocytic, adhesive hemocytes from mosquitoes as plasmatocytes (193, 195), we concur with Hillyer and Christensen (2002) (192) in naming these cells granulocytes since they most closely conform to the characteristics of hemocytes named granulocytes in most other insects (135, 228) (see above). Our functional bioassays and markers also did not indicate that mosquitoes produce more than one adhesive type of hemocyte even though lepidopterans (granulocytes and plasmatocytes) and *Drosophila* (plasmatocytes and lamellocytes) produce two adhesive hemocyte types specialized for phagocytosis and capsule formation, respectively (135, 141, 178, 181, 213, 229). Mosquito oenocytoids are identified by the combination of morphology, MCB labeling, and phenoloxidase activity. The correlation between elevated MCB labeling and phenoloxidase activity is potentially important because MCB reacts with glutathione that is a well known inhibitor of melanization (204).

Since insect PPOs lack signal peptides, they are thought to be stored in the cytoplasm and released when activated oenocytoids lyse following immune challenge (169, 184, 185, 204, 219).

Elevated levels of intracellular glutathione thus may play a role in blocking PPO activation in oenocytoids prior to lysis. Phenoloxidase activity was previously reported to be oenocytoid specific in *Ae. aegypti* (192), but phenoloxidase activity was detected in putative granulocytes from *Anopheles albimanus* (161).

Our results indicate that oenocytoids constitutively exhibit phenoloxidase activity, whereas granulocytes exhibit increased phenoloxidase activity following immune challenge. This suggests that regulation of the phenoloxidase cascade potentially differs between these hemocyte types. Differences in Sp22D expression following immune challenge suggests that functionally distinct sub populations of granulocytes may also exist.

The term prohemocyte has historically been used for putative hemocyte progenitor cells with the capacity to differentiate into other cell types (135, 145, 212). Kaaya and Ratcliffe (1982) concluded that mosquito hemolymph contains prohemocytes, whereas Hillyer and Christensen (2002) suggested these objects were more likely subcellular debris (192, 195) . We also observed contaminants in perfusion samples that could be mistaken for small cells. However, samples collected by probocis clipping and injection/ recovery convince us that *An. gambiae* and *Ae. aegypti* hemolymph contains a hemocyte type that is morphologically and functionally distinct from granulocytes and oenocytoids. The uniform size, rounded morphology, large nuclear to cytoplasmic ratio, and lack of labeling of these cells by the functional markers we tested are consistent with these cells being a type of progenitor cell. However, it is possible these cell could be a more specialized hemocyte type, such as a granulocyte precursor rather than a stem cell capable of differentiating into either granulocytes or oenocytoids. Additional developmental and functional studies will be needed to understand the lineage fate and activity of each hemocyte type present in *Anopheles gambiae* and *Aedes aegypti.*

Our results indicate that the hemocyte types observed in adult females are also present in adult males, pupae, and larvae of both mosquito species. The number of hemocytes collected from adult, sugar-fed mosquitoes also progressively declines with age which has also been implicated in increased susceptibility to septic infection in *Ae. aegypti* (230). However, blood feeding transiently increased the number of hemocytes we were able to collect.

The underlying mechanism for this effect or whether other perturbations, like injection of bacteria, also increase hemocyte numbers is currently unknown. Although, recent studies by Dimoupulos et al 2008 showed that bacterial infection induces a transient increase in hemocyte numbers during bacterial infections in *An. gambiae* mosquitoes (231) . One possibility is that blood feeding transiently increases the number of hemocytes that are in circulation versus sedentary on tissues. Another is that blood feeding stimulates proliferation of one or more hemocyte types. The role of proliferation is more likely and its explored in details in the last chapter.

In summary, our results lay a foundation for collecting and identifying hemocytes from two mosquito species using light and fluorescence microscopy methods. These approaches are also fully compatible with fluorescenceactivated cell sorting (FACS) methods that have proven essential for functional studies of mammalian immune cells. With development of additional markers and sorting methods, studies on lineage relationships, hematopoiesis, and the responses of specific genes in different hemocyte populations following immune challenge should be feasible in mosquitoes**.** The one question that remains unanswered is the importance of the increase of hemocyte numbers after a blood meal, and whether this trait is advantageous to the mosquito. This question will be explored and answered in the next chapters.

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CHAPTER 3

EXPRESSION OF INSULIN-LIKE PEPTIDES AND MOSQUITO INSULIN RECEPTOR (MIR) IN AEDES AEGPYTI HEMOCYTES

3.1 Introduction

Humans and other vertebrates produce up to 10 different ILPs that are subdivided into three classes of molecules: insulin, insulin-like growth factors (IGFs), and relaxins on the basis of their processing, primary structure (presence of C peptide), processing (cleavage of the C peptide and location of the intra and inter-chain disulphide bridges), and receptor binding preferences (119). The basic structure of ILPs consists of four major domains (Pre, B, C, and A), expressed as pro-peptides, which are then proteolytically processed to mature peptides consisting of the B and A chain connected by disulphide bridges linking well conserved cysteine residues. This basic structure is shared in part by relaxins and IGFs.

Most insect ILPs are produced in brain neurosecretory cells. Specific immunostaining for ILPs detects a small cluster of cells in the medial and dorsal regions of the brain in various group of insects, including Orthoptera (232, 233), Hemiptera (234), Lepidoptera (235, 236), Coleoptera (237) and Diptera including *Drosophila melanogaster* (238) and the mosquitoes, *Aedes aegypti* (239) and *Anopheles gambiae* (240). Some ILPs are also expressed in other regions besides the brain including ovaries,

midgut, and fat body tissue (95, 101, 241, 242). ILPs are implicated in the regulation of diverse physiological processes in insects, such as diapuse in *Culex* mosquitoes (99), social behavior in honeybees (243), cell proliferation in hemopoietic organs in Lepidoptera (126) and egg maturation in mosquitoes (119).

In *Drosophila,* gene expression patterns for each of the ILP genes (dilp1-7) have been revealed by *in situ* hybridization and immunocytochemistry of tissues from larvae and adults. mRNA transcripts for dilp-1, -2, -3, and -5 co-localize in the same clusters of brain medial neurosecretory cells, MNCs (244). Other dilps are expressed in various tissues; dilp-4, -5, and -6 are expressed in the midgut, dilp-2 was found expressed in imaginal discs, whereas dilp-7 ad dilp 2 were found expressed in ventral nerve cord, and salivary glands respectively, of larvae, but none in fat body (245). In females, dilp5 was found localized to the follicle cells of oocytes (93) and dilp6 in fat body(244). In *Aedes aegypti*, ILP expression patterns have also been characterized. Five ILPs (-1, -3, -4, -7, -8) are expressed in the brains of larval, pupal, and adult mosquitoes, ILP-2 and -6 (putative IGF) are expressed in head, thorax and abdomen of all stages, whereas ILP-5 is found predominantly in the abdomen only (101). In *Anopheles gambiae*, the expression patterns of the seven different ILPs differ somewhat from *Aedes agypti,* in the sense that ILP-2 and -5 are detected in heads, thoraces, and abdomens of all life stages, and the rest of the ILPs (-1, -3, 4, -6, -7) are detected only in the heads and thoraces of all life stages. This differential expression points to one group being more growth factor-like (ILP-2, -6), given their ubiquitous expression, whereas the other cluster (ILP-1, -3, -4, -7, and -8) could be involved in neuroendocrine functions (240).

Since insulin signaling regulates key processes unique to mosquito physiology, like blood meal processing, nutrition, homeostasis and reproduction, I asked whether these neuroendocrine factors

could also potentially be expressed in hemocytes. My results suggest that some ILPs and the MIR are expressed at the RNA level in hemocytes.

3.2 Materials and Methods

Insects

Aedes aegypti (UGAL strain) was reared in a dedicated insectary in the Department of Entomology as described (246). Adults had access to 5% sucrose solution, and prior to blood feeding, caged mosquitoes were starved and kept in total darkness for at least an hour.

Hemocyte collection

Hemocytes were collected using the high injection/recovery method as described previously (247). In brief adult *Aedes aegypti* females were cold anesthetized on ice for 15 min followed by injection of 8-10 ul of 60% Schneider's medium (Sigma), 10% fetal bovine serum (FBS) (Hyclone) and 30% citrate buffer **(**=anticoagulant) (98 mM NaOH, 186mM NaCl, 1.7mM EDTA and 41 mM citric acid, buffer pH 4.5) (vol/vol) between the last two abdominal schlerites using a glass needle mounted on a micromanipulator and placed on ice for 20 min. I then injected 25 ul of Schneider's:FBS:anticoagulant (60:10:30) into the lateral wall of the mesothorax and collected the diluted hemolymph by capillary action from the original injection site in the abdomen using a second hand-held glass needle. For in situ hybridization (ISH) the diluted hemolymph was collected and placed in Teflon-lined wells on glass slides (see below). After allowing cells to settle, diluted hemolymph was removed and replaced with fresh Schneider's medium plus 10% FBS followed by the ISH procedure.

RNA extraction

Hemocytes were collected from 4- (1 hour post-blood meal) and 5-days old blood fed females (24 h post-blood feeding) and control mosquitoes (1 day old, 4 days old, and 5 days old non blood fed females). A minimum of 20 individuals were bled and hemocytes pooled to obtain at least 200 ng of total RNA per replicate. Hemocytes were pelleted in a microcentrifuge tube and total RNA isolated using the RNAqueous-Micro kit (Ambion) following manufaturer's intructions. In brief, the cell pellet was disrupted in 100 µl of lysis solution by vortexing vigorously. Then 50 µl of 100% ethanol was added and vortexed briefly but thoroughly. The lysate was then passed through a micro-column and centrifuged for 20 seconds at maximum speed; the filters were then washed twice. Finally the filter was dried and RNA eluted in 20 µl of pre-heated elution solution. In addition, co-eluted DNA was removed by incubating the eluted RNA with $1/10^{th}$ volume of 10X DNase I buffer and 1 µl DNase (provided with the kit) and incubated for 20 minutes at 37°C. DNase was inactivated according to the kit's instructions by adding DNase inactivation reagent, incubating and precipitating the mix by centrifugation and transferring the supernatant to a clean tube. RNA amounts were then measured (Nanodrop ND-1000, Thermo Scientific) and stored at -80°C.

cDNA synthesis and RT-PCR using ILP and MIR-specific primers.

For first-strand cDNA synthesis, 200 ng of total RNA per sample was reverse transcribed with Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) and random hexamers in 20 µl reaction mixtures according to the manufacturer's instructions. cDNA was then used for RT-PCR reactions containing 2 µl of template cDNA, 5 µl of 10x HotMaster *Taq* Buffer (with 25 mM Mg^{2+}), 0.2 µM of each forward and reverse primers specific for each *Aedes aegypti* ILP gene and family member and the MIR (for a complete list of primers, see Table 3.1), designed by Riehle at al. (101), 0.2 mM of dNTPs and 2.5 Units Hotmaster Taq DNA polymerase (5 Prime GmbH, Hamburg, Germany)) in 50-µl reaction mixtures.

For all the ILP primers the cycling conditions were: 2 min of initial denaturation at 94°C, followed by 35 cycles of 20 s denaturation step at 94 °C, 30 s annealing at 60°C (57°C for the MIR-specific primers), and 45 s extension at 72°C, and a final extension at 70 °C for 10 minutes. Three separate cohorts were repeated for all ages and blood feeding status. Results reported here are representative RT-PCR amplifications. PCR products were visualized on 0.5% agarose gels stained with ethidium bromide.

Table 3.1
List of Primers used for RT-PCR expression in mosquito hemocytes.

Gene	Forward primer	Reverse primer	Size (bp)
AaegILP-1	GGACCAGTTCCGGTAAAGAGATACCAAC	ATACAGGCACTGCTGGGTCAACCATAGA	635
AaegILP-2	AAGGACCAGTTCCGGTAAAGAGAC	AGGCACTCGGTTTGCCATCTAAAG	576
AaegILP-3	TATTACATCACACCCGTTCCCGGT	TTGAGTTCGGCGTACGAACAGCTT	271
AaegILP-4	CTTCATTCATCCAACCATTCAATCTTCAC	CGACTTCTAAATTTGATAAGCGTAGGG	531
AaegILP-5	GTGCTAATGCGCTACCCTTC	ACACCTTCAACGACTCCATCCGAA	312
AaegILP-6	GACAACGGCGTGTCAGCAAATTGA	CAATCACAGCCTAAGGATTTCTCCC	595
AaegILP-7	TGCTTACGGATACACTCACCGCTT	ATCCGGAGCCAACTCATCGTCATT	278
AaegILP-8	TGCCTCGGTGGACTTCTAGT	ATTCATGGGCCAGGCCAGTAGGAA	253

Cloning of PCR products and plasmid preparation

Selected regions of the coding sequences for ILP1-8 and MIR (Table 3.2) were cloned into the TOPO (pCR4.0) TA cloning dual promoter vector (Invitrogen, Carlsbad, CA) or Strataclone PCR cloning vector (pSC-A amp/kan))(Stratagene). Selected clones were then grown in 20 ml cultures overnight in Luria-Bertani (LB) broth (Fisher Scientific) supplemented with 100 mg/ml ampicilin (Sigma-Aldrich). Midi preparations of Plasmid DNA were performed using the GenElute™ Endotoxin-free Plasmid Midiprep Kit (Sigma-Aldrich) according to manufature's recommendations. Plasmid DNA was quantified and insert orientation was determine by PCR using a combination of gene- and promoter-specific primers, and the

presence or absence of a band in a given two primer pair combination indicated whether the insert is inserted clock-wise or counterclock-wise (sense/antisense).

Table 3.2.
List of Primers used for to generate probes used in situ hybridization and RNAi.

