APOLIPOPHORIN GENE SILENCING BY RNAi INHIBITS LIPID TRANSPORT TO THE OVARIES DURING VITELLOGENESIS IN *Aedes aegypti*

By

CLAUDIO ARAFAT GALVEZ-SAGASTUME

Bachelor of Science in Biochemistry

Universidad de San Carlos de Guatemala

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Thesis Approved:

Dr. Jose Soulages

Thesis Adviser

Dr. Estela Arrese

Dr. Haobo Jiang

Name: CLAUDIO ARAFAT GALVEZ-SAGASTUME

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Aedes aegypti is an anautogenous species and serves as vector for several viruses that cause infection to hundreds of millions of people around the world, causing enormous economic burden. Lipids are essential for *Aedes aegypti* survival and reproduction. Their role during vitellogenesis is critical as primary energy source to support embryo and egg development. Considering the importance of lipids for this species and the epidemiological relevance of the diseases the species can carry and transmit to humans, this study was focused on lipid transport during vitellogenesis and the function of apolipophorin gene in this process.

This project was focused on studying lipid levels in fat body, hemolymph and ovaries during vitellogenesis. Lipophorin is the main lipid carrier for all insects. It is synthesized mainly in the fat body cells and secreted to the hemolymph to act as a shuttle loading and unloading lipids among tissues. Fat body synthesizes and accumulates lipids during the first 24 hours after a blood meal, then lipids are secreted to the hemolymph in the form of high density lipophorin and taken up by the ovaries for oocyte development, while lipid concentration in fat body returns to basal or below basal concentration. Lipid concentration in hemolymph increases gradually until 36 hours post blood meal, while ovaries accumulate lipids and reach a maximum concentration between 48 and 72 hours post blood meal, correlating with their size.

Using dsRNA as the inducer for RNA interference, apolipophorin gene was successfully silenced, causing lipid and protein accumulation in fat body, and lipid shortage in hemolymph and ovaries after blood feeding. Apolipophorin silencing also affected the size and shape of the ovaries, preventing their normal development. Other lipid transport related genes were also affected. Vitellogenin protein abundance in hemolymph and ovaries was increased, while mRNA did not show any significant change. Lipid transfer particle gene appeared to be downregulated at transcript level in fat body cells.

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CHAPTER I

BACKGROUND

1. Introduction

Lipids are essential for insects' survival and reproduction. Their role during vitellogenesis is critical as primary energy source to support embryo and egg development. *Aedes aegypti* is an anautogenous species that serves as vector for several viral infections that affect hundreds of millions of people around the world, causing enormous economic burden. Considering the importance of lipids for this species, and the epidemiological relevance of the diseases it can carry and transmit to humans, the lab focused this study on lipid transport during vitellogenesis, particularly trying to address the function of apolipophorin gene for this process.

The goals for this study were: 1) to evaluate the expression of apolipophorin gene in the fat body of female *Aedes aegytpi* mosquitoes before and after blood feeding, and its correlation with lipid transport during vitellogenesis. 2) To determine the possibility of silencing the apolipophorin gene by RNA interference, using dsRNA as an RNAi inducer. 3) To evaluate the effect of apolipophorin silencing on the process of lipid transport to the ovaries, and its effect on the expression of other lipid transport related genes.

2. *Aedes aegypti*

Aedes aegypti (Diptera: Culicidae), commonly known as the yellow fever mosquito, is one of best studied species of mosquitoes, in part due to the simplicity of its rearing inside the laboratory environment (Nene et al., 2007). In nature, both males and females can feed on plant nectar, but females need to feed on vertebrate blood (primarily on human hosts) to obtain nutrients that are used for egg development (McMeniman et al., 2014). After a blood meal, adult females can lay approximately 100 eggs. These eggs can survive desiccation for several months in a freezing-resistant diapaused stage, until the appropriate climate conditions occur, and they hatch as larvae. Before becoming an adult mosquito, *A. aegypti* has four larval stages and a pupal stage, all of them aquatic. Larval stages last around four days, and they feed on algae and other microorganisms. The pupal stage is a non-feeding stage which lasts approximately two days. Finally, the pupae emerge as adult mosquitoes whose life span ranges from two weeks to a month (Clemons et al., 2010) (Figure 1).

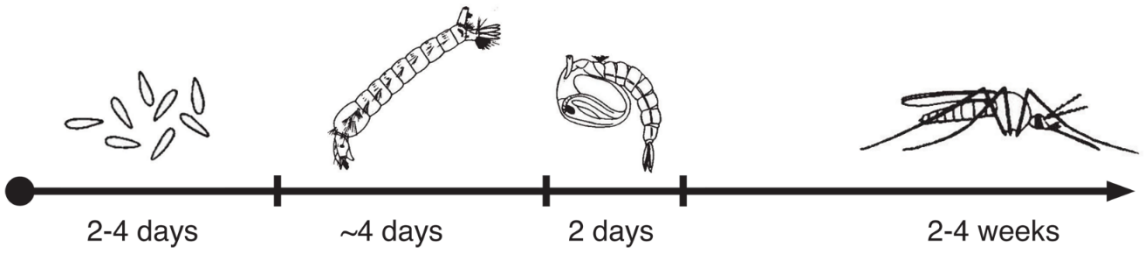

Fig. 1. Timeline of *Aedes aegypti*. Four stages of the life cycle of *Aedes aegypti.* In favorable conditions an egg will hatch into a larva in 2 to 4 days. The larva will develop further for around 4 days until it becomes a pupa. After 2 days, the adult mosquito emerges from the pupa and lives up to four weeks.
Source: http://necsi.edu/research/social/pandemics/transmission.html

Because of their blood-feeding behavior, female *A. aegypti* mosquitoes can efficiently spread blood-borne diseases. *A. aegypti* is the primary vector of chikungunya, yellow fever, dengue and zika viruses, and it is responsible for millions of human infections annually throughout the tropical and subtropical regions of the world (Bhatt et al., 2013).

The prevalence of diseases transmitted by *A. aegypti* and the need to search for alternative methods to control mosquito populations have led to the support of scientific projects directed to understand the biology of this mosquito.

3. Importance of lipids for insects

Lipids are important to insects because they can be used as energy source for reproduction, embryogenesis, metamorphosis and flight. The amount of lipids in insects is influenced by several factors, such as developmental and nutritional states, sex, temperature and flight. Among species, the lipid content can vary from 1-50% of the wet weight. In general, female insects possess more lipids than males because they are used for egg production. The fat body is the principal storage site of lipids for insects. This organ consists groups of cells forming layers of tissue, which is spread throughout the body and encloses the internal organs. It is especially abundant in the abdomen, where large fat body deposits are found in close association with the midgut, facilitating the uptake of dietary nutrients. The fat body is a major center for intermediary metabolism playing physiological roles that are analogous to both vertebrate liver and adipose tissue. This organ is central to the synthesis and also to the storage of carbohydrates, lipids and proteins (Arrese and Soulages, 2010).

4. Lipophorin structure and function

Lipophorin (Lp) is the main hemolymph lipoprotein in insects and as such, it plays a major role in the transport of lipid molecules among tissues. The studies on the structure and function of lipophorin have been reviewed (Soulages and Wells, 1994; Van der Horst and Ryan, 2012). As other lipoproteins, lipophorin is a non-covalent assembly of lipids and proteins, organized as a largely spherical particle. All lipophorin particles contain two apolipoproteins: apolipophorin-I (apoLp-I) and apolipophorin-II (apoLp-II). These apolipoproteins are integral components of lipophorin and cannot be removed without destroying the lipophorin particle. The size and

density of lipophorin depend on its lipid content. In general, these lipoproteins have several hundreds of lipid molecules and a lipid content that represents 40-60% of the particle mass. The core of the lipoprotein molecule is highly hydrophobic, and it is made up of diacylglycerol (DG), triacylglycerol (TG), hydrocarbons, and carotenoids. Phospholipids are also present at the lipophorin particle surface, positioned with their fatty acyl chains interacting with the hydrophobic core of the particle, while their polar head groups interact with the aqueous environment (Soulages & Brenner, 1991).

Lipophorin is a reusable particle that normally loads and unloads lipid molecules multiple times before being degraded (van Heusden et al., 1991). In several insect species, lipophorin particles can also contain a third apolipoprotein, apolipophorin-III (apoLp-III). This extra apoLp molecule is found in lipophorin particles that have a larger than normal content of DG, and normally fall in the category of low or very low density lipoproteins (densities lower than ~1.10g/cm3). It is often found that when the insect needs to mobilize large amounts of fatty acids, for instance to support flight, the fat body will release large amounts of DG that will be loaded into the lipophorin particles together with apoLp-III (Soulages & Wells, 1994). DG is the most common way in which fatty acids are transported among the insect tissues, However, it must be noted that some insects, including mosquitoes, transport fatty acids in the form of TG (Pennington and Wells, 2002)

5. Lipophorin of *Aedes aegypti*

Aedes aegypti lipophorin is considered a high density lipophorin and, like most insects' lipophorin particles, it contains two apoproteins, apoLp-I, with a molecular weight around 230 to 250kDa, and apolipophorin-II, with a molecular weight 70 to 85kDa (Capurro et al., 1994; Sun et al., 2000; Van Heusden et al., 1998). However, its lipid composition differs from other insects' lipophorins, because it contains triacylglycerol as the most abundant neutral lipid, in contrast to

lipophorin from other insect species, in which usually diacylglycerol is the major neutral lipid (Ford and Van Heusden, 1994). Insect lipophorin can function in most cases as a lipid shuttle that can be reused by continuously loading and unloading lipids at different target tissues, without displaying major changes in the production and degradation rates of the apoproteins (Downer and Chino, 1985; Van Heusden et al., 1987) *A. aegypti* lipophorin concentrations increase after a blood meal, responding to the activation of vitellogenesis and embryogenesis, in which the mosquito may need an increased rate of lipid transport to the developing ovaries. *A. aegypti* responds to an increased need for lipid transport by synthesizing and secreting more lipophorin into the hemolymph, instead of loading more lipids into the pre-existing lipophorin (Capurro et al., 1994; Van Heusden et al., 1998). This suggests that lipophorin in this species may not act as a reusable lipid shuttle. Indeed, in vitro studies have shown that the TG-rich lipophorin of *A. aegypti* does not load glycerides from the fat body as efficiently as it does a DG-rich lipophorin like the lipophorin of *Manduca sexta* (Pennington et al., 1996). In this case, it would be expected that *A. aegypti* lipophorin is synthesized and secreted with its full load of lipid as suggested by Van Heusden et al. (1998).

6. Apolipophorin structure homology and assembly

The large lipid transfer protein superfamily (LLTP) includes vertebrate apolipoprotein B (apoB), microsomal triglyceride transfer protein (MTP), and vitellogenin (Baker, 1988). The LLTP superfamily proteins contain the large lipid transfer (LLT) domain that comprises a large lipid-binding cavity of about 1000 amino acids located at the N-terminal domain ApoLp-I and apoLp-II are products of the same gene that originate through post-translational cleavage of their common precursor protein (Sundermeyer et al., 1996; Weers et al., 1993).