Gene	Forward primer	Reverse primer	Size (bp)
AaegILP-1 ISH	ACGGAAACTCAATTTCATCG	TGGCTCTTGGTAGGATCTTG	291
AaegILP-2 ISH	GATTTTGTGGGAAGCAACTG	GTGGAATCAGATCGTTACGG	294
AaegILP-3 ISH	TATTACATCACACCCGTTCCCGGT	AAGCTGTTCGTACGCCGAACTCAA	271
AaegILP-4 ISH	ACGACCCAGTGATGCTAAAG	GGCAACATTCCTCTACGATG	257
AaegILP-5 ISH	ACAAACTAATCCGGCACCTT	GATTGATCCGTTTGTTCGAG	288
AaegILP-6 ISH	TCCCAACTAACCTCTGTGGA	GCACAGTTCCAAATTCCATC	265
AaegILP-7 ISH	TGCTTACGGATACACTCACCGCTT	CAATGACGATGAGTTGGCTCCGGAT	483
AaegILP-8 ISH	TGCCTCGGTGGACTTCTAGTAGT	TTCCTACTGGCCTGGCCCATGAAT	336
MIR 3305F/3561R	CCCCGGTTATGAAACAGTTC	TCTCGATGGACAAACTTCTT	256

***In situ* hybridization**

Purified plasmids (10 µg) were linearized using restriction endonucleases specific for each vector combination. Genes cloned in the pCR4.0 vector samples were digested using *NotI*/*SpeI* (New England Biolabs) combination whereas samples cloned in the pSC-A amp/kan were digested using *NotI*/*KpnI* (New England Biolabs). Digested products were then verified on 0.5% agarose gels. Digested plasmids were purified and concentrated by phenol/chloroform and alcohol precipitation. Linearized plasmids were labeled with digoxigenin using the DIG RNA labeling kit (SP6/T7) (Roche) following the manufacture's instructions. For labeling using the pSC-A amp/kan linearized vector SP6 RNA polymerase was replaced by T3 RNA polymerase (Ambion) due to the lack of SP6 promoter region to drive transcription. In brief, separate reactions were set for each strand/digested-linearized plasmid and 1 µg of purified plasmid DNA template was added to a clean RNAse-free tube and mixed with 2 µl of each of

the following components: 10X NTP labeling mix, 10X Transcription buffer, RNA polymerase (SP6, T7 or T3) and 1 µl of protector RNase inhibitor. The reaction was mixed gently and incubated for 2 hours at 37°C, followed by DNAse I treatment (2 µl) for 15 minutes at 37°C. Newly synthesized probes were then precipitated using absolute ethanol and sodium acetate and dissolved in 25 µl nuclease-free water. Probes were then quantified by dot blot. Serial dilutions of each probe were made and spotted; RNA probe standards were also included. Probes (2 µL) were diluted in 80 µl of RNA dilution buffer (DEPC water: 20X SSC: formaldehyde-5:3:2). Each dilution was spotted on an amphoteric membrane (1 µL probe) (Biodyne A, Pall Corporation), allowed to air dry and cross linked on a Stratagene UV crosslinker on standard mode. Once crosslinked the membranes were washed in wash buffer (maleic acid buffer (0.1 M maleic acid pH7.5, 0.15 M NaCl) + 0.1% Tween 20) for 5 minutes, then blocked using blocking buffer (1X blocking reagent (cat No. 11 096 176 001, Roche) in maleic acid buffer) for 30 minutes and incubated with 1:5000 anti-DIG for 1 hour. Membranes were then washed , equilibrated in detection buffer (0.1 M Tris-HCL, pH 9.5; 0.1 M NaCl) for 5 minutes and detected with NBT-BCIP in detection buffer for 30 minutes; to finish the membranes were washed with distilled water, dried and labeling efficiency was determined by comparison with known RNA standard concentrations spotted. Approximately ≥400 ng/µl of probe was generated per labeling reaction per sample

Whole-mount In situ hydridization of mosquito hemocytes

Hemocytes from 1 day old females were extracted as described earlier and plated in a 96 well plate, allowed to settle for 30 minutes and fixed in freshly prepared 4% paraformaldehyde (PFA) in DEPC-treated phosphate buffer saline (PBS) at 4°C for 20 minutes. Cells were permeabilized with PBS-T (PBS+0.1% tween-20) for 15 minutes followed by Proteinase K digestion (0.1 µg/ml) for less than 2 minutes (watch). The wells were then washed 1X with PBS and fixed again in 4% PFA at 4°C for 15 minutes, then washed 1X with PBS for 5 minutes and incubated once with 0.1 M triethanolamine (TEA)

buffer, pH 8.0 and again with TEA buffer with 0.25% acetic anhydride added (acetylation, both for 5 minutes each. Cells were pre-hybridized in 75 µl of hybridization buffer (2X SSC, 50% deionized formamide (Sigma), 10% dextran sulfate (Sigma) and 0.1 mg/ml salmon sperm (Ambion)) for 1 hour at 55 °C, followed by the addition of the probes at a concentration of 100 ng/ml for 24 h. Probes were diluted in water (1:4), denatured at 80°C for 5 minutes, and chilled on ice for 3 minutes. After that ¼ of the diluted probe was mixed with 75 µl of hybridization buffer pre-warmed to 55 °C and incubated overnight at 55 °C. Next day, hybridized cells were washed Five times with decreasing concentrations of warm (55 °C) SSC buffer (2X, 1X, 0.5X, 0.2X, 0.2X) for 30 minutes each. Cells were blocked for 1 h at 37 °C followed by signal detection using an anti-digoxigenin Fab fragment conjugated to alkaline phosphatase (Roche) in blocking solution (1:5000) for 2 hours at 37°C. To finish, the cells were washed and signal detected by incubating with NBT/BCIP in detection buffer for 5 minutes (check for signal development), according to the instructions' manual. Once the signal developed, the reaction was stopped by washing with TE buffer pH 8.0. Cells were examined using a Leica Inverted Fluorescent microscope. All captured images were exported to Adobe Photoshop CS3 (Adobe) as .tif files for assembly of figures.

3.3 Results

Mosquito insulin like peptides and the MIR are expressed in hemocytes.

To assess whether the MIR and any ILP genes are expressed in hemocytes, I collected samples from non-blood fed (NBF) (1, 4, and 5 days of age) and blood fed (BF) adult females (2, 3, 9, 24 h post blood meal). The expression of ILPs -3, -4, -6, -7, -8 was detected in NBF and BF animals (Fig. 3.1). By contrast, ILP-7 and -8 were expressed constantly at high levels in non-blood fed and blood fed mosquitoes but after 24 h post blood meal they were no longer expressed and in 5 day old females their expression level was very low whereas the expression levels for ILP-4, -7 and-8 disappeared in BF (24 h

pbm). ILP-5 transcript levels disappeared after 9 hours post blood meal and could not be detected at any time point after day 4. In addition, ILP-3 and -6 were expressed at all times with almost no change for ILP-3 but a reduction in expression for ILP-6 at the oldest age tested and at 24 h post blood meal. The detection of ILP-5 was interesting because it was previously shown to be expressed in the abdomen (248), but not surprising given that some hemocytes remain attached to abdominal walls. Previously it was shown that ILPs -3, -4, -7, and -8 were preferentially expressed in the brain of mosquitoes, but my results also showed that hemocytes also produce some of the same ILPs produced in the neuroendocrine system.

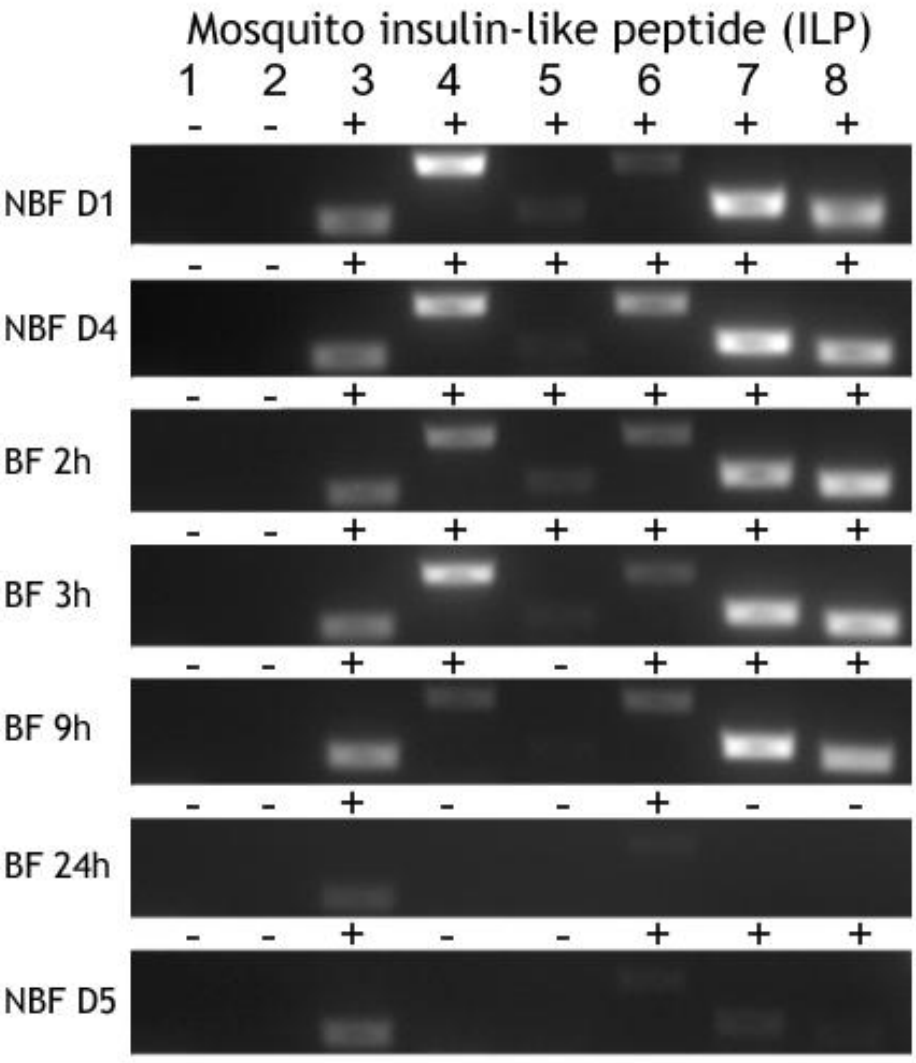


Figure 3.1. Transcript expression of ILP genes in hemocytes of *Aedes aegypti*. Total hemocyte RNA from non-blood fed and blood fed *Aedes aegypti* females at different ages and times after blood meal ingestion was isolated and used as template for RT-PCR (35 cycles). Plus(+)/minus(-) sign denotes presence/ absence of a particular transcript. At least three separate cohorts were tested. Amplification using actin primers was used to check for RNA quality and amplification reliability. NBF/BF= (non) blood fed; D= day.

After showing that mosquito ILPs are expressed in hemocytes, I wanted to determine whether the MIR was also expressed. My results indicated that the MIR was expressed in hemocyte from both NBF and BF adult females (Fig. 3.2), suggesting that the MIR expression is higher in BF that NBF females.

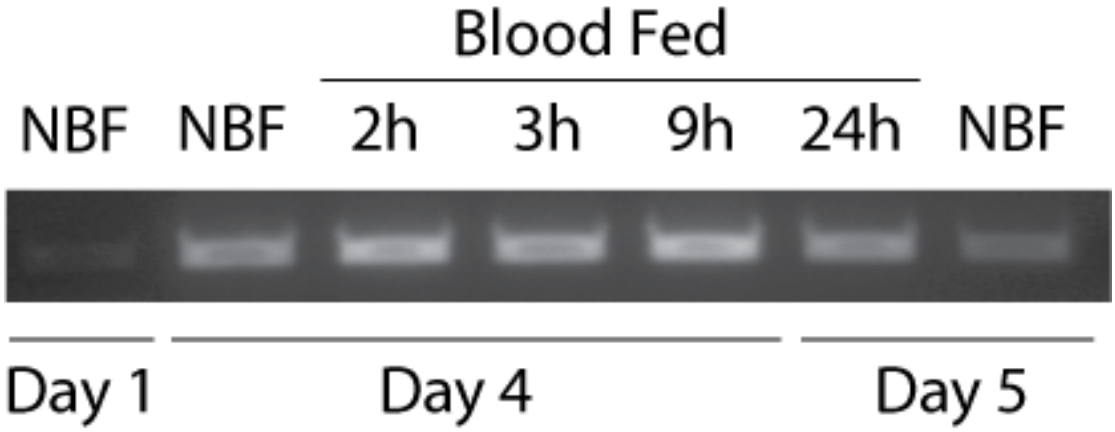


Figure 3.2. Expression of MIR in hemocytes of blood fed and non blood fed females. Detection of the MIR transcript by RT-PCR (40 cycles) was done in blood fed and non-blood fed females at day 4 and 5 of age. The MIR expression levels are slighly higher in blood fed females than females of the same age (day 5 NBF).

ILP family members and the MIR are expressed in hemocytes.

Whole mount *in situ* hibridization was used to validate the expression of each ILP in mosquito hemocytes (day 1) as well as to determine whether the different ILPs are produced in different hemocyte types. ILP-specific RNA probes were designed and the specific staining is shown in Fig 3.3. As previously determined by RT-PCR, ILP-1 and ILP-2 were not expressed in mosquitoes whereas the other ones were expressed at different levels. ILP-3 is expressed at an intermediate level, according to the intensity of the staining. ILPs -4, -6, -7, and -8 are expressed at relatively higher levels whereas ILP-5

(E) expression was similar to ILP-3. The intensity of sense probes was similar for all samples with zero to very little signal. Mosquito ILPs were preferentially detected in granulocytes (Fig. 3.3) with less intensity in oenocytoids and prohemocytes (data not shown). To assess the spatial expression of the mosquito insulin receptor in the different hemocyte types, an MIR-specific RNA probe was used combined with *in situ* hybridization. My results indicated that the MIR is expressed in mosquito hemocytes (Fig. 4) and that its expression is consistent with RT-PCR results generated. Furthermore, the expression was universal, meaning that all cell types expressed the MIR and not a particular subset of hemocytes. On the other hand, granulocytes and oenocytoids showed strong staining, whereas prohemocytes show little staining which was indistinguishable from background staining (Fig. 3.4C-E).