The insect apolipophorin precursors belong to the LLTP superfamily, which shares a high homology with the ancestral N-terminal domain of vitellogenin (Babin et al., 1999; Smolenaars

et al., 2007b). The cleavage of insect apoLp-II/I into apoLp-II and apoLp-I is mediated by an insect furin, acting at a consensus sequence (R-X-K/R-R) of the LLT domain. Since protein cleavage by furin homologs is performed late in the secretory pathway, mainly in the Golgi apparatus, insect lipoprotein biosynthesis was proposed to proceed by initial lipidation of apoLp-II/I to a lipoprotein, while cleavage of apoLp-II/I into apoLp-I and -II would occur at a later stage. The uncleaved LLT domain in apoLp-II/I comprises regions of both apoLp-I and apoLp-II, and it is likely to be essential to allow lipidation. The occurrence of a cleavage step prior to lipidation might result in the impairment of lipoprotein biosynthesis. It was previously shown that if cleavage was impaired by a furin inhibitor or mutagenesis of the consensus substrate sequence for furin, uncleaved apoLp-II/I appeared to be properly lipidated and functional, similar to a normal lipophorin particle (Smolenaars et al., 2005).

As it was mentioned previously, insect apolipophorin precursor is homolog of apolipoprotein B from vertebrates, sharing a similar N-terminal LLT domain. Putative apoB structure is organized as a cluster containing a combination of alpha-helical domains and amphipathic beta-strand domains (N-α1-β1-α2-β2-α3-C) (Segrest et al., 2001), whereas, apoLp-II/I has been proposed to exhibit a similar but smaller structure, organized as N-α1-β-α2-C. The C-terminal β1-α2-β2-α3 domain clusters are thought to stabilize the expansion of the initial lipid core in the LLT module and accommodate most of the lipid-binding capacity. Recombinant expression experiments proved that the β cluster accommodates the apoLp-II/I lipid-binding capacity (Smolenaars et al., 2007a). After cleavage of apoLp-II/I, the β domain is almost entirely situated in apoLp-I, suggesting that apoLp-I, and not apoLp-II, binds most of the lipids carried by the lipophorin particle (Smolenaars et al., 2007a).

Lipophorin biogenesis in insects shows similarities with lipoprotein biogenesis in mammals. Lipoprotein assembly in mammals requires MTP for the initial binding of lipids to the amphipathic lipid-associating segment of apoB (Ledford et al., 2006). An MTP homolog was also

discovered in *Drosophila melanogaster*, which was able to promote the assembly and secretion of human apoB (Sellers et al., 2003). Thus, insect lipoprotein assembly has been proposed to occur similarly (Smolenaars et al., 2005). Regardless of specific modifications, the assembly of lipoproteins both in mammals and insects requires amphipathic structures in the apolipoprotein carriers, as well as MTP for initial lipidation (Smolenaars et al., 2007a).

6.1 Apolipophorin gene of *Aedes aegypti*

The ApoLp-II/I gene (accession number AAEL009955) is located on the forward strand of the short arm of chromosome 3, between nucleotide residues 77,687,304 and 77,708,562 (21,258 bp). It contains 10 exons, with a transcript length of 10,482 bp translated to a peptide with 3,343 amino acid residues (Giraldo-Calderón et al., 2015) (Figure 2). The coded peptide shows a lipid transport protein conserved domain between amino acids 43 and 647 (homolog to vitellogenin) (Madden, 2003) (Figure 3). Post-translational processing is thought to occur between amino acid residues 25 and 26 where the signal peptide sequence is cleaved; and between amino acids 715 and 716 where a putative cleavage site for furin is located, according to the presence of a consensus sequence "R-F-R-R" between amino acids 712 – 715 (Smolenaars et al., 2005). Final products of the gene comprise apoLp-I with a predicted length of 2,628 amino acid residues, and apoLp-II with 690 amino acid residues (Fig. 2-4).

7. Lipid mobilization in vitellogenesis

Lipids are required for oocyte development in insects (Sun et al., 2000). For *A. aegypti*, a blood meal triggers ovary development and oocyte maturation, stimulating the accumulation of yolk proteins and lipids. Oocytes of *A. aegypti* contain around 35% of their dry weight in the form of lipids, which are used to supply energy to the developing embryo (Troy et al., 1975). Lipid content of the oocytes could be synthesized in the ovaries, although it has been previously shown that only minor fatty acid synthesis takes places in the ovaries, which then are incorporated in

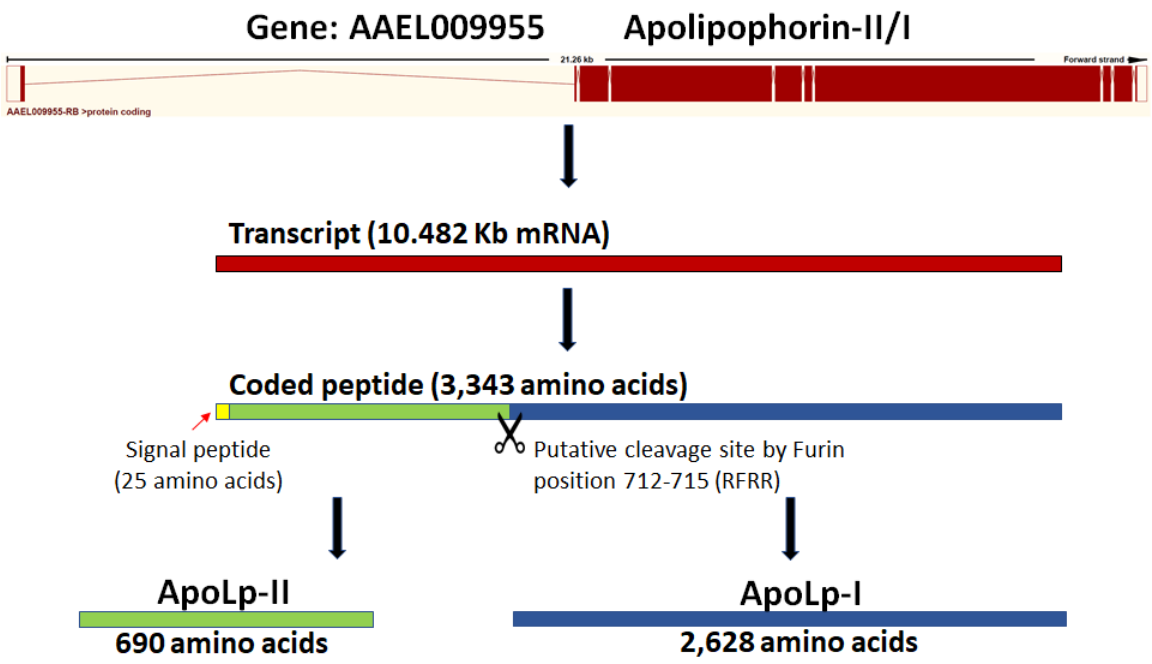

Fig. 2. *Aedes aegytpi* apolipophorin gene structure and coded peptides. Apolipophorin gene gives rise to both apolipohorin proteins by post-translational cleavage of the initial coded peptide. The putative cut site was identified by a consensus sequence recognized by furin protease, located among residues 712-715.

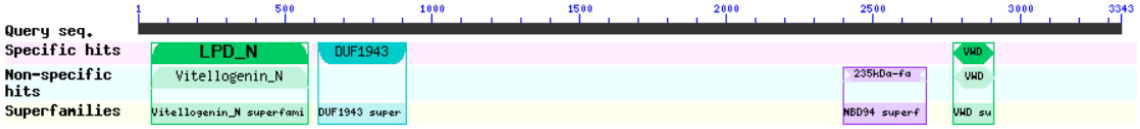

Fig. 3. Conserved domains in *Aedes aegypti* apolipophorin peptide. Conserved domains found on apolipophorin nascent peptide show a large vitellogenin superfamily domain near the N-terminal region, and a von Willebrand factor type D domain close to the C-terminal region (Maden, 2003).

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L.migratoria    KSLYNRITERFEKTFRQKRSVSKDAVDNIRQQAYKSLLPSQRDRSLDVDLSLKTFGSELA  761
M.sexta         QESKK----KVEDSLSRGRRSIKSEIDVFDKNL--KAESAPYNNELDLDIYVKLFGTDAV  749
A.aegypti       TKL-------SEKAQSRFRRGLKEDVRAFAKGV--NM-RNDALEDFNLDVSVKFFGSELF  752
D.melanogaster  GNN-------GVAAGGRARRSIVDDVSKISKKY--KMYGVKNVQDLNLDVSLKLFGSELA  744
                 .          :  : *^   . :  : :    .       ..:::*: :* **::
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Fig. 4. Putative cleavage site in apolipophorin peptides from four insect species determined by Multiple Sequence Alignment. Consensus sequences recognized by furin protease for cleavage follow the general structure R-X-K/R-R and are conserved among different insect species. This cleavage produces both apolipophorin proteins found in circulating lipophorin particles.

phospholipids not triglycerides (Ziegler, 1997). Most of the lipids found in the oocytes come from the fat body and are delivered to the ovaries by lipophorin particles or, to a lesser extent, by vitellogenin (Vg). According to previous studies, lipophorin can deliver lipids as a reusable low density lipophorin particle (LDLp) or as high density lipophorin particle (HDLp) that is internalized and deposited in the developing oocytes via receptor-mediated endocytosis (Kawooya and Law, 1988; Liu and Ryan, 1991). Female *Aedes aegypti* mosquitoes which have fed only on blood are able to mature some eggs. However, it has been reported that it is an advantageous condition for the mosquitoes' fecundity to feed on sugar before the blood meal, since they are able to lay more eggs and the lipid content in their fat body and ovaries is higher. In females that fed on sugar before the blood meal, about 80% of the eggs lipids is derived from fat body lipid stores accumulated before blood feeding (Ziegler and Ibrahim, 2001). Lipids are extremely important for the proper development of embryos, as it was shown in the mosquito *Culex quinquefasciatus,* where 90% of the metabolic energy required for this process is provided by lipids (Van Handel, 1993).

8. Lipophorin receptors

Lipophorin receptors have been identified and reported for several insect species including *A. aegypti* (Cheon et al., 2001). These receptors mediate the intracellular uptake of lipophorin particles in different tissues, depending on the metabolic state of the insect. Two tissue specific splice variants of the *A. aegypti* lipophorin receptor gene were reported to be found in the fat body and ovaries (Cheon et al., 2001). The expression of ovary-specific lipophorin receptor is activated by a hormonal cascade initiated by a blood meal, with the terminal signal being 20-hydroxyecdyson that triggers a vitellogenic state in the fat body, by activating the yolk protein precursor genes. The expression of the fat body-specific lipophorin receptor is restricted to a post-

vitellogenic state, where production of yolk protein precursors is over, and the fat body becomes a storage tissue again (Raikhel et al., 2002).

Domain organization of insect lipophorin receptors is identical to mammalian low density lipoprotein receptors (LDLR). However, the ligand-binding domain of the insect lipophorin receptor contains an additional cysteine-rich repeat compared to LDLR, similar to the human very-low density lipoprotein receptor VLDLR (Van der Horst and Ryan, 2012). Despite their structural similarities, their mechanism of function is different and the ligand specificity of lipophorin receptors and low-density lipoprotein receptor are mutually exclusive (Van Hoof et al., 2002). It has been reported that human low density lipoprotein (LDL) binding to its receptor depends on the C-terminal portion of ApoB, which despite of being homolog to insect ApoLp-II/I, the C terminal part does not resemble that homology; leaving the receptor binding domain of lipophorin unknown (Babin et al., 1999).

Considering that the expression of lipophorin receptors in fat body is triggered by a specific metabolic or developmental state where the fat body lipid reserves are depleted, it has been proposed that lipophorin receptor expression is regulated by lipid deficiency (Van Hoof et al., 2003). Results of experiments using partially-empty HDLp particles with a density of 1.17g/ml suggested that lipophorin receptor binds to these partially-empty HDLp with more affinity than normal density HDLp. Therefore, the lipid loading of the particle results in decreased affinity for lipophorin receptor, facilitating the process of recycling instead of destruction of the particle (Roosendaal et al., 2009). Downregulation of lipophorin receptor expression in fat body of locust newly ecdysed adults suggests that this receptor is not involved in the lipophorin reusable shuttle mechanism seen in the flying insect (Van der Horst and Ryan, 2012).

The pathway followed by internalized HDLp is different than mammalian LDL-receptor lysosomal pathway. Mammalian LDL receptor-mediated endocytosis releases the particle into

lysosomes promoted by the acidic pH, and it is completely degraded (Goldstein et al., 1985). Whereas insect HDLp remains coupled to the lipophorin receptor and is transported to a non-lysosomal compartment called endocytic recycling compartment (ERC), from which HDLp is eventually re-secreted, avoiding lysosomal degradation (Van Hoof et al., 2002). This phenomenon contradicts the general fate of ligands endocytosed by all other LDLR family members. However, it is well known that lipophorin interacts with a specific high-affinity binding site of the lipophorin receptor and remains bound even at endosomal pH (Roosendaal et al., 2008).

Immunocytochemical data revealed that around 3% of the total yolk protein accumulated by developed oocytes in *A. aegypti* comes from HDLp internalization. It was concluded that it is unlikely that internalization of HDLp could be the major route of lipid delivery to the oocyte (Sun et al., 2000). As previously stated, a dual mechanism was proposed for lipid transport; a lipophorin shuttle involving LDLp and internalization of HDLp and recycling. Although, no LDLp molecules have been reported for *A. aegypti* in any developmental or nutritional state. The mechanism of lipophorin receptor-mediated endocytosis of HDLp and the fate of the lipophorin in the oocytes of *A. aegypti* still remain unknown.

9. Other lipid binding proteins

9.1 Lipid transfer particle

Lipid transfer particle (LTP) was first isolated from the hemolymph of *M. sexta* larvae in 1986. It was reported to facilitate the distribution of lipids among lipophorin subspecies (Ryan et al., 1986). Later studies in *M. sexta* showed that LTP was also present in the conversion of HDLp to LDLp in response to adipokinetic hormone (Van Heusden and Law, 1989). LTP is high molecular weight lipoprotein complex that displays a semi-spherical head region and a cylindrical tail. It

contains about 14% lipids (mostly phospholypids and diacylglycerides) and three apoproteins: apoLTP-I of 320 kDa, apoLTP-II of 85kDa, and apoLTP-III of 55 kDa (Ryan et al., 1988).

LTP helps not only interconverting lipophorin subspecies, but also helps transferring DG from the midgut to lipophorin particles, and from lipophorin to ovarioles (Canavoso and Wells, 2001; Jouni et al., 2003). As it was proved that an LTP antibody can block the net transfer of DG from the midgut to lipophorin and to the ovarioles. LTP is also capable of transferring hydrocarbon molecules, carotenoids, and phospholipids among lipoproteins, but much slower than transferring DG (Golodne et al., 2001; Takeuchi and Chino, 1993; Tsuchida et al., 1998).

Interestingly, the lipid transfer particle, which is unique to insects (Kanost et al., 2016), is also a member of the apoB - large lipid transfer protein family (Yokoyama et al., 2013).

9.2 Vitellogenin

Another protein that transports lipids to the ovaries during oocyte development is vitellogenin (Vg), although its contribution is only 5% of the total amount of lipids stored (Sun et al., 2000). Vitellogenin is the most abundant yolk protein precursor in all insect species. Similar to lipophorin, it is synthesized in the fat body, then secreted to the hemolymph and taken up by the ovaries. Although the primary function of vitellogenin is to provide a pool of amino acids for the embryo, it also functions as a carrier of carbohydrates, lipids, phosphates, vitamins, metals, and hormones (Sappington & Raikhel, 1998). Vitellogenin only carries around 10% of its molecular mass in the form of lipids, mainly phospholipids and glycerolipids (Van der Horst and Ryan, 2012). It is internalized to the oocytes by interacting with receptors from the LDLR family independent of and different from lipophorin receptors (Sappington et al., 1996). *A. aegypti* oocyte size increases more than 300-fold within 48 hours of a blood meal, mainly because of the specific accumulation of the major yolk protein precursor vitellogenin. This biological feature depends on the proper interaction of vitellogenin with its receptor on the oocyte surface. In

addition to its exceptional value as a model for studying receptor-mediated endocytosis, this system is also a promising target for future novel control strategies (Sappington et al., 1996).

A. aegypti vitellogenin is composed of two subunits, a large subunit of ~200kDa and a small subunit of ~66kDa. Both subunits are glycosylated and phosphorylated and derive from a common high molecular weight precursor (Dhadialla and Raikhel, 1990). Insect vitellogenins are cleaved once in the fat body to produce both subunits. The cleavage sites are immediately preceded by a motif or a consensus sequence, (R/K)-X-(R/K)-R or R-X-X-R, specifically recognized by the subtilisin-like proprotein endoproteases, convertases (Sappington & Raikhel, 1998).

Transcription of the vitellogenin gene is highly upregulated after amino acid transporters located in the fat body plasma membrane sense the present of amino acids in the hemolymph. This signal then activates target of rapamycin (TOR) pathway that stimulate a specific transcriptional activator-induced synthesis of vitellogenin that is highly abundant around 30 hours after blood feeding (Park et al., 2006). Rapamycin treatment leads to a disaggregation of polysomes in *S. cerevisiae*. However, in mammalian cells rapamycin inhibits the translation of certain classes of mRNAs. The regulation of these mRNAs seems to be mediated by TOR, which modulates translation via the regulation of the phosphorylation state of several different translation effector proteins (Raught et al., 2001).

10. RNA interference

Double-stranded RNA, or dsRNA, triggers a post-transcriptional gene silencing mechanism that induces degradation of specific mRNA containing sequences that are complementary to the dsRNA (Mello and Conte, 2004). This mechanism has been termed RNA interference or RNAi, and it is thought to be an active response against viral infection and mobilized transposable elements, as well as playing a role in translational suppression (Ding, 2010). RNAi also refers to

a sequence-specific silencing of target genes by reducing transcription, destabilizing mRNAs, or inhibiting protein translation. Since its discovery in *Caenorhabditis elegans* (Fire et al., 1998), RNAi has been successfully applied several insect orders, including Hymenoptera, Lepidoptera, Coleoptera, and Diptera (Yu et al., 2013).

RNA interference specificity is determined by small RNAs and their complementarity to the target mRNA (Nandety et al., 2015). There are at least three small RNA classes playing a role in RNAi: small interfering RNAs or siRNAs, microRNAs or miRNAs, and Piwi-interacting RNAs or piRNAs. These 3 types of RNA are generated by independent biogenesis pathways (Siomi and Siomi, 2009). The sizes of siRNAs and miRNAs vary between 19-24 nucleotides in length and are generated from longer dsRNAs by the action of ribonuclease III type Dicer enzymes. While piRNAs are 24-30 nucleotides in length and are generated in a Dicer-independent manner. However, the 3 classes of RNAs interact with Argonaute proteins to mediate the RNA interference mechanism, which includes target RNA cleavage, translational repression, RNA destabilization or epigenetic modifications (Siomi and Siomi, 2009).

Post-transcriptional gene silencing is triggered by a long dsRNA, a short hairpin RNA, a short dsRNA, or a short single-stranded siRNA. Whereas in nematodes and flies, long dsRNA is effectively used to induce gene silencing, in most mammalian cells, long dsRNA activates the interferon response and cell death. Therefore, short hairpin RNAs or single stranded siRNAs are used as triggers of RNAi in mammalian systems (Agrawal et al., 2003). Long dsRNAs and hairpin RNAs are processed by the RNaseIII enzyme Dicer into short double-stranded siRNAs. These siRNAs are incorporated into Argonaute proteins, which are part of the multi-protein complex known as RISC or RNA Induced Silencing Complex. Only one of the strands of the siRNA remains bound to Argonaute, called guide strand, while the other is degraded. RISC complex, with the help of the guide strand, will locate and cleave mRNAs whose sequences are complementary to the guide strand. In general, exogenous dsRNA triggers the degradation of

target RNA by the Argonaute protein, through base pairing between the siRNA and mRNA (Nandety et al., 2015) (Figure 5).

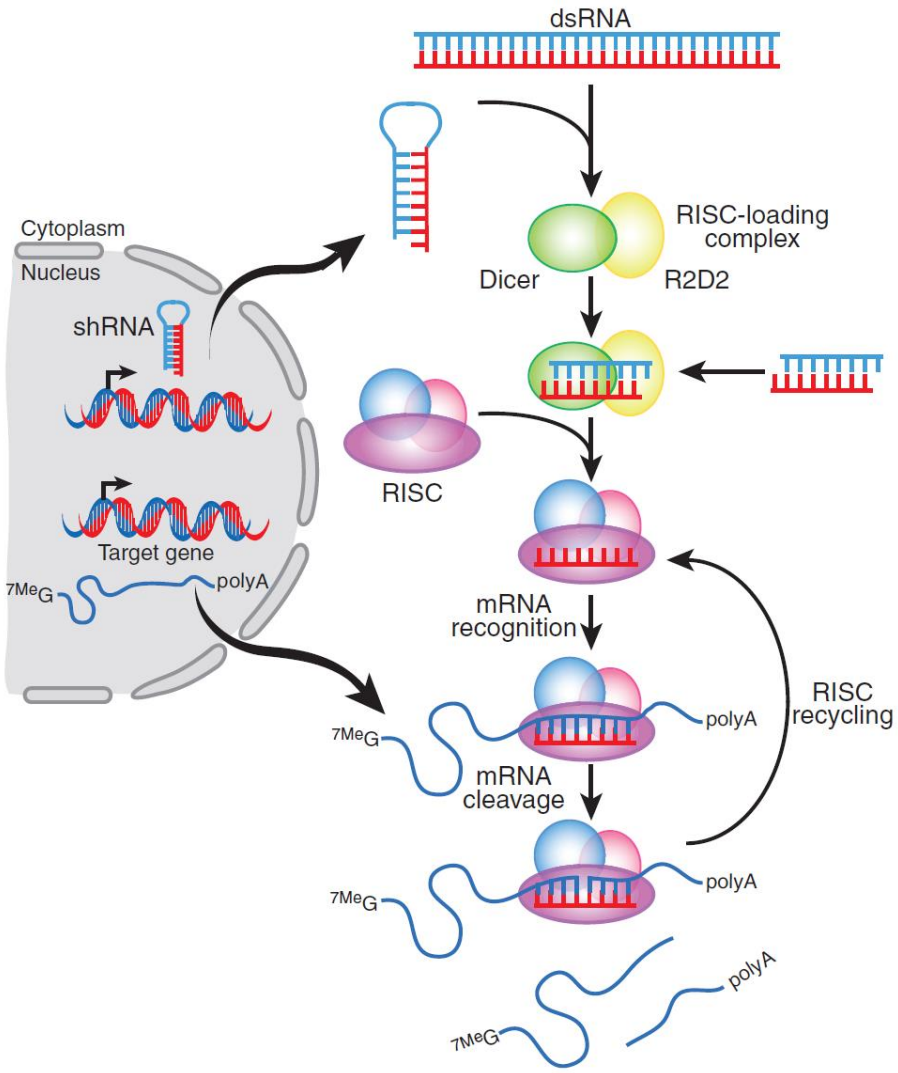

Fig. 5. Gene silencing mechanism by RNA interference. Long double stranded RNA (dsRNA) and short hairpin RNA (shRNA) are processed in the cytoplasm by Dicer in concert with the dsRNA-binding protein R2D2 into small interfering RNA (siRNA). The resulting siRNAs are taken up by the RNA-induced silencing complex (RISC). The duplexed siRNA is unwound, and the passenger strand dissociates. The antisense RNA strand guides RISC to the complementary site in the target mRNA, which engages the endonucleolytic activity of argonaute (Ago2), resulting in mRNA cleavage. (Dominska & Dykxhoorn, 2010).