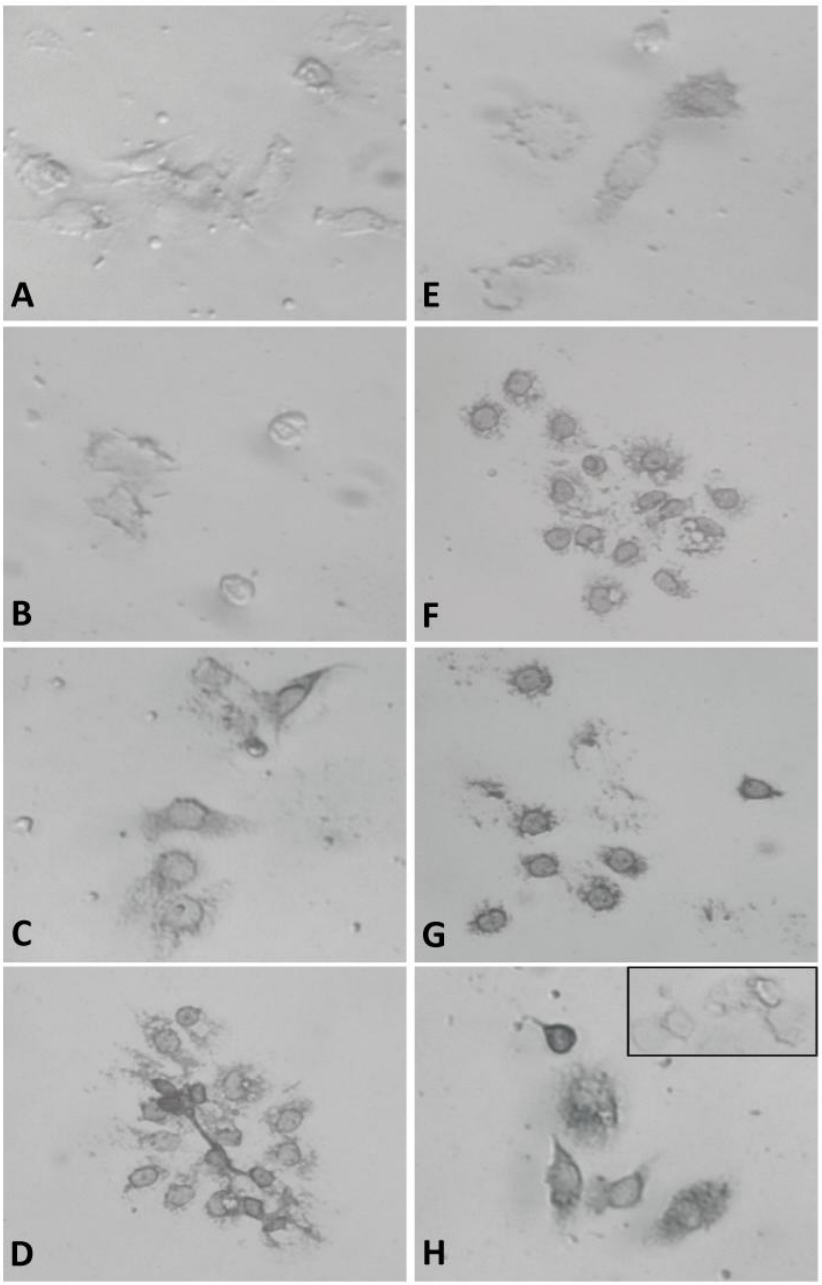


Figure 3.3. Insulin-like peptides are expressed in all hemocyte types. In situ hybridization results are presented, the letters on each box correspond to each of the ILPs : ILP-1 **(A)**, ILP-2 **(B)**, ILP-3 **(C)**, ILP-4 **(D)**, ILP-5 **(E)**, ILP-6 **(F)**, ILP-7 **(G)**, ILP-8 **(H)**. The box in pannel H represent hemocytes incubated with the sense probe, illustrating the level of background (zero). Sense/antisense probes were run side by side.

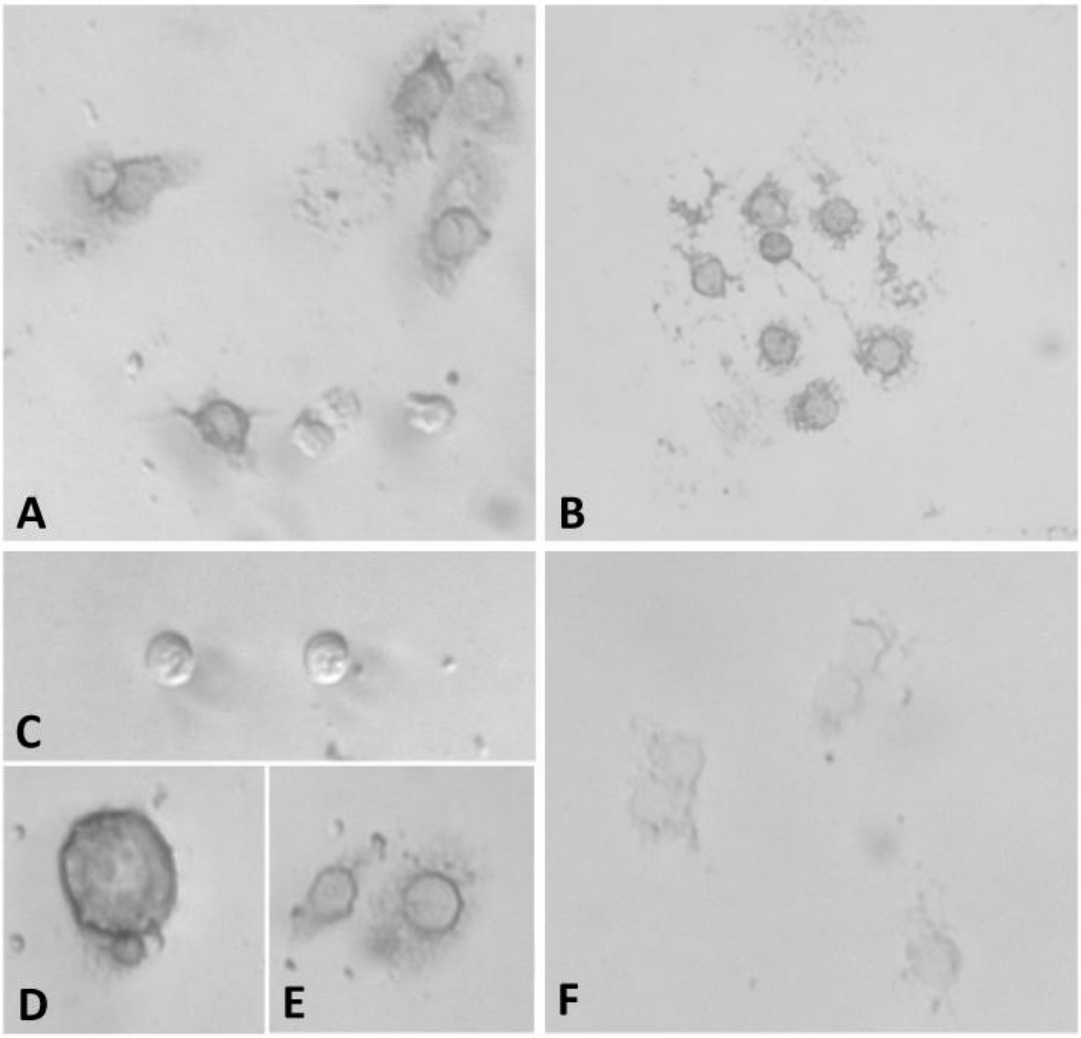


Figure 3.4. The MIR is expressed in all hemocyte types. Expression of MIR **(A-B)** in mosquito hemocytes is shown here with a good signal detection. Panels **C**, **D** and **E** show expression in the different cell types : prohemocytes **(C)**, Oenocytoid **(D)**, and granulocytes **(E)**. Panel **(F)** shows the signal generated by the sense probe (low background, no signal).

3.4 Discussion

Mosquito ILPs are essential for insulin signaling. Their particular expression pattern could help in assigning roles to each of the ILP variants. Previously, the expression of the different ILPs and the MIR was characterized and showed differences in expression in time and space in tissues of the mosquitoes *Aedes aegypti* and *Anopheles gambiae* (103, 241, 248). To explore the potential role that the insulin signaling cascade might play in insect immunity and hemocyte physiology, I aimed at characterizing the expression of the different ILPs and the MIR in mosquito hemocyte. Although ILP expression has been characterized in different insect species and tissues, hemocytes have never been examined. This is the first report indicating that both ILPs and the insulin receptor (MIR) are expressed in hemocytes.

I compared the expression of the different ILPs in mosquitoes of different ages and blood fed vs sugar fed and found that 6 out of 8 ILPs are expressed in mosquitoes. This study suggests that ILP-4, -7, and -8 are preferentially expressed in non blood fed females and in blood fed females from 2-9 h pbm. In adult females, these three ILPs were previously detected only in the heads of female mosquitoes (ILP-4, -7 are also expressed in ovaries) (248). The fact that ILP-3, -4, -7, and -8 (brain ILPs) are expressed in in hemocytes could mean that these four ILPs may have pleiotropic roles, regulating the neuroendocrine and immune systems by interacting with multiple insulin receptor substrates (IRS) and therefore signaling through different branches of the insulin signaling cascade. In humans, the differential activation of different IRSs engages various downstream signaling factors during insulin signal transmission, causing different biological effects (249). The co-expression of multiple ILPs in the same tissue/organ is not uncommon. For instance, in *Drosophila*, dilp-2, -3, and -5 are expressed in the same cluster of m-NSCs but with differences in the time of expression; dilp-2 was expressed in the first instar stage, whereas dilp-2 and dilp-5 expression is detectable in second instar larvae, and dilp3 expressed from mid to late third instar stage (250). In the case of mosquito hemocytes it could be that different

ILPs are necessary to carry individual functions, much like ILP-3 regulating egg maturation and ILP-4 regulating ecdysteroid production (251). I was surprised by the absence of ILP-2, given that it is ubiquitously expressed in head, midgut and ovaries (101). My results suggest that ILPs and the MIR are differentially expressed in blood fed vs non-blood fed females, in concordance with previous studies that showed that MIR expression changes after a blood meal (123).

The role that ILPs play in regulating mosquito physiology has been intriguing. As mentioned above, ILP-3 was shown to stimulate yolk deposition and to have a true insulin-like function in regulating sugar and lipid levels in female mosquitoes (251). In addition, receptor binding experiments showed that ILP-3 is able to directly interact with the insulin receptor (MIR) (102) and thus stimulate downstream responses. ILP-4 on the other hand, has no role in metabolic activity but does stimulate the production of ecdysteroids and yolk deposition in ovaries (102). The expression of ILPs in mosquito hemocytes could be testament to the tight connection that exist between the neuroendocrine and immune systems (252, 253).

Gene expression analysis can suffer from undesired contaminants that could alter the detection of specific transcripts. Although hemocytes preparations were done with extra care not to include fat body cells or any other kind of debris, I cannot rule out that contamination potentially affected my RT-PCR results. My *in situ* hybridization, however, does support the expression profile obtained by RT-PCR, suggesting that contamination is not responsible for my RT-PCR results. The expression of the MIR further suggests that it is constitutively expressed in hemocytes with no change in expression levels before and after a blood meal. Although the MIR did not seemed inducible upon a blood meal, it could be that, basal levels of MIR are enough for basic cell signaling. Furthermore, given that some ILPs exhibited differences in biological activity (ILP-3 and -4), the need for several ILPs to be expressed in hemocytes could mean that these ILPs are involved in several functions.

The expression of different ILPs and the MIR in hemocytes is consistent with studies in vertebrates in where both insulin and IGFshave are expressed in immune cells like macrophages, and lymphocytes (254). In vertebrates, insulin-like growth factor-I (IGF-I), which is known to be responsible for the growth promoting effect of growth hormone, has also been shown to directly affect several immune function (255, 256), including lymphopoiesis *in vitro* (257, 258). The expression of ILPs in hemocytes could mean that insulin signaling might be involved in the maintenance of hemocyte populations and hemocyte proliferation. Although only one mosquito ILP resembles an IGF molecule (ILP-6), other ILPs might have mitogenic properties that could induce cell proliferation and maintenance of cell numbers by controling apoptosis (259). The expression of the MIR in mosquito hemocytes is not surprising either, given insulin receptor expression has been detected in T-lymphocytes (260) and related cells.

To summarize diffferent ILPs are expressed in adult female *Aedes aegypti*. ILP-3 presents itself as a promising molecule capable of regulating many different processes and its role in hemocytes is yet to be determined and discussed in the next chapter. Although the biological function of the other ILPs remain unclear, future research needs to be done to determine whether each ILP is unique in function or whether their secretion is redundant in function.

CHAPTER 4

AN ENDOGENOUS INSULIN-LIKEE PEPTIDE STIMULATES HEMOCYTE PROLIFERATION IN THE YELLOW FEVER MOSQUITO *AEDES AEGYPTI*

4.1 Introduction

Hemocytes play an important role in immune surveillance and are essential for cell-based clearance of pathogens, such as bacteria, parasites, and parasitoids. *Drosophila* and the mosquitoes *Aedes aegypti* and *Anopheles gambie* produce distinct hemocyte types with similar functions but different nomenclature. Plasmatocytes, lamellocytes and crystal cells are present in *Drosophila* (171), whereas granulocytes, oenocytoids and pro-hemocytes, are the cell types produced by mosquitoes (174, 246, 261, 262). In *Drosophila*, hemocytes are produced during embryogenesis from a subset of cells located in the head mesoderm and later from the lymp gland (hemopoietic organ) during larval stages (263). However, in other dipteran insects such as mosquitoes, the existence of a hemopoietic organ *per se* is unknown.

In insects, different types of hemocytes are produced via cell lineage differentiation within dedicated hematopoietic organs (132, 264). Although there are some studies suggesting that circulating pro-hemocytes can differentiate via linear transition of pro-hemocytes to plasmatocytes andinto other cell types (265-267), the current accepted dogma is that hemocytes are mostly produced during embryogenesis. The number of hemocytes in circulation markedly changes during development (180), and after immune responses to stress, wounding or infection (164, 268-270). Maintenance of hemocyte populations is thought to be regulated by mitotic division of circulating hemocytes (131, 271), by production and release of hemocytes from the hematopoietic organs (132, 272), and by attachment

and detachment of hemocytes to organs in the body cavity (273) that enter and leave circulation. Significant progress has recently accumulated in understanding the genetic basis and control of hemocyte differentiation during development (274, 275). In insects, cell proliferation is controlled by many different pathways (151, 276) including insulin signaling (125). The insulin signaling cascade is well conserved in insects (1), and controls many aspects of their physiology. In mosquitoes, ILPs are implicated in regulating oogenesis (103), yolk deposition, carbohydrate metabolism (251), diapause (277, 278), and ecdysteroid production (279). Studies with non-dipteran insects further show that insulin signaling is involved in cell proliferation (126, 280-282) and cell growth *(129)*. Kim et al. (125) also demonstrated that mammalian insulin induces proliferation by *Drosophila* S2 cells by activating the insulin signaling cascade and effector genes regulated by the MAP kinase signaling cascade.

Mosquitoes have a unique physiology that requires a blood meal to initiate a developmental program that ultimately leads to egg production. Several hormonal factors are released following blood feeding which are responsible for major physiological changes, including several ILPs, ecdysteroids and ovarian ecdysterogenic hormone (OEH). In Chapter II, I reported that blood feeding induces a dramatic increase in the number of hemocytes present in circulation in *Anopheles gambiae* and *Aedes aegypti* (10). The unique array of physiological events that occur after a blood meal, the particular activity of insulin during during this process, and other precedents illustrating the proliferative properties of ILPs led me to explore the role of insulin signaling in mosquito blood cell proliferation.

4.2 Materials and methods

Insects

Aedes aegypti (UGAL strain) was reared in a dedicated insectary in the Department of Entomology as described (246). Adults had access to 5% sucrose solution, and blood fed as needed.

Hemocyte collection

Hemocytes were collected using the high injection/recovery method as described previously (247). In brief, adult *Aedes aegypti* females were cold anesthetized on ice for 15 min followed by injection of 8-10 ul of 60% Schneider's medium (Sigma), 10% fetal bovine serum (FBS) (Hyclone) and 30% citrate buffer **(**=anticoagulant) (98 mM NaOH, 186mM NaCl, 1.7mM EDTA and 41 mM citric acid, buffer pH 4.5) (vol/vol) between the last two abdominal sclerites using a glass needle mounted on a micromanipulator and placed on ice for 20 min. I then injected 25 ul of Schneider's:FBS:anticoagulant (60:10:30) into the lateral wall of the mesothorax and collected the diluted hemolymph by capillary action from the original injection site in the abdomen using a second hand-held glass needle. For in situ hybridization experiments, diluted hemolymph was collected and placed in Teflon-lined wells on glass slides (see below). After allowing cells to settle, diluted hemolymph was removed and replaced with fresh Schneider's medium plus 10% FBS followed by the ISH procedure. For RNA extraction, samples were pooled in eppendorf tubes and extracted as indicated in the next section.

Mosquito decapitation and rescue bioassay

For the purpose of determining whether insulin signaling has a role in circulating hemocytes after a blood meal, I performed decapitation experiments. *Aedes aegypt* produces different ILPs expressed in medial neurosecretory cells (MNSCs) in the brain, with ILP-3 knownto regulate reproduction and metabolism (119). I decided to test whether ILP-3 can stimulate hemocyte

proliferation in females. Four day old females were blood fed and decapitated at different time points 10 minutes-24 hours post blood meal (pbm). After decapitation animals were put in small cages for 24h, followed by collection of hemocytes. ILP-3 was chemically synthesized using Fmoc chemistry as described in (119). For *in vivo* ILP-mediated rescue experiments in decapitated females, blood-fed females (4 days of age) were decapitated 9 h pbm, injected (0.5 µl) with graded amounts of ILP-3 (0.5, 5.0, 25.0, and 50.0 pmol), followed by collection and counting of hemocytes 24 h later. Decapitated females injected with saline served as a negative control while intact blood-fed and non blood fed females served as positive controls. As a control for monitoring ILP bioactivity, yolk deposition was determined in all treatments according to methods described by Brown et al. (119).

RNAi knock down assays

The mosquito insulin receptor (MIR) cDNA was amplified using forward and reverse primers (*T7-AaMIR-CFwd-* TAATACGACTCACTATAGGGCCGGAGGTGAATCCAGACTA and *T7-AaMIR-CRev-* TAATACGACTCACTAT AGGGCCTTCTTTGCCGAAAGTACGC) previously designed (119) corresponding to the carboxy-terminus (nucleotides 3079–3546) and T7 promoter sequence overhangs (highlighted) at their 5′ ends. The amplified fragment was purified (Qiaprep PCR purification kit, Qiagen) cloned (TOPO Eppendorf Westbury, NY). Plasmid DNA was used as templates for dsRNA synthesis using the MEGAscript T7 RNAi kit (Ambion). Briefly, 1 µg of plasmid DNA was transcribed *in vitro* by using T7 polymerase. dsRNA was then digested with DNaseI and RNaseI, precipitated, and resuspended in nuclease-free water. GFP dsRNA was generated as a control. Integrity of dsRNAs was assessed by gel electrophoresis. Concentration was then measured (Nanodrop, Thermo scientific) and adjusted to 4 µg/µl in water and stored at −80°C. dsRNAs (0.5 µl of a 4 µg/µl stock) was injected into newly eclosed females (less than 12h post emergence) and for the purpose of bioassays, dsRNA-treated females were blood fed at day 4 post injection and hemocytes extracted and counted 24 h post blood meal. Control

samples included non blood fed females treated with GFP-dsRNA and MIR-dsRNA. Body carcasses were processed for qRT-PCR (Biorad Sybr green mix) and RT-PCR to assess knock down using MIR-specific primers designed to amplify a fragment outside the region used to generate dsRNA.

Hemocyte proliferation assay

A stock solution of BrdU (BromoDeoxyUridine, Sigma) was prepared in sterile water, heated at 37°C to dissolve, filtered, and added to the sugar-water solution (8% sucrose) used to feed adult mosquitoes. Newly eclosed mosquitoes were fed BrdU at a final concentration of 1.0 mg/ml in sugar water. BrdU-fed females were blood fed at day 4 of age and subsets of those were decapitated immediately after blood feeding. Control samples included non-blood fed/decapitated and non-blood fed/intact females. Hemocytes were placed into L-lysine coated slides and incubated in Schneider's medium for 40 minutes to allow the cells to settle. Hemocytes were then fixed in 4% freshly prepared ice-cold formalin for 30 min at 4°C and permeabilized for 20 min in PBS-T (1x PBS, 0.2% Triton X-100). After rinsing in PBS for 5 minutes, cells were incubated in 2 N HCl for 40 min at 37°C to denature the DNA, and then neutralized in 0.1 M sodium borate (pH 8.5) for 12 min (twice) at room temperature (RT). After this step, cells were washed several times in PBS-T, incubated in blocking solution (PBS-T, 1X Blocking reagent, Roche) for 1 hour, and then incubated overnight with anti-BrdU antibody (murine) (Cat.No 033900, Invitrogen) at a 1:100 dilution at 4°C. The primary antibody was visualized by incubation with an Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (Invitrogen) at a 1:200 dilution for 2 hours at 37°C. Finally samples were washed in PBST and mounted for observation. For each treatment, cells were examined, counted and scored for BrdU-positive labeling. Cells were scored as positive if a distinct positive nuclear (green) signal was visible under dark field.

Bacterial Infection Assays

Escherichia coli (Migula) (ATCC# 25254), carrying streptomycin resistance was grown in brain heart infusion (BHI) medium supplemented with streptomycin (50 µg/ml) at 37°C to an OD 600=1.0 (10^6 cfu/ml). Bacterial density was adjusted to 1.0× 10^3 bacteria ml^{-1} (0.5 µl) as determined by colony forming unit (CFU) counting on BHI+streptomycing (50 µg/ml) plates. I injected 1.07-1.5X10^3 cfu/ml into 4 days old blood fed mosquitoes (n=25 and n=17) for samples collected at 3 and 24 h respectively (20 minutes post blood meal) and in non-blood fed females (n=24 and n=26) for samples collected at 3 and 24 h respectively. Bacterial titers from whole mosquitoes where calculated by crushing whole mosquitoes at 3 and 24 h post infection. In brief, whole animals were ground in 100 µl of BHI (+streptomycin) medium and used to prepare serial dilutions from 10^{-1} to 10^{-4} from the lysate. Dilutions were plated in plated in BHI plates supplemented with streptomycin (50 µg/ml), incubated for 18 hours and colony forming units calculated. The data collected is the result of three independent replicates.

Data analysis and Image processing

All data were analysed using Sigmaplot 11 (Systat Software Inc., San Jose, CA, USA) and a one way analysis of variance (ANOVA). Data transformations were used; arscin transformation was used to normalize cell ratios (percentage of BrdU-labeled cells) and natural logarithm (ln) was used for all the other cell count data. When the dataset did not meet the rigor of interval data associated with normality, the Kruskal-Wallis (H) test was used (non-parametric, ANOVA on ranks). All pair-wise comparisons of means were conducted using Dunnett's or Dunn's methods for post hoc comparisons. Differences between group means versus control were considered significant at $P<0.05$. Images were processed and plates made using Adobe Photoshop CS3 software package (Adobe Systems Inc.).

4.3 Results

Decapitation affects hemocyte abundance after a blood meal.

Humoral or neurofactors from the head are necessary for ovarian maturation and many other physiological processes in mosquitoes, so I assessed whether the release of a head factor was also involved in mediating the increased in hemocyte numbers observed in blood fed females. For this purpose I used multiple cohorts of synchronously reared *Aedes aegypti* females (4 days old), blood fed and decapitated (BF/D) them within 30 minutes post blood meal (pbm). Blood fed/non decapitated (BF/ND) females served as positive controls and non blood fed females [decapitated (NBF/D) and non decapitated NBF/ND)] were also included at 24 h pbm. A total of 30 animals were bled per treatment and hemocytes counted. Non-blood fed females showed no sign of increase when compared to blood fed females with an average number of 1647 and 1167 hemocytes in NBF/ND and NBF/D respectively (Figure 1). Interestingly, females that were blood fed and decapitated after taking a blood meal also showed no increase in hemocyte numbers, with an average number of 1233 versus 2130 hemocytes per mosquito reported in blood fed and non decapitated (BF/ND). The difference observed in my data clearly indicates that decapitation prevented circulatory blood cells from increasing in abundance after blood feeding ($F_{3, 119} = 30.4$; $p \leq 0.001$).

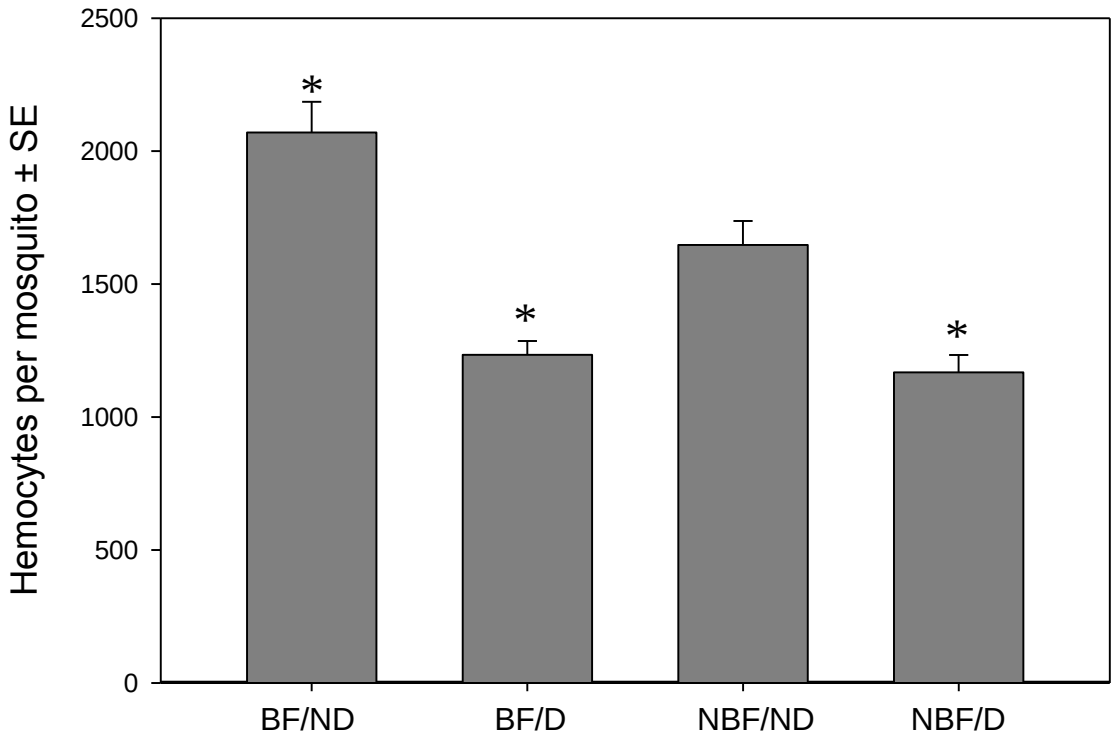


Figure 4.1. Decapitation affects total hemocyte abundance after a blood meal. Total Hemocyte counts in blood fed and blood fed/ decapitated mosquitoes. A cohort of 4 day old mosquitoes was blood fed and a subsample of those was decapitated 30 minutes after blood feeding. Control samples were non blood fed females of the same age as well as a group of decapitated non blood fed females. Decapitated blood fed females showed no changes in hemocyte abundance after a blood meal. This result is significantly different from that of blood fed/non decapitated females. Asterisks indicate a treatment statistically different from the negative (NBF/ND) control ($F_{3,\ 119}$ = 30.4; p≤0.001). [BF=blood fed; NBF=non-blood fed; D=decapitated and ND=non-decapitated].

Decapitation up to 9 h pbm blocks increased hemocyte abundance. After establishing that decapitation prevented the increase in hemocyte abundance after a blood meal, I asked whether this phenotype is dependent on time of decapitation by conducting a series of decapitation experiments at different time points (Fig. 4.2). The time at which decapitation happened was critical to blocking an increase in the abundance of circulatory hemocytes ($F_{11,\ 163}$=6.1; p≤0.001). Female mosquitoes decapitatated between 10 minutes and 9 hours after blood feeding exhibited no increase in hemocyte abundance , whereas females decapitated 12h or later after blood feeding exhibited hemocyte numbers similar to decapitated control (24 h pbm) and blood fed/non-decapitated individuals (Fig 4.2.).