The successful use of RNA interference in insects depends on several parameters including: the types of RNAi inducers to be used (siRNAs, piRNAs or miRNAs), dose dependency of the RNAi

inducers, and their mode of delivery. Other factors that must be considered include size of the RNAi inducer (short vs long), location of the target match (5' end, 3' end, or in the central region of the target), and their ability to spread inside insects (Nandety et al., 2015).

At least 5 different methods have been used to deliver RNAi inducers to insects. Microinjection has been widely used for RNAi studies in whole insects, because it can ensure the RNAi inducers are delivered to the tissue of choice, or into the hemolymph. Furthermore, when specific volumes of known concentration are injected, the dosages used can be accurately compared, and different RNAi inducers can be mixed and simultaneously injected (Liu, Ding, Zhang, Yang, & Liu, 2010). In addition to microinjection, other methods like soaking, spraying, and electroporation have been used to deliver RNAi inducers. Like microinjection, these are excellent approaches for fundamental RNAi studies, but not for practical applications like pest control (Nandety et al., 2015). Oral delivery of RNAi inducers has been successful in many, but not all insects studied so far. A variety of approaches have been attempted including adding RNAi inducers to artificial diets, baits, and in some examples for plant-feeding insects, the plant has been used to deliver the RNAi inducers. Oral delivery of RNAi inducers is not as straightforward as is injection because inconsistent doses of RNAi inducers may be taken up by individual insects, frequency of feeding can be variable, and stability of the RNAi inducers in the oral delivery medium might be problematic (Christiaens et al., 2014).

CHAPTER II

METHODS

1. Lipid quantification from fat body, hemolymph and ovaries of *Aedes aegypti* female mosquitoes after blood feeding.

1.1 Rearing of insects

Aedes aegypti Liverpool strain (LIV-1B12, MRA-735) eggs were placed to hatch in groups of approximately 100 in plastic trays with 500ml of distilled water under low oxygen conditions. Their daily diet consisted in 0.1g of 1:1 yeast/albumin powder and 0.25g of Tetramin (Tetra-Werke, Germany). Plastic trays with larvae were kept at 27º C with 14 hours of light and 10 hours of darkness. Pupae were removed daily from trays and transferred to plastic cages for molting, where adult mosquitoes with no more than 24 hours of difference since molting were grouped in batches for the experiments. Adult mosquitoes were maintained with no food during the first day after molting, then they had free access to 10% sucrose solution for the next 2 days. Only 2 to 3-day-old sucrose fed females were used for this part of the study.

1.2 Blood feeding

Four to five-day-old female mosquitoes with 48 hours of starvation were offered a blood meal with defibrinated sheep's blood (Hemostat Laboratory, California, USA) for a maximum period of 2 hours. Three milliliters of blood were placed inside a glass feeder covered with Parafilm-M® sealing membrane (San Diego, CA) and heated at 37º C with a water bath circulator connected to the feeder. Blood fed and starved female mosquitoes were used for the experiments.

1.3 Insect dissection and sample collection

Samples for lipid quantification were obtained from starved and blood fed mosquitoes during the first 72 hours after blood feeding, following the designed time course for the experiment. Samples were collected at 48 hours of starvation as reference point, then 2, 4, 6, 12, 24, 36, 48, 60 and 72h post blood meal (PBM) to evaluate the effect of blood feeding. 10 mosquitoes per time point were dissected under a stereoscope. Photographs of ovaries were taken at 30x magnification with a digital 12 MP camera. For lipid quantification, fat body and ovaries were collected in 10µL per insect of phosphate-buffered saline solution (PBS) containing 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, with additional 0.25M sucrose, and pH 7.4 in sterile 1.5mL eppendorf tubes. Hemolymph was collected from the thorax by centrifugation. Thoraxes from 5 insects were placed inside a sterile 10µL barrier sterile tip (on top of the barrier) and centrifuged inside a 1.5mL eppendorf tube for 5 minutes at 2,500 rcf using a 0.5mL tube as a tip holder. The barrier of the tip was then rinsed with 10µL of PBS buffer containing 0.25M of sucrose to elute any remaining hemolymph.

1.4 Sample homogenization and processing

Fat body and ovaries from insects were pooled and homogenized on ice, using plastic pestles with conical tip. Homogenate was centrifuged for 20 minutes at 18,000 rpm and 4^o C. Soluble fraction and fat layer were collected and divided into aliquots equivalent to one insect per 15µL of homogenate for lipid quantification. Hemolymph samples were centrifuged at 14,000 rpm and 4^o C to eliminate hemocytes. Soluble fraction was transferred to a clean 1.5mL eppendorf tube. Samples were stored at -20^o C until lipid quantification.

1.5 Lipid quantification

Glyceride content was measured using Wako Diagnostics L-type Triglyceride kit, following manufacturer's instructions. PBS buffer with 0.05% v/v reduced triton was used as blank.

Different concentrations of triolein in PBS buffer with reduced triton were used as standards to prepare the calibration curve. Glyceride content was estimated as nmol of glycerides per mosquito. Fat body and ovary samples were measured per duplicate. Hemolymph from 5 insects was pooled and used for a single measurement. Three independent experiments were carried out for data collection.

2. Apolipophorin-I detection in fat body, hemolymph and ovaries of *Aedes aegypti* female mosquitoes after blood feeding

2.1 Rearing of insects

Same procedure as described previously in Methods, section 1.1.

2.2 Blood feeding

Same procedure as described in Methods, section 1.2

2.3 Insect dissection and sample collection

Samples for SDS-PAGE and western blot analyses were obtained from starved and blood fed mosquitoes during the first 72 hours after blood feeding, following the designed time course for the experiment. Samples were collected at 48 hours of starvation as reference point, then 2, 4, 6, 12, 24, 36, 48, 60 and 72h PBM to evaluate the effect of blood feeding. 10 mosquitoes per time point were dissected under a stereoscope. For SDS-PAGE and Western Blot analyses, fat body and ovaries were collected in 10 µL of homogenization buffer 50mM Tris, 0.25M sucrose, 10mM ethylenediamine tetra-acetic acid (EDTA), 0.1mM Benzamidine, 5mM dithiothreitol (DTT) and 5mM phenylmethylsulfonyl fluoride (PMSF) per insect, in sterile 1.5mL eppendorf tubes. Hemolymph was collected from the thorax by centrifugation. Thorax from 5 insects were placed inside a sterile 10µL barrier sterile tip (on top of the barrier) and centrifuged inside a 1.5mL collecting Eppendorf tube for 5 minutes at 2,500rcf using a 0.5 mL tube as a tip holder. The

collecting tube had 5µL of homogenization and the barrier of the tip was then rinsed with 5µL of the same buffer to elute any remaining hemolymph.

2.4 Sample homogenization and processing

Fat body and ovaries were pooled and homogenized on ice, using plastic pestles with conical tip. Homogenate was centrifuged for 20 minutes at 18,000 rpm and 4º C. Soluble fraction and fat layer were collected on a 1.5 mL Eppendorf tube, then an equal volume of Laemmli 2x sample buffer was added. Hemolymph samples were centrifuged at 14,000 rpm and 4º C to eliminate hemocytes. Soluble fraction was transferred to a clean 1.5 mL Eppendorf tube and an equal volume of Laemmli 2x sample buffer was added. Samples were boiled for one minute and then stored at -20º C until used.

2.5 SDS-PAGE and Western Blot

For fat body and hemolymph samples, a volume equivalent to 0.5 mosquito was loaded per well, on a 4 – 15% precast gradient acrylamide gel (Bio-Rad). Proteins were separated by SDS-PAGE during 100 minutes at a constant current of 90 volts, and then transferred to nitrocellulose membranes (Bio-Rad) for 30 minutes using a semi-dry Trans-Blot Turbo Transfer System (Bio-Rad). Nitrocellulose membranes were then stained with Ponceau S. dye to verify the transfer. Acrylamide gels were colored with Coomassie brilliant blue G-250 stain to corroborate the successful transfer of proteins to the membranes.

The membranes were blocked for 5 hours with 5% non-fat milk diluted in tris-buffered saline solution containing 0.1% Tween 20 (v/v) (TBST). Rabbit anti-apolipophorin-I from *Manduca sexta* was used as primary antibody in a 1:5,000 dilution. The identity of the protein detected by the antibody was confirmed by mass spectrometry (data not shown). Primary antibody incubation was performed overnight, under constant agitation at 4º C. After 15 minutes of washing with TBST, the membranes were incubated with Goat anti-rabbit IgG-HRP antibody (1:10,000) for 1.5

hours under constant agitation at room temperature. After 15 minutes of washing with TBST, secondary antibody binding reaction was developed with ECL chemiluminescence reagents (Amersham Pharmacia, Piscataway) exposing on X-ray films.

3. Apolipophorin mRNA levels in fat body and ovaries of *Aedes aegypti* female mosquitoes after blood feeding.

3.1 Rearing of insects

Same procedure as described previously in Methods, section 1.1.

3.2 Blood feeding

Same procedure as described previously in Methods, section 1.2.

3.3 Insect dissection and sample collection

Samples for RNA extraction were obtained from starved and blood fed mosquitoes during the first 72 hours after blood feeding, following the designed time course for the experiment. Samples were collected at 48 hours of starvation as reference point, then 2, 4, 6, 12, 24, 36, 48, 60 and 72h PBM to evaluate the effect of blood feeding on the expression of apolipophorin gene. 10 mosquitoes per time point were dissected under a stereoscope. For RNA extraction, fat body and ovaries were collected in 200µL of Trizol reagent in separate sterile, nuclease-free 1.5mL Eppendorf tubes.