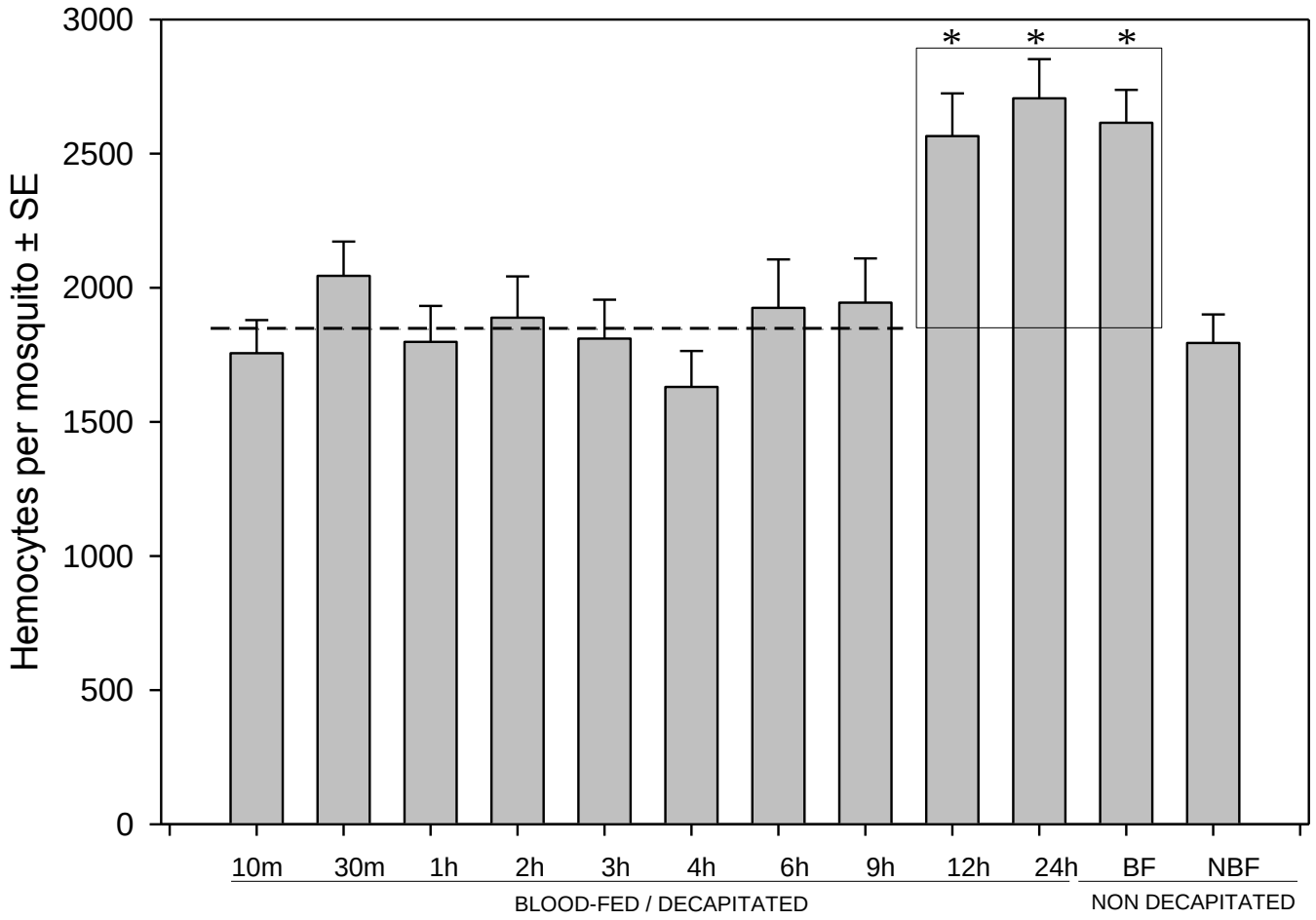
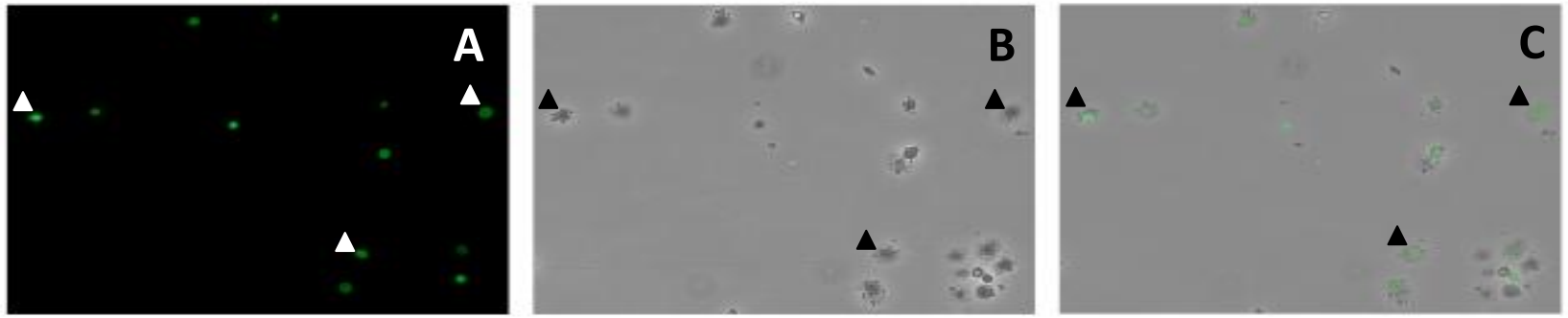
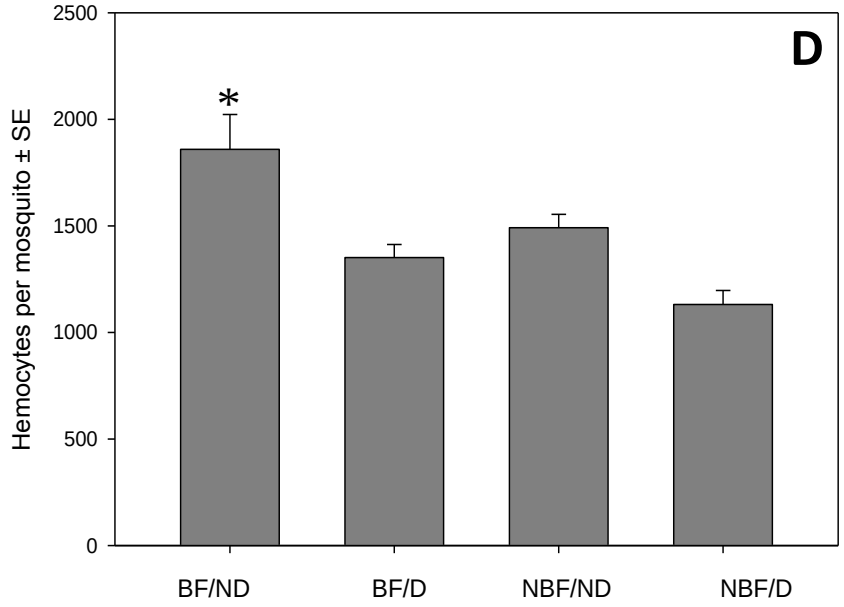

Hemocytes per mosquito ± SE
3000
2500
2000
1500
1000
500
0
*
*
*
10m
30m
1h
2h
3h
4h
6h
9h
12h
24h
BF
NBF
BLOOD-FED / DECAPITATED
NON DECAPITATED

Figure 4.2. Decapitation up to 9 h pbm blocks increased hemocyte abundance. Four day old mosquitoes were blood fed and decapitated at the different time points (10 minutes-24 hours pbm) and sampled at 24 h pbm. Controls included non decapitated and blood fed females. Significant differences between pair-wise means and control (NBF/ND) are indicated by an asterisk (*)($F_{11, 163}$=6.1; p≤0.001), followed by Dunnett's method). The dotted line indicates the average number of circulatory hemocytes per mosquito from 10 minutes to 9 hours pbm.

The increase in hemocyte numbers post blood meal is due to cell proliferation.

The increase in hemocyte numbers after a blood meal could reflect either increased proliferation or mobilization of sessile hemocytes to enter circulation. To determine whether hemocytes are proliferating in blood fed females we used an in vivo labeling approach in which we fed BrdU to adult mosquitoes. Blood fed/non decapitated females as well as control animals (NBF/D, NBF/ND and BF/D) were bled at 24 h pbmand hemocytes were processed to detect BrdU incorporation into dividing daughter cells. BrdU positive cells were detected in hemocyte preparations, as evidenced by strong nuclear staining, and low background as shown in figure 4.3A-C. As indicated in Fig 4.3D, blood fed/non-decapitated females showed the highest number of hemocytes, in contrast with blood fed/ decapitated females ($H = 20.065$, 3 d.f., $P \leq 0.001$). To learn more about the specific proportion of BrdU-positive cells present in the total sample pool, BrdU labeled cells were counted and expressed as a proportion (percentage, arcsin transformed) of the total number of hemocyte collected from each mosquito. The percentage of labeled cells was higher in blood fed females than non-blood fed females. This suggests increased proliferation contributes to the higher number of circulatory hemocytes obserbed after blood feeding. The percentage of BrdU-positive cells in blood fed /non-decapitated (BF/ND) animals (Fig 4.3D) was roughly 41% (based on transformed data), significantly higher when compared to the percentage found in blood fed/decapitated females (BF/D) (30.2%), ($F_{3,43} = 11.92$; $p \leq 0.001$). On the other hand, NBF/D and BF/D samples had very similar labeling percentages (25.67 vs 25.26%, respectively), with a small margin of difference between them (only 0.41% difference) and 7.55% less cells than NBF/ND control, ($P<0.05$, Dunnet 's method).


A
B
C


D
Hemocytes per mosquito ± SE
2500
2000
1500
1000
500
0
*
BF/ND
BF/D
NBF/ND
NBF/D

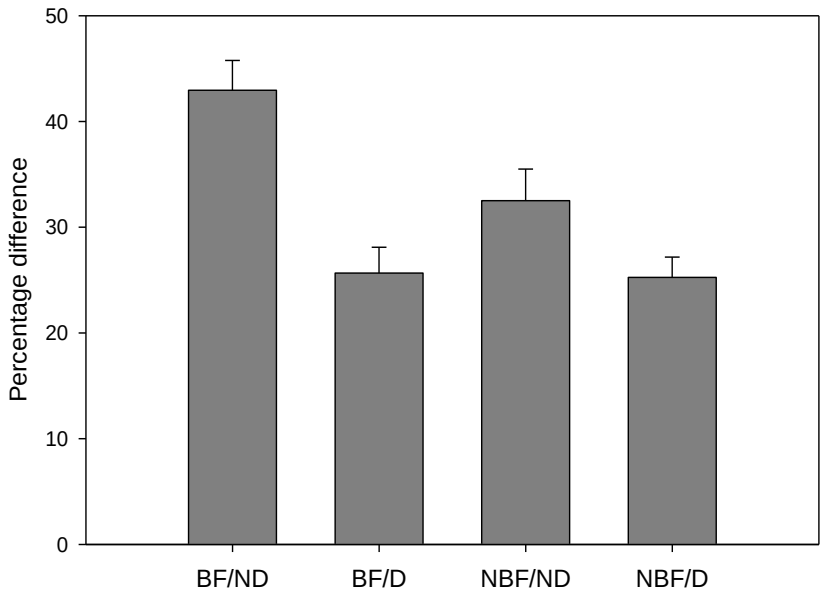
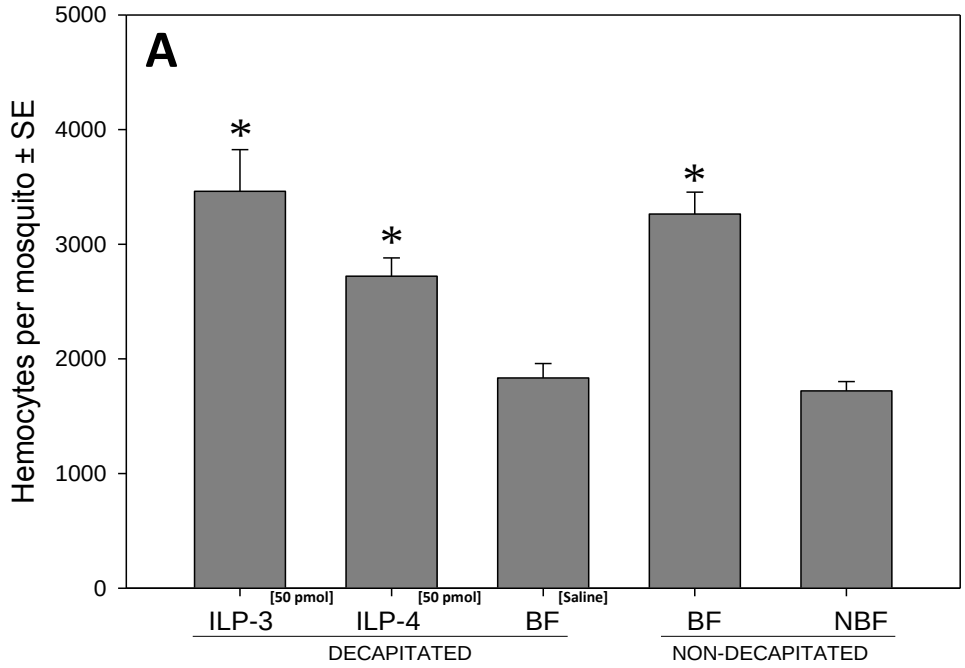

Percentage difference
50
40
30
20
10
0
BF/ND
BF/D
NBF/ND
NBF/D

Figure 4.3 The increase in hemocyte numbers post blood meal is due to cell proliferation. (A-C) Visualization of BrdU incorporation in hemocytes from blood fed mosquitoes 24 hours post blood meal. Colored triangles are pointing to labeled cells. **(A)** Fluorescent, DIC images **(B),** and overlay **(C)** images of BrdU- positive hemocytes showing nucleus-specific staining in proliferating hemocytes. Most of the cells in this image are granulocytes, although all cell types seemed to be labeled equally (data not shown). **(D)** Total hemocyte numbers in BrdU-treated female *A. aegypti*. Newly eclosed female mosquitoes were fed BrdU in water for 4 days. A cohort of non-decapitated control females were sampled on day 5 (no blood feeding). A second cohort of females was blood fed on day 4 and sampled on day 5 (no decapitation). A third cohort was blood fed on day 4, decapitated 1 hour post blood meal and sampled 24 hours later (day 5). A fourth non blood fed decapitated control was also added; animals were decapitated at day 4 and sampled on day 5. Hemocytes were collected using the high injection/recovery method, and BrdU incorporation detected by Immunocytochemistry. Significant differences between pair-wise means and control (NBF/ND) are indicated by an asterisk (*) ($H=20.065$, $d.f=3$, $P\leq0.001$). Decapitation inhibits the increase (proliferation) in hemocyte numbers in blood fed animals when compared to non-decapitated animals. In blood fed and decapitated animals the percentage of labeled cells was similar to that of non blood fed/ decapitated females. **(E)** Percentage of BrdU positive cells in BrdU fed (and/or decapitated) mosquitoes. Blood fed/non decapitated mosquitoes had ~43% positive BrdU cells vs 25% in blood fed/decapitated females ($F_{3,43} = 11.92$; $p\leq0.001$). On the other hand, NBF/D and NBF/ND had 25 and 32.5% positive cells, significantly lower lower than the percentage observed in BF/ND animals. (BF=blood fed, D=decapitated, NBF=non blood fed, ND=non-decapitated.

ILP-3 and -4 stimulate hemocyte proliferation in *Aedes aegypti*.

ILP-3 and ILP4 regulates yolk deposition and/or carbohydrate and lipid metabolism in *Aedes aegypti* (102, 119). ILP-3 exerts its biological activity by binding the mosquito insulin receptor (MIR) which initiates downstream signaling events. To determine whether ILP-3 and/or ILP-4 has a role in stimulating hemocyte proliferation I injected synthetic ILP-3 and ILP-4 into decapitated *Aedes aegypti* (Fig. 4.4A). Mosquitoes fed at day 4 of age and decapitated 9 hours post blood meal were injected with 50 pmol of ILP-3 or ILP-4 (n=11 mosquitoes) and hemocytes collected 24 h pbm. Females treated with ILP-3, ILP-4 and blood fed/non-decapitated control mosquitoes were significantly higher from hemycte numbers in controls (NBF/ND) ($F_{4,54}$ = 21.95; p≤0.001) as indicated with asterisks. The results indicate ILP-3 is more biologically active than ILP-4. The level of stimulation of proliferation induced by ILP-3 was comparable to the threshold level set by the positive control (BF/ND) (P<0.05, Dunnet t's method) and fully rescued cell increase. To verify that the biological response we were getting was due to biologically active ILP, we conducted a yolk deposition assay as positive control (n=10 mosquitoes) (FIG. 4.4B). ILP-3 stimulates yolk deposition (biologically active ILP-3) whereas ILP-4 does not (P<0.05, Dunnett's method) when compared to blood fed/decapitated (PBS injected) controls.


A
Hemocytes per mosquito ± SE
5000
4000
3000
2000
1000
0
*
*
*
[50 pmol]
[50 pmol]
[Saline]
ILP-3
ILP-4
BF
BF
NBF
DECAPITATED
NON-DECAPITATED

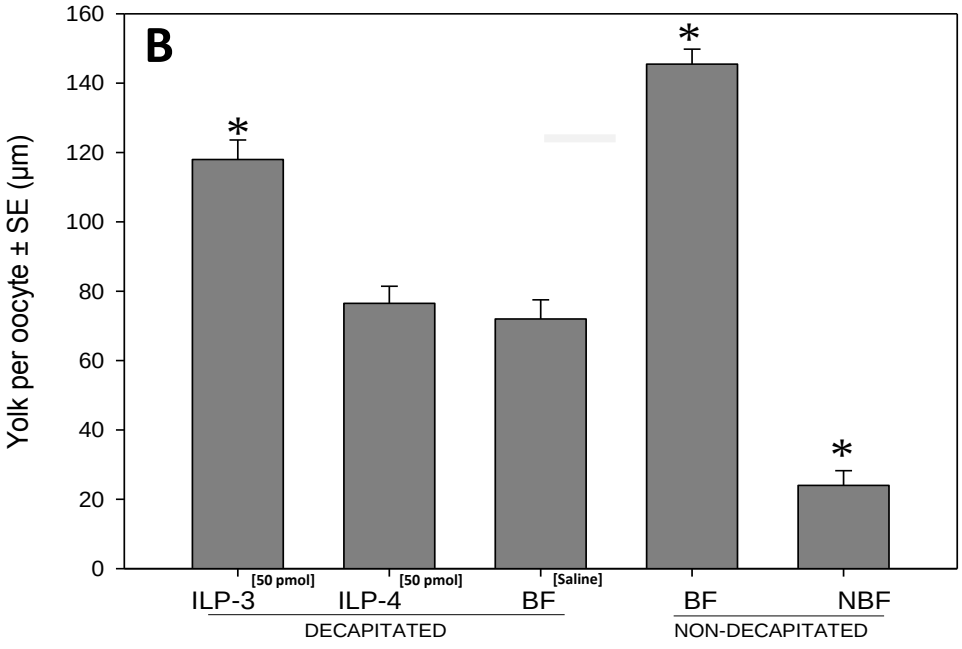
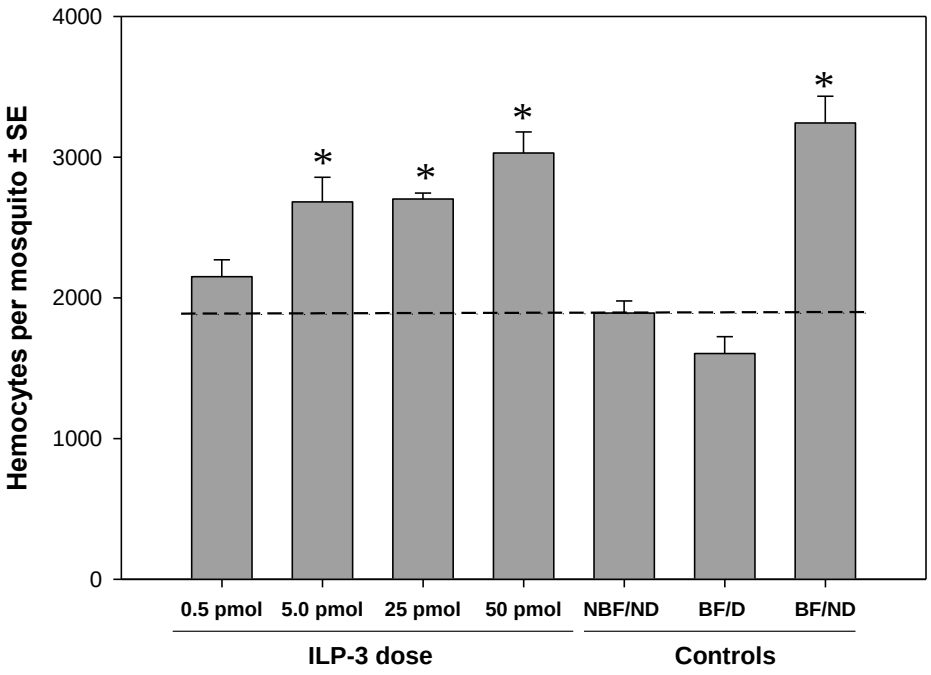

B
Yolk per oocyte ± SE (µm)
160
140
120
100
80
60
40
20
0
*
*
*
[50 pmol]
[50 pmol]
[Saline]
ILP-3
ILP-4
BF
BF
NBF
DECAPITATED
NON-DECAPITATED

Figure 4.4. ILP-3 and -4 stimulate hemocyte proliferation in *Aedes aegypti*. Four day old female mosquitoes were blood fed, decapitated 9 hours PBM and injected with 50 pmol of ILP-3 or ILP-4. Mock controls were injected with PBS. Another cohort was fed sugar water (and decapitated). Control samples included non-decapitated blood fed and non-blood fed females. **(A)** Hemocyte numbers in ILP-3 and -4 injected mosquitoes. ILP-3 and ILP-4 stimulated the production hemocytes comparable to the BF/D control, but ILP-4's effect was minor compared to ILP-3. Treatments differing significantly from control (NBF/ND) are indicated by an asterisk (*) ($F_{4,54}$ = 21.95; p≤0.001), followed by Dunnetts method). **(B)** ILP-3 stimulates yolk deposition as previously described (119). Ovaries from mosquitoes treated with ILP-3 showed increased yolk deposition, equal to that of blood fed/non-decapitated positive control (H= 79.337, d.f.=4, p≤0.001).

ILP-3 dose-dependently stimulates hemocyte proliferation in *Aedes aegypti*.

I further tested the effects of ILP-3 on hemocyte proliferation (Fig. 4.5), by decapitating blood-fed females at 9 hours post blood meal and injecting them with different amounts of the peptide, and counting the number of circulating hemocyte 24 h later. My results showed that 0.5 pmol of ILP-3 per mosquito had no effect on hemocyte proliferation, whereas injections of 5.0-50.0 pmoles stimulated proliferation to levels equivalent to my positive control (BF/ND) ($F_{6, 76}$=18.55 ; p≤0.001). Similarly, yolk deposition was only stimulated in females treated with 50.0 pmol of ILP-3. Concentrations below 50 pmol could not stimulate yolk deposition. In addition, only the 50 pmol dose was able to stimulate yolk deposition, doses between 05-25 pmol were insufficient to stimulate yolk packing into oocytes ($F_{6, 77}$=25.80 ; p≤0.001).


Hemocytes per mosquito ± SE
4000
3000
2000
1000
0
*
*
*
*
0.5 pmol
5.0 pmol
25 pmol
50 pmol
NBF/ND
BF/D
BF/ND
ILP-3 dose
Controls

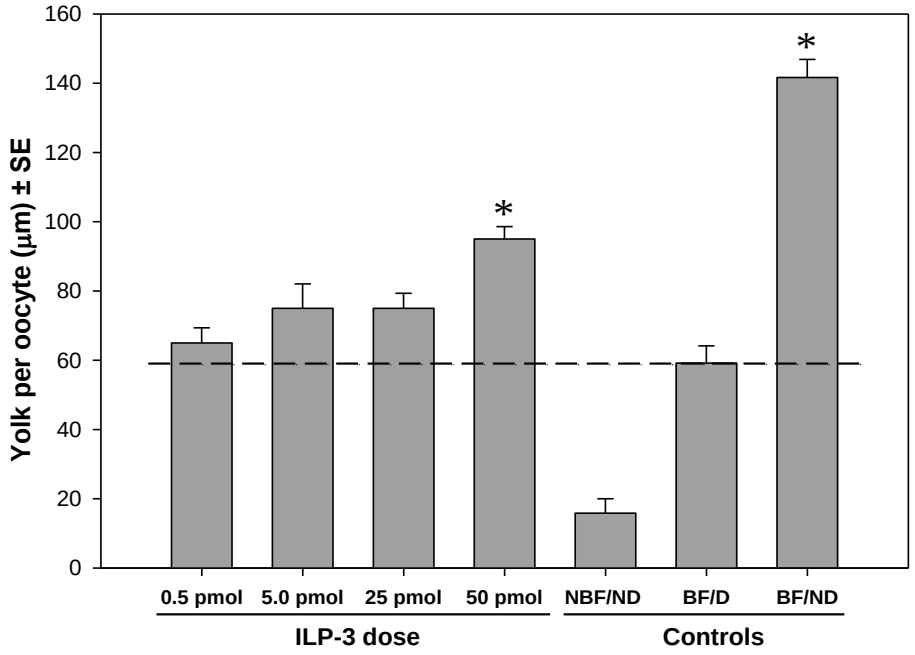

Yolk per oocyte (μm) ± SE
160
140
120
100
80
60
40
20
0
*
*
0.5 pmol
5.0 pmol
25 pmol
50 pmol
NBF/ND
BF/D
BF/ND
ILP-3 dose
Controls

Figure 4.5. ILP-3 dose-dependently stimulates hemocyte proliferation in *Aedes aegypti*.

Mosquitoes (n=11) were decapitated 9 hours post blood feeding and injected with increasing concentrations of ILP3 (0.5–50 pmol) or saline only (blood fed/decapitated=negative control). Blood fed, normal (nondecapitated) females served as the positive control and non-blood fed/non-decapitated as the negative control. Total hemocytes were extracted at 24 h pbm, and counted. Eleven mosquitoes were assayed per treatment. **(A)** Hemocyte numbers (± SE) per mosquito after injection of ILP3 24 h pbm. Treatments significantly differing from the negative control (NBF/ND) are indicated by an asterisk (*)($F_{6,76}$ = 18.55 ; p≤0.001) **(B)** Yolk deposition (μg ± SE) amounts per mosquito 24 h when injected with ILP3. Treatment significantly differing from the negative control (BF/D) are indicated by an asterisk (*) (H = 63.527, d.f.=6, P≤0.001).

MIR knock down inhibits hemocyte proliferation in *Aedes aegypti*.

To determine whether ILP-3 induces proliferation by activating the insulin signaling cascade (ISC), I knocked down expression of the mosquito insulin receptor (MIR) by RNA interference (Fig 4.6). RT-PCR and qRT-PCR analysis indicated that dsRNA treatment reduced MIR transcript abundance by 50% relative to female mosquitoes treated with dsRNA-EGFP (Fig. 4.6B). Concomitantly, my results indicated that dsRNA-treated mosquitoes exhibited no increase in hemocyte abundance following blood feeding (Fig. 4.6C). Interestingly, another phenotype observed is that RNAi knock down also caused a reduction in the total number of cells present in non-blood fed and blood fed/decapitated controls, and this result was significantly different from non-blood fed/non-decapitated dsRNA-EGFP treated females ($F_{3, 68}$=31.152 ; P≤0.001).

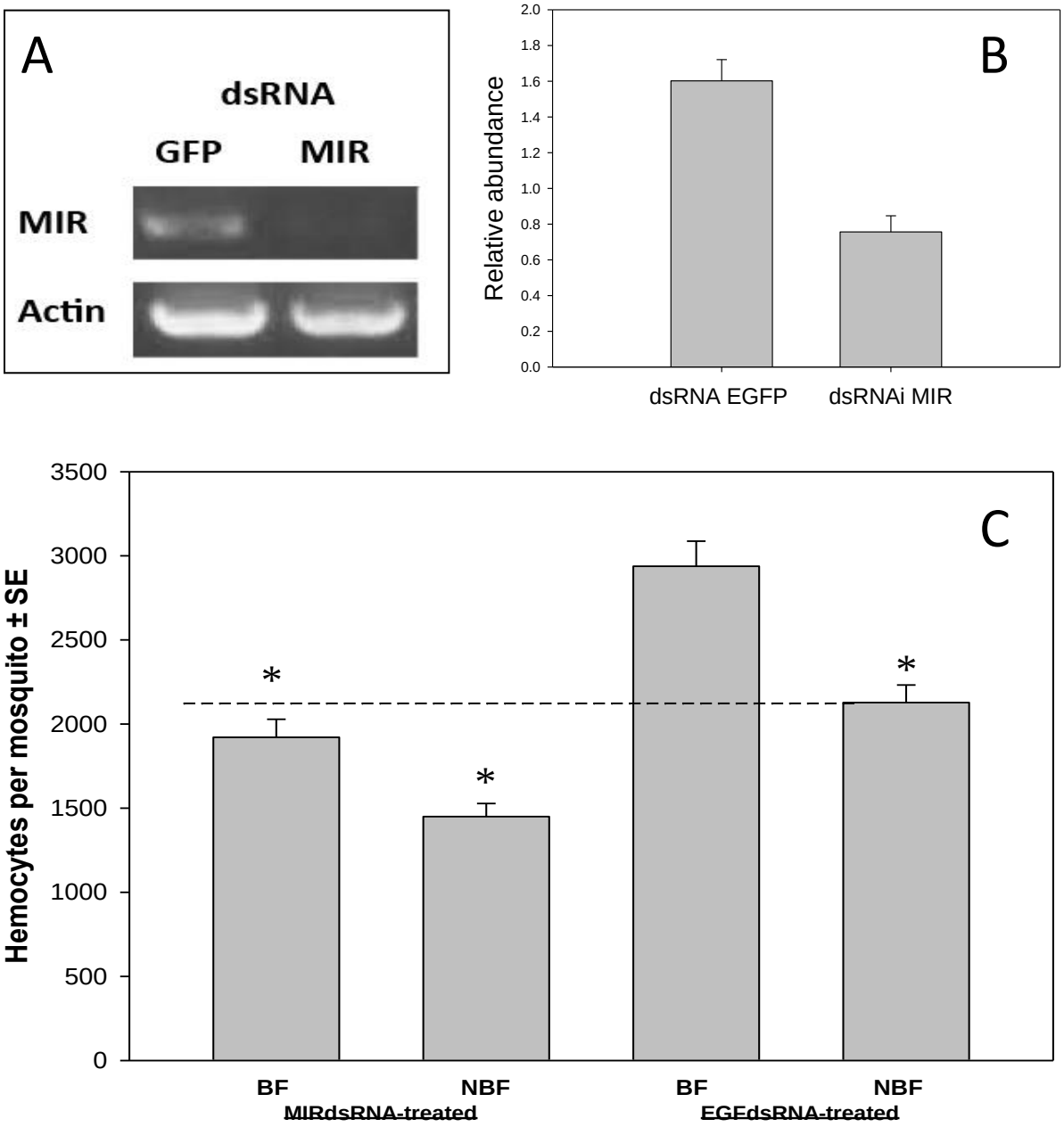
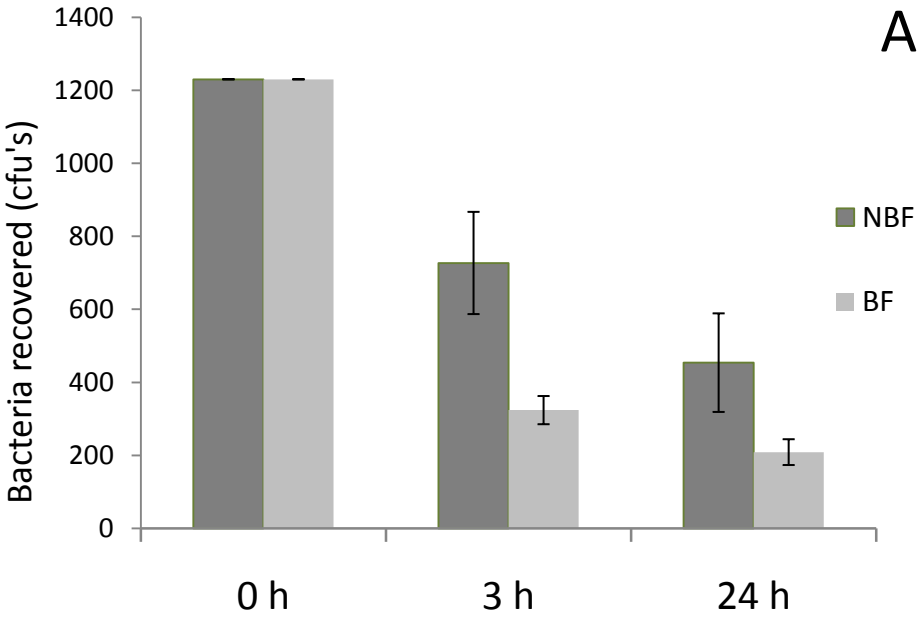