3.4 RNA extraction and cDNA synthesis

Total RNA was extracted from fat body and ovary cells (4 insects per time point) using Direct-zol RNA MiniPrep kit (Zymo Research Corp., USA) following manufacturer's instructions. Extracted RNA concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). Subsequently, cDNA was synthesized from 1µg of total RNA using

iScript cDNA Synthesis Kit (Bio-Rad Laboratories Inc., California, USA) according to the manufacturer's directions.

3.5 Quantitative PCR

Relative mRNA levels of apolipophorin gene were measured against mRNA levels of Ribosomal protein RpL8 gene by real time PCR (qRT-PCR) with the previously synthesized cDNA samples from fat body and ovaries. CFX Connect ™ Real-Time PCR Detection System (Bio-Rad) and iQ™ SYBR Green Supermix (Bio-Rad) were used for the qPCR reaction. The primers used for the amplification reaction of the apolipophorin gene were: ApoLp-II/I F- Primer 5'CTG ACG AGT AAC TAC AAC AGC CAG CA3' and ApoLp-II/I R- Primer 5'CGG TGT ACG TCA GTT TGG TGG TGA3'. The primers used for the amplification reaction of RpL8 gene were: RpL8 F-Primer 5'CCC TCG GGT GCC AAG AAG G3' and RpL8 R-Primer 5'GGC CAG CAG TTA CGC TTC ACC T3'.

Thermocycler protocol had the following steps: 95° C for one minute, followed by 45 cycles of 95° C for 5 seconds and 60° C for 45 seconds. Melting curve of PCR products confirmed that only one product was amplified on each reaction. Relative abundance of apolipophorin mRNA was evaluated with three technical replicates. ($2^{-\Delta Cq}$) method was used to calculate relative mRNA levels of target gene normalizing against RpL8 mRNA.

4. Apolipophorin silencing by RNA interference

4.1 Rearing of insects

Same procedure as described previously in Methods, section 1.1.

4.2 Double stranded RNA synthesis

The target for RNA interference was apolipophorin gene from *Aedes aegypti*. Double stranded RNA was used as the RNA interference inducer, and it was synthesized through Reverse-

Transcription Polymerase Chain Reaction (RT-PCR) from a cDNA template of *Aedes aegypti* containing T7 promoters. Luciferase gene from the firefly *Photinus pyralis* was used as an exogenous injection control. Double stranded RNA was synthesized as described in Lu et al. (2006). Primers for dsRNA synthesis can be found in Table 1.

For the first PCR reaction, High Fidelity Taq polymerase (Invitrogen) was used, with the following thermocycler program: 94° C for one minute, followed by 30 cycles of 94° C 30 seconds, 65° C for 30 seconds, and 68° C 30 seconds. 2.5ng of the first PCR product were used as a template for the second reaction, using primers with the T7 RNA polymerase promoter sequence (5'TAA TAC GAC TCA CTA TAG GG 3') added to the 5' end of each primer. Performing the amplification in two steps increases the yield of PCR products bearing complete T7 sites.

In vitro dsRNA synthesis was performed using the MEGAscript RNAi Kit (Ambion, Austin, TX) in an overnight reaction at 37° C using 2µg of the purified PCR product of the second reaction. The resulting dsRNA product was validated by 1.5% agarose gel electrophoresis and then purified using MEGA-clear purification column (Ambion). The dsRNA was precipitated using 300mM sodium acetate, pH 5.5, washed with sterile 75% ethanol, and resuspended in nuclease-free water at 2µg/µl.

4.3 dsRNA injections and blood feeding

Sugar fed 2 to 3-day-old females were cold-anesthetized and injected in the right mesothoracic spiracle with 0.3 - 0.5µg of dsRNA, either with ApoLp-II/I dsRNA as the target, or Luc dsRNA as the control, delivered in 138nL of aqueous dsRNA solution. Injections were carried out using a Nanoject II microinjector (Drummond Scientific Company, Broomall, PA) equipped with a capillary glass needle. RNAi-treated and control mosquitoes were kept in separate plastic cages with 10% sucrose solution for 36 hours after the injection, then left in starvation for 36 hours and

finally fed with defibrinated sheep's blood for no more than 2 hours as previously described. No sucrose or any other diet was offered after the blood meal.

4.4 Insect dissection and sample collection

Samples were obtained from blood-fed injected and control mosquitoes at 12, 24, 36, 48 and 72h PBM to evaluate the effect of dsRNA injection on the expression of apolipophorin gene. Ten injected females per time point were dissected from RNAi-treated group and control group. Photographs of ovaries were taken at 30x magnification with a digital 12 MP camera. Fat body from abdominal carcass and ovaries were collected in 3 different solutions. For RNA extraction, fat body and ovaries were collected in 200µL of TRIzol reagent in sterile, nuclease-free 1.5mL Eppendorf tubes. For lipid and protein quantification, fat body and ovaries were collected in PBS buffer containing 0.25M sucrose (10µL per insect) in sterile 1.5mL eppendorf tubes. For lipid analysis the hemolymph was collected from the thorax by centrifugation. Thorax from 5 insects were placed inside a sterile 10µL barrier tip (on top of the barrier) and centrifuged inside a 1.5 mL eppendorf tube for 5 minutes at 2,500 rcf. The barrier of the tip was then rinsed with 10µL of PBS buffer containing 0.25M of sucrose to elute any remaining hemolymph. For SDS-PAGE and Western Blot analyses, fat body and ovaries were collected in 10µL per insect of homogenization buffer (50mM Tris, 0.25M sucrose, 10mM EDTA, 0.1mM Benzamidine, 5mM dithiothreitol and 5mM PMSF) per insect, in sterile 1.5mL Eppendorf tubes. Hemolymph was collected as described above with some modifications. The collecting tube had 5µL of homogenization buffer and the barrier of the tip was then rinsed with 5µL of the same buffer to elute any remaining hemolymph.

4.5 RNA extraction and cDNA synthesis

Same procedure as described previously in Methods, section 3.4.

4.6 Quantitative PCR

Same procedure as described previously in Methods, section 3.5.

Table 1. Primers used for dsRNA synthesis and qPCR reactions

Primer	Sequence (5' to 3')	TM (°C)	Amplicon size
ApoLp dsRNA-F	CAG CCA GAA CAA TGT GGG TAA GCT	67.5	150bp
ApoLp dsRNA-R	GAC CTT ACG TGC GAG CAA CTT GTT C	66	
ApoLp qPCR-F	CTG ACG AGT AAC TAC AAC AGC CAG CA	66.2	144bp
ApoLp qPCR-R	CGG TGT ACG TCA GTT TGG TGG TGA	66.3	
Vg qPCR-F	CTG AAC CCA GAA GAC GTC AGC ATT CA	66.2	152bp
Vg qPCR-R	TTG GCG CAG ATG ATA GAA CAG TCC AC	66.2	
LTP qPCR-F	GCT GTA GTA AAC CTG ATG ACG CCA ATG G	67.5	152bp
LTP qPCR-R	GTC TTC ACT CTC AAA CGA AAC AGC TAT CG	66	

(TM) melting temperature, (ApoLp) apolipophorin, (dsRNA) double stranded ribonucleic acid, (qPCR) quantitative polymerase chain reaction, (Vg) vitellogenin, (LTP) lipid transfer particle.

4.7 SDS PAGE and Western blot

Same procedure as described previously in Methods, section 2.5.

4.8 Lipid quantification

Same procedure as described previously in Methods, section 1.5.

4.9 Protein quantification

The protein concentration of fat body and ovary homogenates was measured by UV-absorption spectroscopy in the presence of 10% SDS. For this purpose, we used the difference between the second derivative values of the absorbance at 288 nm and 291 nm. This value was applied to a modified Beer-Lambert equation along with a molar extinction coefficient (Ɛ'') of 0.017 and the

dilution factor for the total reaction volume. A volume equivalent to 1 mosquito was used for each reading. Two technical replicates of each sample were measured. Data from three independent experiments was collected.

CHAPTER III

RESULTS AND DISCUSION

1. Changes in lipid content in fat body, hemolymph and ovaries of *Aedes aegypti* after blood feeding:

The purpose of this study was to determine the normal values and patterns of change of the glyceride content of fat body, hemolymph and ovaries that take place after a blood meal. We define as normal the lab experimental conditions described in methods, and the use of 2 to 3-day old sugar-fed adult female mosquitoes as starting point. We start the experiments by subjecting these animals to a 48h period of starvation. The mosquitoes are then offered a blood meal and kept without food for the rest of the experiment. For each time point, 10 insects were dissected to obtain ovaries and fat body tissue (abdominal carcass) and collect hemolymph from thorax.

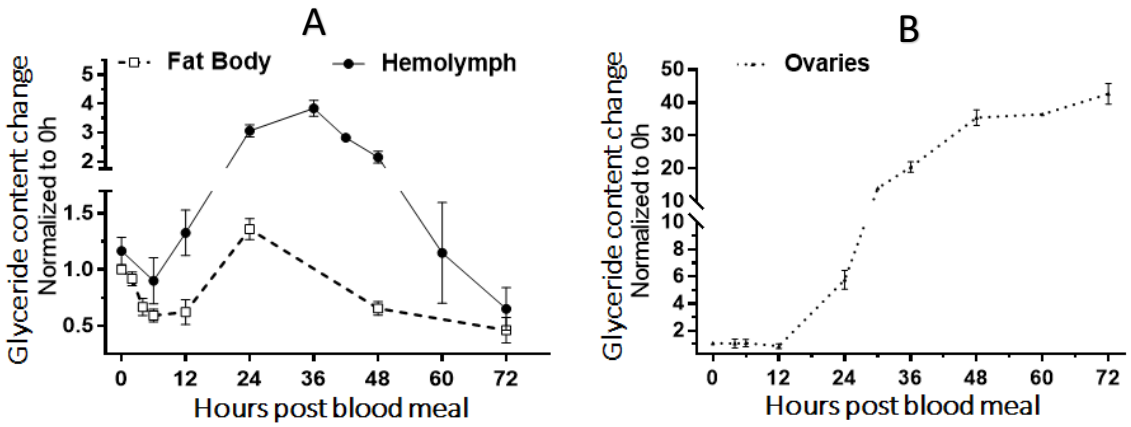

Fig. 6. Changes in lipid content in fat body, hemolymph and ovaries of Aedes aegypti after blood feeding. **A)** Average glyceride content change ±SD in fat body and hemolymph after a blood meal normalized to 0h. **B)** Average glyceride content change ±SD in ovaries after a blood meal normalized to 0h. Data from 3 independent experiments.

In *Aedes aegypti* the gonotrophic cycle is triggered by the blood meal. The mobilization of lipids from the fat body to the ovaries is carried out mainly via lipophorin particles through the hemolymph, although vitellogenin can also carry a small amount (Sun et al., 2000). The temporal variation of the glyceride content in fat body, hemolymph and ovaries after a blood meal was monitored to characterize the process under our experimental conditions. This information was needed for subsequent studies. The results from three independent experiments are shown in Figure 6. The "0h" time represents the time following the 48h of starvation period and marks the beginning of data collection.

As shown on the Figure 6A, the fat body accumulates lipids during the first 24 hours post blood meal (PBM) and at this time, a maximum in the glyceride content is observed. After the 24h PBM the fat body lipid content decreases and a concomitant increase in the glyceride content of the hemolymph and ovaries is observed. The lipid content in hemolymph reaches a maximum concentration around 36 hours PBM and then decreases to a basal level. On the other hand, the lipid content of the ovaries reaches a maximum between 48 and 72 hours PBM (Figure 6-B). Consistently with the change in lipid content, following the blood meal the ovaries also show a progressive increase in size (Figure 7).

2. Lipophorin synthesis in fat body cells of *Aedes aegypti* after blood feeding

To characterize further the role of lipophorin in the vitellogenic process we also determined the changes in mRNA levels of apolipophorin gene that are triggered by the blood meal. The mRNA levels of apolipophorin-II/I gene in fat body cells were determined by qPCR as described in Methods, section 3.5. Representative results from 3 independent experiments are presented (Figure 8).

As previously stated, lipophorin apoproteins are synthetized mainly in the fat body cells and then secreted to the hemolymph in the form of lipid-loaded high density lipophorin. A blood meal

triggers the transcription of the ApoLp-II/I gene, rapidly increasing the level of apolipohorin mRNA, which at 24 hours PBM reaches values 20-fold higher than those observed in the sugar-fed state. Afterwards, the apolipophorin mRNA levels rapidly decrease until reaching basal levels around 60 to 72 hours PBM (Figure 8). This phenomenon corresponds to the vitellogenic state of the ovaries, which are accumulating lipids for egg development.

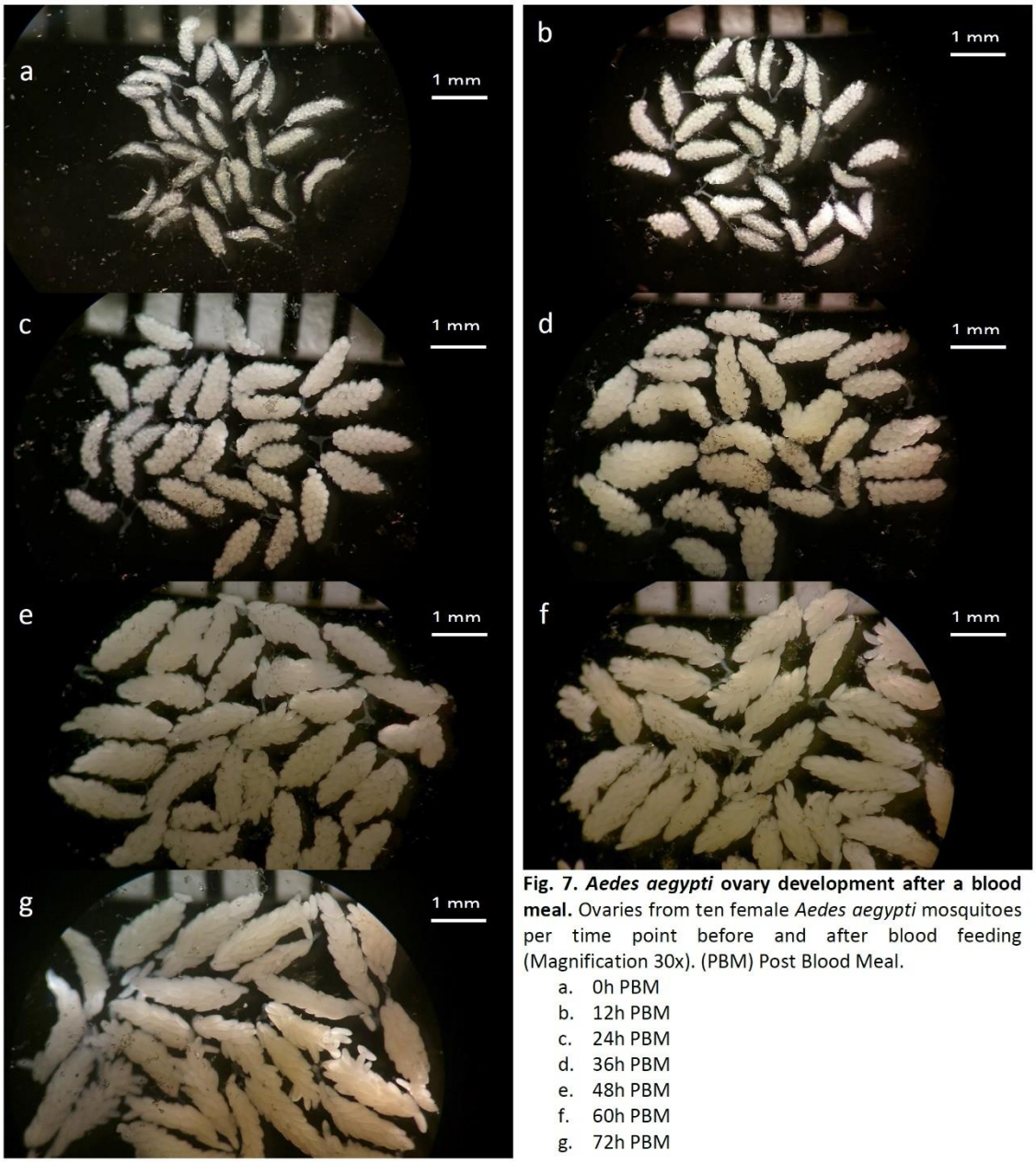

Fig. 7. *Aedes aegypti* ovary development after a blood meal. Ovaries from ten female *Aedes aegypti* mosquitoes per time point before and after blood feeding (Magnification 30x). (PBM) Post Blood Meal.

a. 0h PBM
b. 12h PBM
c. 24h PBM
d. 36h PBM
e. 48h PBM
f. 60h PBM
g. 72h PBM

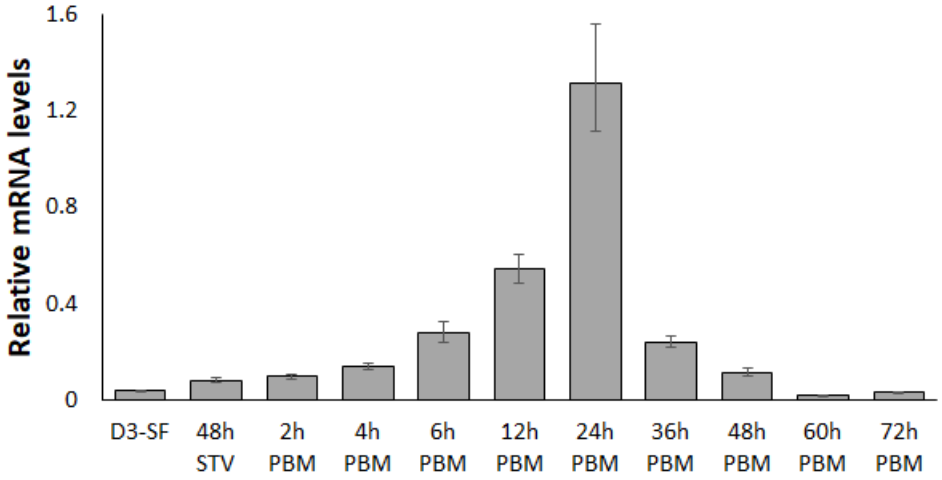

Fig. 8. Apolipophorin-II/I mRNA levels in fat body after blood feeding. ApoLp-II/I mRNA levels were determined by qRT-PCR using ribosomal protein RpL8 mRNA as reference. Values for each time point are plotted as mean ± SD (n=3). Day-3 sugar fed (D3-SF), starvation (STV), Post blood meal (PBM).

3. Lipophorin synthesis in ovaries of *Aedes aegypti* after blood feeding

Expression of ApoLp-II/I gene in the ovaries was also evaluated. The highest level of mRNA in ovaries was detected at 24 hours PBM, and it is only 1% of the lowest level of mRNA detected in the fat body, which corresponds to the sugar fed state (Figure 9). These results corroborate that apolipophorin proteins are synthesized mainly in the fat body cells.

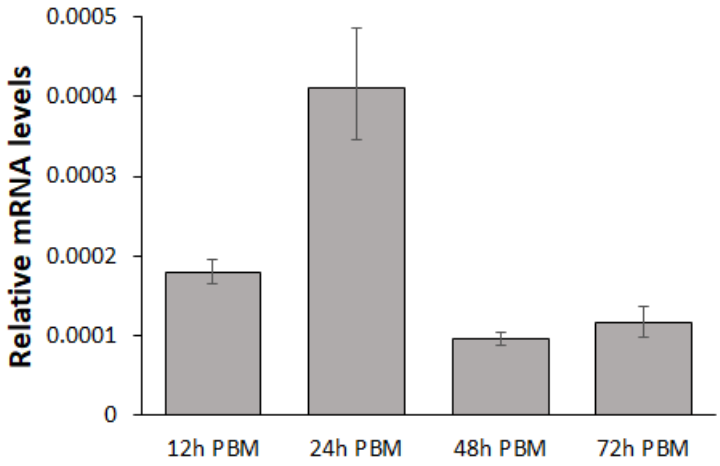

Fig. 9. Apolipophorin-II/I mRNA levels in ovaries after blood feeding. ApoLp-II/I mRNA levels in ovaries were determined by qRT-PCR using ribosomal protein RpL8 mRNA as reference. Values for each time point are plotted as mean ± SD (n=3). Post blood meal (PBM).

4. Abundance of apolipophorin-I protein in fat body, hemolymph and ovaries after a blood meal.

The abundance of apolipophorin circulating protein in fat body, hemolymph and ovaries was determined by western blot analyses as described in Methods, section 2.5. Representative results from 3 independent experiments are presented (Figures 10, 11 and 12)

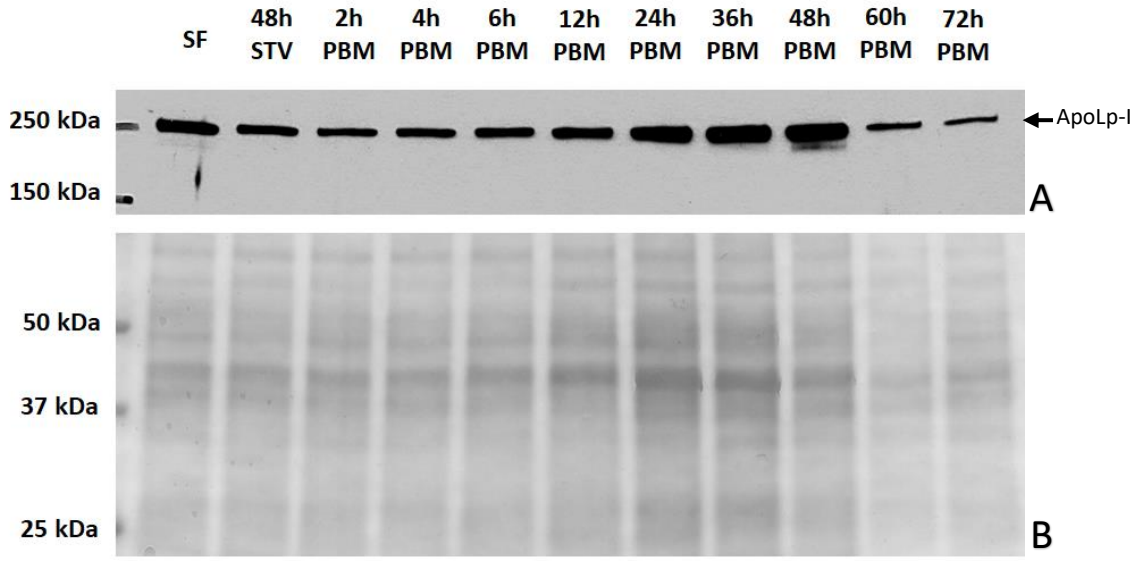

Fig. 10. Apolipophorin-I in fat body of *Aedes aegypti* before and after blood feeding. A) Western blot: Apolipophorin-I (ApoLp-I) is detected as a band around 250 kDa. **B)** Ponceau S staining of the membrane used in western blot. Each lane was loaded with the equivalent to 0.5 mosquito. Sugar fed (SF), Starvation (STV), Post blood meal (PBM).