A
dsRNA
GFP
MIR
MIR
Actin
B
Relative abundance
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
dsRNA EGFP
dsRNAi MIR
C
Hemocytes per mosquito ± SE
3500
3000
2500
2000
1500
1000
500
0
*
*
*
BF
NBF
BF
NBF
MIRdsRNA-treated
EGFdsRNA-treated

Figure 4.6. MIR knock down inhibits hemocyte proliferation in *Aedes aegypti*. Newly eclosed female *Aedes aegypti* were treated with MIRdsRNA (C-terminus region of the MIR cDNA sequence) blood fed at day 6 post dsRNA injection and hemocytes were collected 24 h pbm. Control mosquitoes were treated with EGFPdsRNA. **(A)** RT-PCR (whole boby) results indicating expression levels in dsRNA-MIR and dsRNA-EGFP treated females, showing reduced transcript levels in dsRNA-MIR injected samples and control (dsRNA-EGFP). **(B)** qRT-PCR expression analysis showing 50% reduction in transcript levels in dsRNA-MIR treated samples vs EGFP control. **(C)** MIR knock down reduces the number of proliferating hemocytes in blood fed female mosquitoes. The number of hemocytes in MIRdsRNA-treated/blood fed mosquitoes were considerably lower when compared to EGFPdsRNA- treated/blood fed animals. In addition MIRdsRNA-treated samples (both blood fed and non-blood fed) exhibited lower cell counts than blood fed controls ($F_{3, 68}$=31.152 ; P≤0.001).

Increases in circulatory hemocytes enhances clearance of bacteria.

Insulin in regulates reproductive and nutritional physiology. Because it is likely release after food intake and has a role in stimulating hemocyte proliferation, I wanted to test the hypothesis that insulin-induced hemocyte proliferation after a blood meal confers protective immunity to infection due to the increase in cell numbers, and thereby enhancing bacterial clearance. For that purpose, I injected live *E.coli* ($1.23X10^3$ cfu's) into 4 day old blood fed mosquitoes and counted the number of dividing bacteria (cfu's/per mosquito) at 3 h and 24 h post infection. Mosquitoes infected with *E. coli*, seemed to be able to clear the infection faster than non blood fed females. The rate of clearance was significantly faster in blood fed mosquitoes. At 24 h post blood meal 83% of the total bacteria injected was cleared, versus only 63% in non blood fed animals (Fig. 4.7A). Also, blood fed animals showed an increased rate of bacterial clearence during the first 3 hours post infection, with a rate of clearance of 73.66% vs 40.89% in non blood fed animals. Blood fed mosquitoes injected with a higher and medium doses died sooner than non blood fed mosquitoes. However, there was no difference between non-blood fed and blood fed females injected with a low dose of bacteria (Fig. 4.7B). The higher mortality in blood fed animals could be explained by the increase in nutrients present in the hemolymph of blood fed females that could potentially promote bacterial growth. Blood feeding does seem to confer an increase rate of bacterial clearance but do not seem to increase the survival of blood fed females injected with medium or high doses. It could be that in blood fed females, digestion of the blood meal, and egg production have a big impact in immunity.


A
1400
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Bacteria recovered (cfu's)
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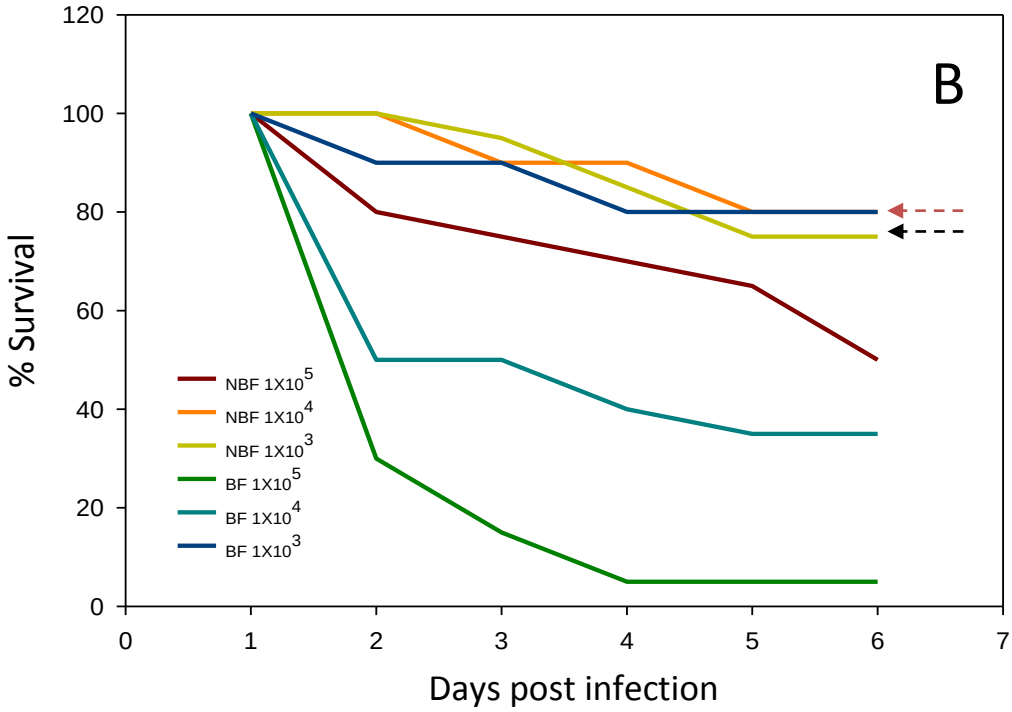
B
120
100
80
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20
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% Survival
NBF 1X10^5
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NBF 1X10^3
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BF 1X10^4
BF 1X10^3
0
1
2
3
4
5
6
7
Days post infection

Figure 4.7. Blood feeding confers resistance to bacterial infection in *A. aegypti* females. *E.coli*-injected blood fed and non-blood fed female mosquitoes showed lower numbers of internal bacteria when compared to the initial dose injected. **(A)** Blood fed mosquitoes were injected with $1.23X10^3$ cfu's intra-abdominally and bacteria was recovered at 3 and 24 hours post infection. Blood fed animals showed a more pronounced reduction in bacterial titers 24 h post infection with roughly 83% of the total inoculum cleared versus 73.66% clearance in non-blood fed controls. Moreover, the rate of bacterial elimination was also faster in blood fed animals with 63% cleared by 3 hours post infection in blood fed animals versus 40.89% in non-blood fed animals. **(B)** Survival plot of blood fed and non-blood fed *E.coli* injected mosquitoes. Blood fed mosquitoes are more susceptible to infection when injected with a high or medium low dose whereas non blood fed exhibited less mortality over time. When mosquitoes where injected with a low dose of bacteria, both blood fed and non blood fed displayed similar survival rates. The red arrow points at the line corresponding to blood fed animals and the black line corresponds to non blood fed animals, both injected with a low dose of bacteria.

4.4 Discussion

Mosquito physiology after a blood meal is controlled by factors secreted from the brain (ILPs), and nutritional signals affect their release (283), resulting in the activation of the insulin cascade and a plethora of biological effects. For instance, decapitation experiments in blood fed females impairs yolk deposition, but when ILP-3 is injected, it rescues yolk deposition and those mosquitoes are then able to produce and pack yolk in newly formed eggs (119). After learning that mosquito ILPs and the MIR are expressed in hemocytes (see Chapter 3) I wanted to determine whether a head-produced signal mediated the proliferative change observed in hemocyte populations. In order to do that, I first needed to establish whether a signal from the head also mediates the increase in circulatory hemocytes. My decapitation experiments showed that a factor(s) produced in the mosquito head mediates hemocytes proliferation

Decapitation within 30 minutes to 9 hours post blood meal prevents an increase in cell numbers. Factors from the head seem to be necessary only during the first 9–12 h; after this period decapitation did not prevent the normal increase in abundance. One possibility that could explain the late response could be the need of preparatory time necessary for nutrients from the blood to be released in the hemolymph and be sensed by the mTOR branch of the insulin pathway (284, 285) responsible for nutrient sensing. Moreover, the time of response also appears to be linked to the time needed for digestion of the blood meal. During digestion, the release of proteolitic enzymes necessary to initiate digestion and assimilation of nutrients that are necessary for biosynthesis (286, 287) concurs with the time of induction of hemocyte increase (9-12 h post blood meal) suggesting that hemocyte increase is dependent on a signal, that takes time to amplify and therefore activate hemocyte proliferation. Another alternative worth considering is the peak in ecdysteroids resulting from insulin-like peptides

stimulation that occurs after a blood meal. Ecdysteroids as discussed earlier have been shown to increase after blood feeding and this release is regulated by the insulin signaling cascade.

The ILP variants expressed in hemocytes (see Chapter 3) that exhibited high levels of expression are all ILPs that have been previously found expressed in the brain. My previous results (chapter 3) showed that brain ILPs are not exclusively expressed in the brain and that decapitation removes the source of endogenous ILPs. These results are quite contradictory. On one hand, decapitation removes brain factors (ILPs) and on the other hand, hemocytes express the same ILPs that are produced in the head. Three alternative explainations for the fact that ILPs expressed in hemocytes are not able to rescue decapitated blood fed mosquitoes may be suggested. First, relatively higher levels of ILP-7 and ILP-8 could outcompete ILP-3 for the MIR. ILP-3 has been shown (this study) to stimulate hemocyte proliferation. Other ILPs expressed in hemocytes such as, ILP-7 and ILP-8 might have another function, maybe acting as a survival signal necessary for hemocyte maintenance. Furthermore, IGF's and insulin are known for competing for the insulin receptor. Differential expression of this two ligands and variation in the receptor isoform expressed are responsible for making cells switch from a metabolic to a mitogenic program, inducing cancer (288). Second, the fact that the main source of ILPs is the brain (medial neurosecretory cells, mnscs), head removal (decapitation) may cause a systemic insulin depletion phenotype. In *Drosophila* cell-directed ablation of insulin-producing medial neurosecretory cells *(289, 290)* caused a remarkable reduction in Dilp-2 expression, but did not affect its expression in other tissues. Moreover, ILP expression in other tissues was not able to rescue the animals from the lack of DILP-2. Our results are in agreement with these results, and despite the fact that ILPs are produced in hemocytes they are not able to rescue decapitated animals to their normal physiological state.

Third, ILPs might be activily transcribed in hemocytes but not translated so even though the mRNA is present in hemocytes, the functional peptide might be absent. For instance, in *Drosophila*,

transcripts of Forkhead box type O (dFoxO) transcription factor and the dIR are not always translated ant during fasting conditions the mRNA's 5′UTR sequences has the ability to initiate IRES (Internal Ribosomal Entry Sites)-mediated translation in S2 cells allowing cells to translate the messenger using a cap-independent mRNA translation strategy (291).

Brain ILPs and more specifically ILP-3 might be responsible for initiating cell proliferation program in intact females and my results supports the idea that ILP-3 is responsible for cell proliferation. ILP-3 is capable of interacting with the insulin receptor and stimulates yolk deposition and changes in carbohydrates and lipids (251). Chemically synthesized-ILP-3 (119) injected to decapitated mosquitoes effectively rescued proliferation of hemocytes to levels observed in normal blood fed intact controls. ILP-4 was also able to induce changes in hemocyte numbers although its effect was minor. Reverse genetics studies using RNA interference also show that mosquito MIR knock down, much like the phenotype seen in decapitated mosquitoes, also inhibits hemocyte proliferation and this effect could not be rescued by injecting ILP-3 (data not shown), suggesting that the MIR is necessary for ILP-3 activity. Furthermore, I also observed that total hemocytes numbers substantially decreased in dsRNA-MIR treated mosquitoes. This result circumstantialy suggest that the insulin signaling cascade might also be involved in hemocyte survival given that the reduction in cell numbers was observed in blood fed and non blood fed females treated with dsRNA-MIR. Previous experiments done in *Drosophila* support the role of insulin as a survival signal (292). In *Culex pipiens*, ILP-1 (similar to *Aedes aegypti*'s ILP-1) is responsible for halting ovarian development to evoke a diapause response (278); in *Aedes aegypti*, ILP-3 regulates processes that lead to egg maturation and carbobydrate and lipid metabolism (251) whereas ILP-4 has gonadotropic properties. Additionally, *Aedes aegypti* ILP-3 is shown (this study) to be responsible of regulating hemocyte proliferation, which makes it a pleiotropic molecule with diverse roles, from having a role in reproduction and nutrition to being deemed a growth factor.

Immune challenge experiments aimed at determining whether hemocyte proliferation (in blood fed animals) confers an adaptive advantage over sugar fed mosquitoes in resisting infection were conducted in female *Aedes aegypti*. Our results circumstantially suggest that hemocyte proliferation might confer increased protection during bacterial infections, as evidenced by a faster rate of clearance of injected *E.coli* when compared to sugar fed controls. However, my results show no difference in survival over time, but instead faster clearance in blood fed animals. I believe that this could be due to additional stress put on the mosquito by the divertion of energy towards digestion and reproduction in addition to been shown to increase of nutrients in the hemocoel that could potentially promote the growth of bacteria injected.

My findings highlight the role of proliferation in regulating blood cell increase in mosquitoes and also it is one of the few reports of such a phenomenon in adult insects. The current dogma is that hemocytes are mostly produced during embryogenesis and larval stages (144, 171, 293). However, there are a few instances in which cell proliferation have been shown to occur in adults. For instance, Ryan et al. (294) concluded that bacterial infection in the American cockroach *Periplaneta americana* induces hemocyte proliferation in adult cockroaches injected with the bacterium *Enterobacter cloacae*. Post-embryonic and post-larval increases in cell population also have been demonstrated to occur through a different mechanism: re-circulation of sessile hemocytes that are attached to tissues or in special compartments present in the adult insect, in this case *Drosophila* (295). Proliferation of adult hemocytes has not been previously demonstrated in hematophagous insects. My study is the first to demonstrate hemocyte proliferation in an adult insect under conditions not related to infection or development. In addition, this proliferation event is stimulated by blood feeding which is a novel

hemopoietic strategy that has evolved in mosquitoes; whether it is widespread in other hematophagous insects is unknown.