The amount of apolipophorin-I detected by western blot in fat body homogenate shows how the protein starts increasing as early as 4 hours PBM. ApoLp-I reaches a maximum between 24 and 48 hours PBM and then decreases to basal levels at 60-72h PBM, at which time the vitellogenesis is nearly complete (Figure 10). These results show a good correlation with the changes in mRNA levels (Figure 8). Although it appears that the transcript, after peaking at 24h PBM, disappears sooner than the protein.

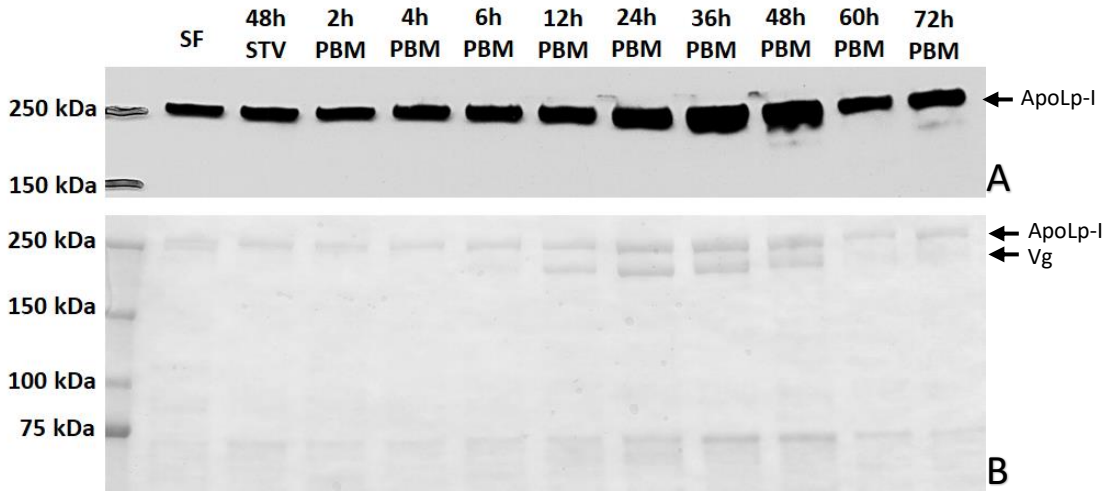

Fig. 11. Apolipophorin-I in hemolymph of *Aedes aegypti* before and after blood feeding. A) Western blot: Apolipophorin-I (ApoLp-I) is detected as a band around 250 kDa. **B)** Ponceau S staining of the membrane used in western blot. Apolipophorin-I (ApoLp-I) and Vitellogenin large subunit (Vg) bands can be observed. Each lane was loaded with the equivalent to 0.5 mosquito. *Sugar fed (SF), Starvation (STV), Post blood meal (PBM).*

As in the study of apolipophorin content in fat body, the levels of apolipophorin-I in hemolymph samples were determined by Western blotting. Each gel lane was loaded with an amount of hemolymph equivalent to that extracted from 0.5 mosquitoes. Apolipophorin-I was detected as a band at the same position as with the fat body samples (around 250 kDa). Lipophorin is a highly abundant protein in insect hemolymph. The pattern of changes in apolipophorin levels in hemolymph was very similar to the overall pattern of temporal changes observed in fat body. A clear increase in the level of hemolymph apolipophorin is observed as early as 4 h PBM. The maximum concentration of ApoLp-I is found between 36h and 48h PBM. This peak is followed by a sharp decrease in apolipophorin content in the following 12h and thereafter (60-72h PBM) (Figure 11). These results correlate well with the relative glyceride changes found in hemolymph (Figure 6-A), where the maximum concentration of glycerides was found at 36 hours PBM, decreasing gradually afterwards, until 72 hours PBM. These results also correlate with the lipophorin production in fat body, which precedes the release of the lipophorin to the hemolymph. According to these results, the major lipid mobilization takes place between 12 and 48 hours post blood meal.

Another important aspect to mention from the hemolymph analysis is the presence of vitellogenin (Vg) observed between 12 and 48 hours post blood meal. The large subunit of Vg appears as a band of ~200 kDa, which can be clearly observed in the Ponceau staining of the nitrocellulose membrane (Figure 11). The molecular weight of the band matches the weight reported in previous studies (Dhadialla and Raikhel, 1990) and the protein identity was confirmed with mass spectrometry (data not shown). Vitellogenin is the main yolk protein precursor and it is synthesized in the fat body after a blood meal to support oocyte development (Dhadialla and Raikhel, 1990). It is transported through the hemolymph to the developing ovaries along with lipophorin and other minor yolk protein precursors. Vitellogenin can also act as a lipoprotein and carry around 10% of its mass in the form of lipids (Kawooya and Law, 1988). Vitellogenin contains a conserved lipid binding domain near the N-terminal region and it is considered an ancestral lipoprotein. According to phylogenetic studies it has been shown that vitellogenin gave rise to lipophorin from insects and apolipoprotein B from vertebrates (Babin et al., 1999).

Considering the large increases in ovary size that take place after the blood meal because of the protein and lipid uptake, a different gel loading scheme was needed to avoid membrane saturation and to obtain sharp bands. Thus, the equivalent to one ovary per lane was loaded for the first seven lanes (sugar-fed to 24 hours PBM), the equivalent of 0.5 ovary was loaded for 36 and 48 hours post blood meal, and 0.25 ovary was loaded for 60 and 72 hours post blood meal. The amount of apolipophorin-I observed revealed an increasing concentration from 4 to 24 hours PBM, then a steady amount between 24 and 48 hours PBM, and a decrease to nearly undetectable from 60 to 72 hours PBM (Figure 12). These results correlate with the massive uptake of lipids and proteins of the growing ovaries during the first 48 h after blood feeding and the amount of lipids observed in the previous experiment (Figure 6A). This also suggests that lipids are being transported to the ovaries by lipophorin particles synthesized in the fat body and transported via the hemolymph.

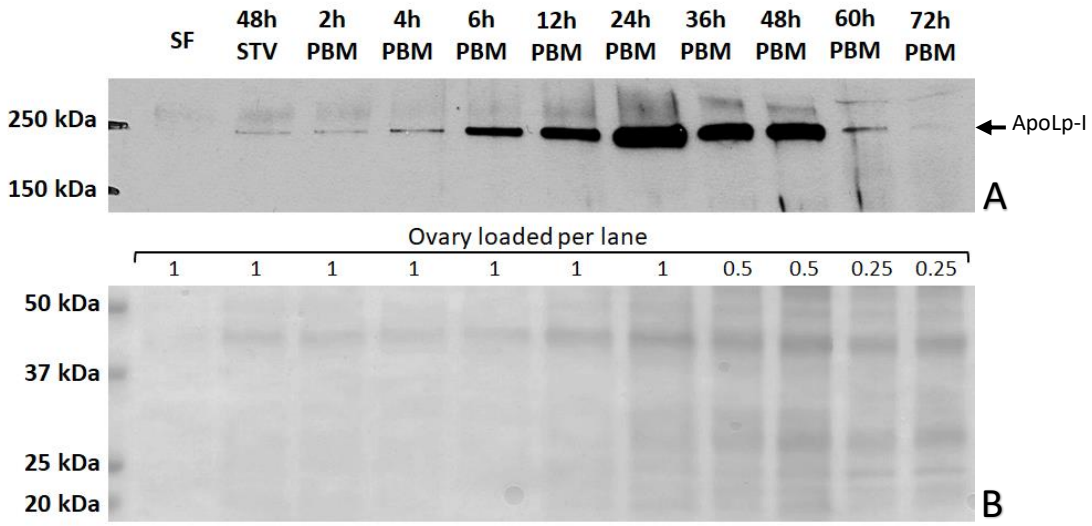

Fig. 12. Apolipophorin-I in ovaries of *Aedes aegypti* before and after blood feeding. A) Western blot: Apolipophorin-I (ApoLp-I) is detected as a band around 250 kDa. **B)** Ponceau S staining of the membrane used in western blot. *Sugar fed (SF), Starvation (STV), Post blood meal (PBM).*

5. Apolipophorin gene silencing by RNA interference

Considering that lipids constitute around 35% of the dry weight of the ovaries, we evaluated the effect of lipophorin knockdown on the transport of lipids from the fat body to the ovaries. The main goal of this study was to assess if dsRNA could be used as an RNA interference inducer to silence the apolipophorin gene and affect the lipid transport in *Aedes aegypti*. For this purpose, 3-day-old sugar fed females were injected with dsRNA targeting ApoLp-II/I, or dsRNA targeting the exogenous Luciferase gene from *Photynus pyralis* as negative control.

To be able to determine the success of the dsRNA injections, fat body samples from abdominal carcass were collected from dsRNA injected females 24 and 48 h PBM (96 and 120 hours post injection respectively). Total RNA was extracted, and cDNA was prepared for quantitative PCR analysis. Details of these procedures can be found in the previous section.

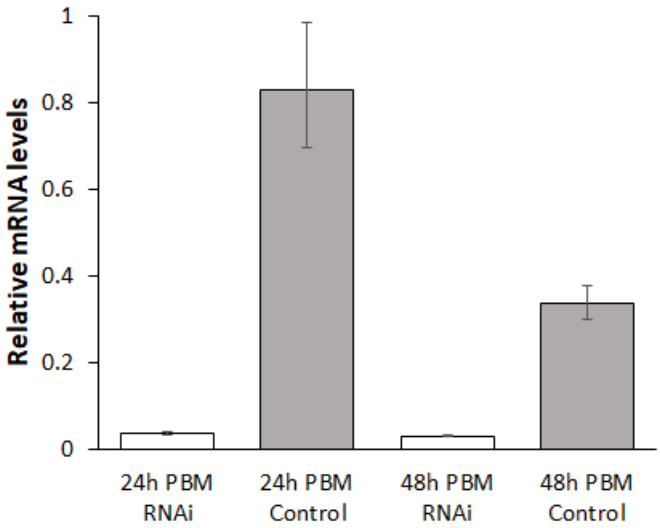

Fig. 13. Apolipophorin-II/I mRNA levels in fat body of RNAi-treated (RNAi) and control mosquitoes post blood meal (PBM). mRNA levels were determined by qRT-PCR using ribosomal protein RpL8 mRNA as reference. Values for each time point are plotted as mean ± SD (n=3).