My results led me to conclude that hemocytes respond to the same signaling pathways responsible for reproduction (insulin signaling cascade), and as a result, hemocytes proliferate in response to a blood meal. It is well documented that the endocrine system integrates energetic changes and immune responses (296), and in the particular case of mosquitoes, their nutritional physiology is directly linked to reproduction and designed to guarantee reproductive success. In order to maximize success, a female mosquito not only has to survive after taking a blood meal (changes in host-seeking behavior), but also has to survive disease processes (especially those caused by pathogens that might be present in the blood meal) in order to successfully produce offspring. Previous studies have come to the conclusion that immune function, like all other biological processes, requires energy and is therefore costly (297, 298), sometimes at the expense of reproductive success (299-302). When the risk of infection is elevated, a physiological shift that prioritizes availability of energy to mount an immune response seems most adaptive, and can ultimately lead to increased fitness (303). However, some circumstances that represent high risk of infection such as blood feeding (considering that warm-blooded vertebrates carry numerous pathogens in their blood, e.g. parasites, viruses, bacteria) and the omnipresence of pathogens in the environment are translated into competing demands for energy. Such energy competition occurs during acute stress responses, commonly known as fight-or-flight responses. Although these changes are energetically conservative in terms of the amount of energy diverted from other processes such as reproduction, they could provide increased short-term protection against infection incurred with the ingestion of blood. Therefore I hypothesize that mosquitoes have evolved to maximize their energy reserves by having a tight control over energy efficiency that leads to a transient hyper immune state that is rather conservative (not detrimental to reproduction) and

synchronized with their nutritional physiology. Thus, some "optimal" level of immune function must be transiently maintained to maximize fitness and this is done in the form of a quick increase in hemocyte numbers.

Recently, the overlapping roles of the immune and the neuroendocrine systems have been explored in *Drosophila melanogaster* (298) demonstrating that activation of the Toll pathway in the fat body leads to immune activation and nutrient competition that are required for immune processes. In other words, the Toll pathway may reduce insulin signaling pathway, by diverting nutrients and resources allocated for nutrition to the immune system in order to fight a systemic infection (septicemia) caused by *E.coli*. Their data suggested a close communication between these two pathways to better serve the needs of the fly in times of infection by sacrificing nutrition and reproduction and supporting immunity.

Further research is required to determine the specific mechanisms mediating hemocyte proliferation and the signaling mechanism that leads to it. These results validate a novel role for insulin in regulating energetic trade-offs among competing physiological systems (reproductive and immune systems) and provide an important step towards understanding the neuroendocrine mechanisms regulating changes in insect immunity in the form of blood cell proliferation. The understanding of this process may prove critical to finding novel vector controls strategies in mosquitoes, by possibly targeting a robust modulation of their cell-based immunity and inherent humoral properties against parasites such as *Plasmodium.*

CHAPTER 5

CONCLUSIONS

Hemocytes are central to cell-based immune responses in metazoans. In insects hemocytes play critical roles in phagocytosis, encapsulation of egg parasitoids and foreign objects and melanization. In addition to their immune functions hemocytes also assist in tissue remodeling, wound healing and maintenance of the basal lamina (304-307). Key to our understanding of cell based immunity is, knowing the types of hemocytes produced by insects. Insect hemocytes have been characterized in different insect species and different classification schemes have been used (174, 265, 308-310). Some of these cells are named arbitrarily and their identification at times suffers from some challenges, particularly due to their small size.

In insects, hemocytes are produced during embryogenesis and in dedicated hemopoietic organs (264). In flies, hemocytes are produced at two times during larval development. First, subsets of hemocytes are produced during embryogenesis from cells in the head mesoderm. Another subset is produced during the larval-pupa transition (311). The lymph gland (hemopoietic organ) is in charge of producing hemocytes and at the onset of pupation it disintegrates releasing hemocytes into the haemocoel, thus providing a source of fully functional hemocytes that will populate the adult fly. On the other hand, lepidopteran insects possess a disctict hemopoietic organ, responsible for producing hemocytes during larval stages and similarly to Drosophila's lymph gland, it disappears before adulthood. Other insects also possess hemopoietic (Orthoptera, Coleoptera, Hemiptera, Thysanura) (312-315) organs but in mosquitoes, the existence of a hemopoietic organ is unknown, and the type of

hemocytes produced have been characterized using morphology and certain functional markers, but the information about their types is quite disperse and at times not suitable for multiple comparisons due to differences in nomenclature. Identifying hemocyte types produced by mosquitoes and understanding about their biology and dynamics is critical to our understanding of insect immunology in this importantgroup of disease vector insects. *Aedes aegypti* and *Anopheles gambiae* are two of the most important vector species responsible for millions of deaths due to dengue, yellow fever and malaria.

To be able to study mosquito hemocytes I first compared methods used to extract hemocytes in other insect species (perfusion, proboscis clipping, low injection/recovery), and after observing different rates of variability in collecting clean and abundant hemocyte preparations, I developed an improved volume displacement collection method (high injection/recovery) to reliably collect hemocytes from mosquitoes, based on adaptations from previous methodology. The high injection/recovery method proved to be reliably useful for obtaining hemocytes in good numbers to be able to characterize them, and conduct experiments that require lots of cells (e.g. expression analyses). Although the perfusion method yielded cell numbers similar to the high injection/recovery method, it also included a lot of cell debris which made cell counting difficult and could represent a major hurdle in gene expression analysis. The other two methods (proboscis and low injection recovery) yielded very low cell counts per mosquito and therefore were not useful to our needs.

Hemocytes from mosquitoes belong to three basic cell types: granulocytes (fibroblastic, adherent), oenocytoids (round, non-adherent), and prohemocytes (small, non-adherent), a small cell type, presumably a progenitor cell of all hemocytes. Granulocytes represented the most abundant of all hemocytes reported for mosquitoes (this study), representing roughly 90% of the total cell population;

oenocytoids and prohemocytes comprised a smaller ratio compared to granulocytes and combined they accounted for 10% of the total number per mosquito. A fourth type was observed only in samples extracted using the perfusion method, which in the literature are called adipohemocytes (192), but I concluded that this cells represent dislodged fat body cells resulting from traumatic extraction procedures (perfusion) and are not observed in samples extracted with the high injection/recovery method. The types and ratio of hemocytes obtained from males, females, larvae, and pupae were all the same, although there were some differences in cell number, indicating that these cell types are the only blood cells produced by mosquitoes. To discriminate the different cells types identified by morphology in blood fed vs sugar fed, I used a battery of probes and functional markers. Granulocytes are the only type capable of engulfing bacteria (phagocytosis), spreading and strongly attaching to surfaces (macrophage-like behabior). Oenocytoids are the only cell type showing strong reactivity (when compared to granulocytes) to components of the melanization cascade (mono-chlorobimane positive, a molecule that reacts with gluthatione during melanization responses). Prohemocytes, for the most part were not reactive to many biochemical and immunocytochemistry probes, which is in concordance to their role as quiescent and metabolically inactive progenitor cells. Specific immunostaining using four mosquito-specific antibodies developed against proteins from *Anopheles gambiae* (anti-PP06, -SP22D, -SRPN6, -SRPN10) showed that oenocytoids from *Anopheles gambiae* are positive for prophenoloxidase and serine proteases involved in the melanization cascade, these antibodies did not stain *Aedes aegypti* hemocytes. Hemocytes from both species (granulocytes and oenocytoids alike) were immuno-stained with antibodies to PSMD3 (DoxA2), a gene closely linked to a QTL for melanotic encapsulation) and LYS c-1 (lyzosyme). Immune challenge increased the immunostaining pattern observed with antibodies to PPO6, and PSMD3. Initially (mock challenged) oenocytoids were the only positive cell type, but immune challenge expanded their staining to granulocytes, indicating that these genes are constitutively expressed in oenocytoids and up-regulated

in granulocytes after bacterial challenge. Last but not least, hemocyte numbers decline with age and blood feeding induces a significant increase in hemocyte numbers. The nature and benefit (if any) of this response, led me to the second part of my dissertation that dealt with finding the answer to that question. After knowing that mosquito hemocytes basically produce three main cell types, and learning that blood feeding induced an increase in hemocyte numbers, I aimed to identify the factor(s) involved in inducing this change in hemocyte abundance.

The insulin signaling cascade is well conserved in insects and directly involved in physiological processes induced by blood feeding. After blood feeding, female mosquitoes initiate a developmental program that leads to the production of eggs. Blood meal digestion and concomitant nutrient release and assimilation provide the nutrients needed for making yolk and complete the oogenic cycle. The insulin signaling cascade has been directly link to the regulation of this process. Brown et al. (2008) showed that ILP-3, one of the 8 ILPs present in *Aedes aegypti* regulates egg maturation and metabolism. Decapitated females could not produce yolk necessary for eggs, but when injected with ILP-3, the production of yolk was restored. Similarly, dsRNA-MIR knock down blocked the production of yolk and showed that the insulin cascade is necessary for the regulation of egg maturation. Furthermore, ILP-3 was shown to directly bind the MIR and to activate downstream signaling events (102, 119).

In insects, previous studies have shown that ILPs (bombyxin) are capable of inducing cell proliferation in hemopoietic organs (126, 316). In vertebrates, insulin and the insulin receptor are expressed in various types of lymphocytes and capable of inducing lymphopoiesis and cell proliferation (IGFs) (317, 318). The expression of ILPs in *Aedes aegypti* hs been characterized, showing that five ILPs are preferentially expressed in the brain, making the brain the main source of endogenous ILPs (101). For the purpose of determining whether insulin signaling has a role in circulating hemocytes after a

blood meal, I first characterized their expression in hemocytes. ILPs and the MIR are expressed in hemocytes and their expression level is slightly up-regulated in blood fed females versus non blood fed controls. Four brain ILPs (-3, -4, -7, and -8) were detected in hemocytes, which suggest that the same neuroendocrine signal released after a blood meal responsible for egg maturation may also regulate hemocyte physiology. Given that ILPs and the MIR are expressed in mosquito hemocytes, and that *Aedes aegypti* produces different ILPs expressed in medial neuro secretory cells (MNSCs) in the brain, I performed decapitation experiments. Decapitated blood fed females showed no changes in hemocyte abundance after a blood meal, suggesting that a brain signal produced in the brain may be involved in the increase in hemocyte numbers. The time of decapitation proved to be critical, since mosquitoes decapitated from 10 minutes-9 hours pbm did not show changes in hemocyte numbers. By contrast, decapitation after 12 hours pbm could not inhibit hemocyte increase, suggesting that the time of preparation for hemocyte increase could be linked to nutritional physiology.

When the expression data for ILPs and MIR were contrasted against the effect of decapitation, I noticed that they did not match in the sense that, on one hand, decapitation removes the source of ILPS (secreted from the brain), and on the other, the same ILPs are expressed in hemocytes, so why can't ILPs produced in hemocytes rescue cell proliferation? Several explanations are proposed ,but it could be due to differences in transcription and translation of the RNA messenger (291), competition between ILPS (e.g. ILP-7 and -8 are expressed at higher levels than ILP-3 in hemocytes) and differences in secretion of ILPs from the brain upon signal stimulation from the fat body. In Drosophila, the fat body is able to sense changes in nutrient levels and produce and unknown humoral factor that affects Dilp/insulin receptor signaling in the rest of the body (319, 320). In humans, insulin and IGF's compete for the IR and modulate the physiological response from metabolic functions to the induction of mitogenic effects on cancer cells (288), or the amount of ILPs produced by this cells is not enough to initiate the activation of

proliferation. The expression of ILP-7 and -8 at high levels could suggest that these two ILPs might be involved in cell maintenance, by acting as survival signals.

My results are in agreement with the fact that cell proliferation is expensive and therefore there is increase need for energy which explains why the connection of the nutritional physiology with the immune system. To understand the nature of increased cell abundance, I performed proliferation assays using BrdU incorporation. Proliferation assays showed that hemocytes from blood fed females actively proliferate, as evidenced by increased labeling when compared to non blood fed and decapitated controls. After learning that hemocytes proliferate after a blood meal and demonstrating that factors produced in the head of the mosquitoes are needed for this phenotype, I then studied the effect of chemically-synthesized ILP-3 and ILP-4. Both ILPs were capable of inducing cell proliferation in decapitated females, by rescuing the lack of brain signals. This response was dose-dependent and much stronger with ILP-3. Similarly, the injection of ILP-3 (to verify ILP bioactivity) also stimulated yolk deposition whereas ILP-4 did not, demonstrating that the biological effect observed was due to biologically active ILP. As the injection of ILP-3 rescued hemocyte proliferation in decapitated females, I investigated the effect of the absence of insulin signaling (loss-of-function) using RNAi. I injected female mosquitoes with MIR-specific dsRNA to knock down the expression of the mosquito insulin receptor. Cell proliferation was inhibited in RNAi knock down females and this phenotype could not be rescued by injection of ILP-3, suggesting that the MIR is required for ILP activity as previously reported. Moreover, MIR knock in hemocytes also caused a reduction in total hemocyte numbers in dsRNA-MIR treated females, suggesting that insulin signaling may be required for cell survival.

After demonstrating that the increase in cell numbers after a blood meal is due to proliferation and showing that the insulin signaling cascade is directly responsible for mediating this response, I

wanted to learn more about the benefit of having such a response. Immune challenge experiments aimed at determining whether hemocyte proliferation (in blood fed animals) confers an adaptive advantage over sugar fed mosquitoes in resisting infection were conducted in female *Aedes aegypti*. My results circumstantially suggest that hemocyte proliferation may confer increased protection during bacterial infections, as evidenced by a faster rate of clearance of injected *E.coli* when compared to sugar fed controls. However, my results show no difference in survival over time, but instead faster clearance in blood fed animals. I believe that this could be due to additional stress put on the mosquito by the shuffling of energy towards digestion and reproduction in addition to the increase of nutrients in the hemocoel that could potentially promote the growth of bacteria injected. Further studies are needed to explore the biological significance of hemocyte proliferation during pathogen challenge as well as to study their ontogeny.

This dissertation has provided information regarding collecting and identifying hemocytes from mosquito species using light and fluorescence microscopy methods. In addition, I have provided a description of the different hemocytes produced by two mosquito species, *Aedes aegypti* and *Anopheles gambiae*, two of the most important insect vectors. More importantly, my work has demonstrated that hemocytes from adult mosquitoes do proliferate and do so in a way not related or induced by developmental changes or infection, influenced by insulin signaling. This is one of the first reports of hemocyte proliferation in an adult insect, and the first one linking homologous insulin effects and hemocyte function in invertebrates.

Although this dissertation has set some groundwork in our understanding of cell proliferation in insects, additional studies need to be performed so the specific genes under the influence of the insulin signaling cascade and involved in regulating hemocyte proliferation and immune regulation are identified. Our

understanding of the way insulin interacts with the immune system could represent an opportunity to develop strategies to boost the immune system of mosquitoes in order to make it more refractory to parasites.

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