RNA interference mechanism works at post-transcriptional level by destroying the messenger RNA it is targeting, preventing its translation and the synthesis of new protein. After dsRNA injections, the level of messenger RNA (cDNA) coding for apolipophorin proteins decreased significantly (Figure 13). The most noticeable effect was seen at 24 hours post blood meal, where ApoLp-II/I messenger RNA levels decreased 20-fold in ApoLp-II/I dsRNA injected mosquitoes compared to the control (Luc dsRNA injected mosquitoes). Values were adjusted to mRNA levels from the ribosomal protein RpL8 which was used as housekeeping or referencegene. At 48h PBM, the levels of messenger RNA decreased 10-fold in ApoLp-II/I dsRNA injected mosquitoes compared to the control. This confirms the successful silencing of apolipophorin gene using dsRNA as RNAi inducer.

The abundance of apolipophorin-I in fat body, hemolymph and ovaries was determined. Following the same protocol used in previous experiments, 3-day-old sugar fed females were injected with dsRNA targeting ApoLp-II/I, or dsRNA targeting Luciferase gene from *Photynus pyralis* as exogenous non-specific control. Hemolymph, fat body and ovary samples were

collected and processed for SDS-PAGE and western blot as described in the methods section. Representative results from 4 independent experiments are shown in the Figures 14 and 15.

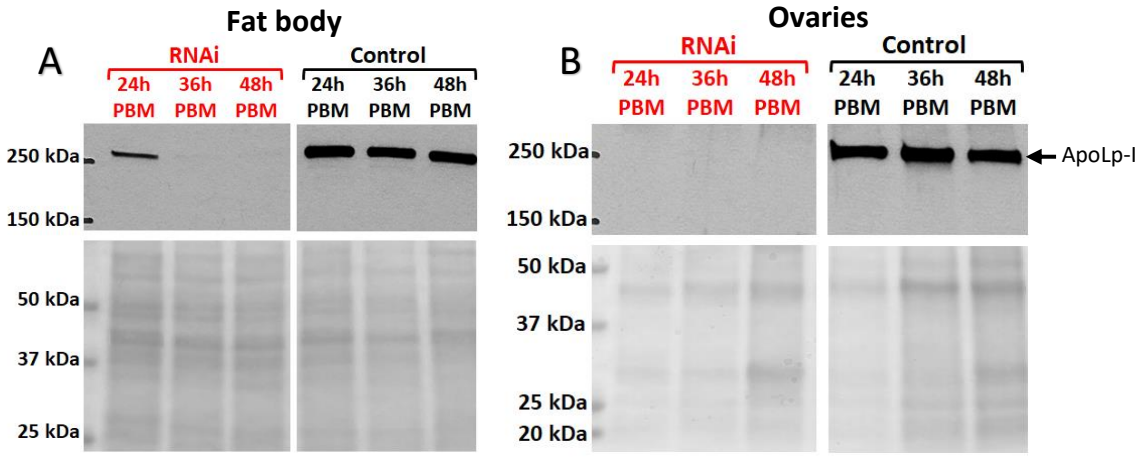

Fig. 14. Apolipophorin-I in fat body and ovaries of RNAi-treated (RNAi) and control mosquitoes post blood meal (PBM). Western blot analysis of **(A)** fat body and **(B)** ovaries from RNAi-treated and control insects. **Top panels:** Apolipophorin-I is detected as a band around 250 kDa. **Bottom panels:** Ponceau S staining of the membranes used in western blot. Samples were loaded as 0.5 mosquito per lane.

Western blot analysis revealed a marked decrease of apolipophorin-I in both tissues, correlating with the reduction of the corresponding mRNA and confirming the successful apolipophorin gene silencing. At 24 h PBM (96 hours post injection), the abundance of apolipophorin-I protein in the fat body of RNAi treated insects was 10-fold lower than the level observed in the non-specific RNAi control (Figure 14A). This weak band observed was probably the remnant of protein pre-existing before the dsRNA injection and its silencing effect took place. Whereas at 36 and 48 h PBM the silencing effect was almost 100% effective, and the protein was almost undetectable. No apolipophorin-I band was detected in the ovary samples, which correlates with the downregulation of the protein synthesis observed in the fat body (Figure 14B).

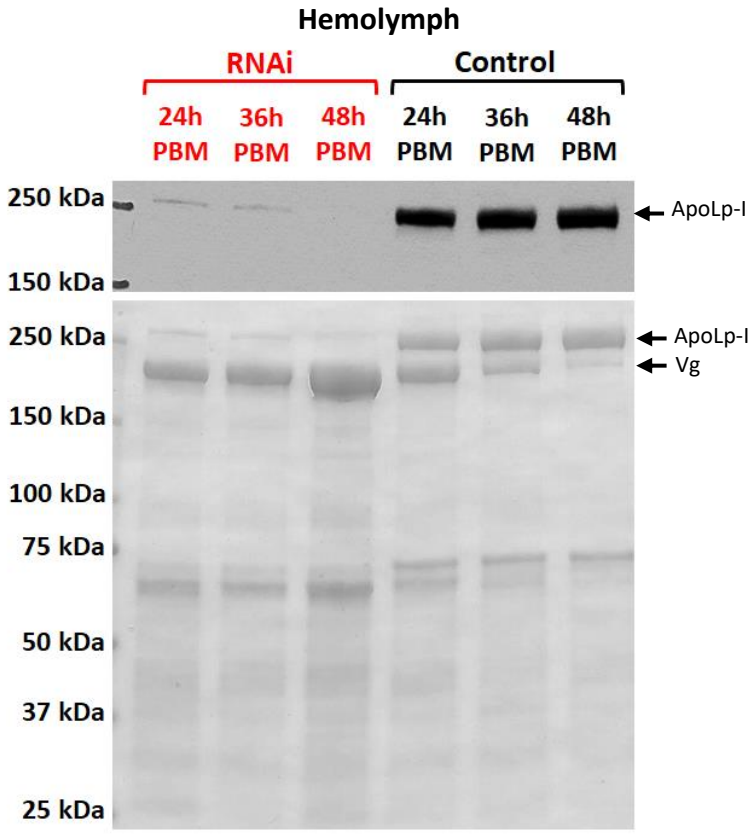

Fig. 15. Apolipophorin-I in hemolymph of RNAi-treated (RNAi) and control mosquitoes post blood meal.
Top panel: Western blot of hemolymph of RNAi-treated and control mosquitoes. Apolipophorin-I (ApoLp-I) is detected as a band around 250 kDa. **Bottom panel:** Ponceau S staining of the membranes used in western blot, showing ApoLp-I and Vitellogenin (Vg) bands.

A marked decrease of apolipophorin-I protein was also observed in hemolymph samples of apoLp-I dsRNA injected insects. The results correlate with the reduction of the corresponding mRNA and with the downregulation of the protein synthesis in fat body cells. The abundance of apolipophorin-I in the hemolymph of RNAi treated insects at 24 and 36 h PBM (96 and 108 hours post dsRNA injection respectively) was close to 50-fold lower than the amount observed in the non-specific RNAi control. Whereas at 48 hours post blood meal, the protein was undetectable (Figure 15).

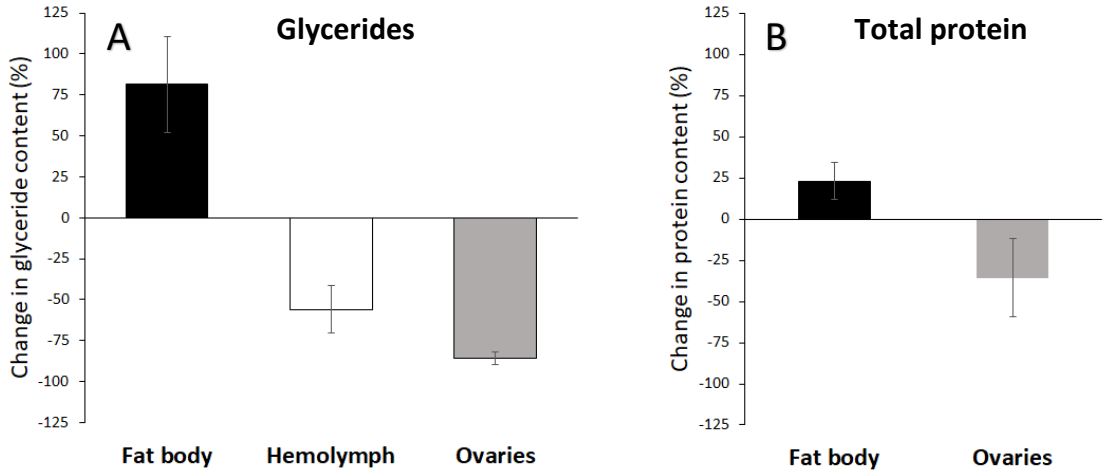

Fig. 16. Lipid and protein content in fat body, hemolymph and ovaries after apolipophorin silencing. A) Mean glyceride content change in fat body (12h – 48h PBM), hemolymph (12 – 48h PBM) and ovaries (24 – 72h PBM) of RNAi-treated insects compared to control insects; average ± SEM (n=9) is presented. **B)** Total protein content change in fat body and ovaries of RNAi-treated insects compared to control insects at 48h PBM; average ± SEM (n=3) is presented.

The successful silencing of ApoLp-II/I gene by RNAi, altered the concentration of glycerides in the three tissues studied. The absence of apolipophorin proteins caused accumulation of lipids in the fat body, due to the incapability of the cells to secret them properly. The concentration of glycerides in the fat body of RNAi treated females increased in average 50 –125% compared to the amount of glycerides found in the fat body cells of the control. Lipids in hemolymph of RNAi treated females decreased 50 to 75% compared to the control, as a result of the insufficient amount of lipophorin being secreted from the fat body cells. The most noticeable effect, regarding lipid content, was observed in the ovaries of RNAi treated females. The amount of lipids detected was 90 to 95% lower than the control (Figure 16A).

Protein concentration was also affected by apolipophorin silencing. Fat body from RNAi treated mosquitoes showed a 25% increase in total protein compared to the control, whereas ovary protein concentration decreased around 35% (Figure 16B). It is important to mention that lipophorin is also a yolk protein precursor during vitellogenesis as previously stated, however it has been reported that its contribution to yolk protein accumulation is modest.

These results indicate the critical function of lipophorin in lipid transport during vitellogenesis. Proper ovary development requires lipid and protein uptake for yolk production as energy reserve for the growing embryos. Apolipophorin gene silencing not only affected lipid and protein uptake by the ovaries, but also their size and morphology. Ovaries from RNAi treated mosquitoes displayed a smaller size compared to the ovaries from the control mosquitoes at 48 and 72 hours PBM. Follicles appeared undeveloped with round shape instead of the regular rice-like shape of normal follicles at 72 hours post blood meal (Figure 17). This suggests that RNAi treated mosquitoes were not ready to lay eggs at the end of the gonotrophic cycle due to insufficient lipid and protein uptake and improper oocyte development.

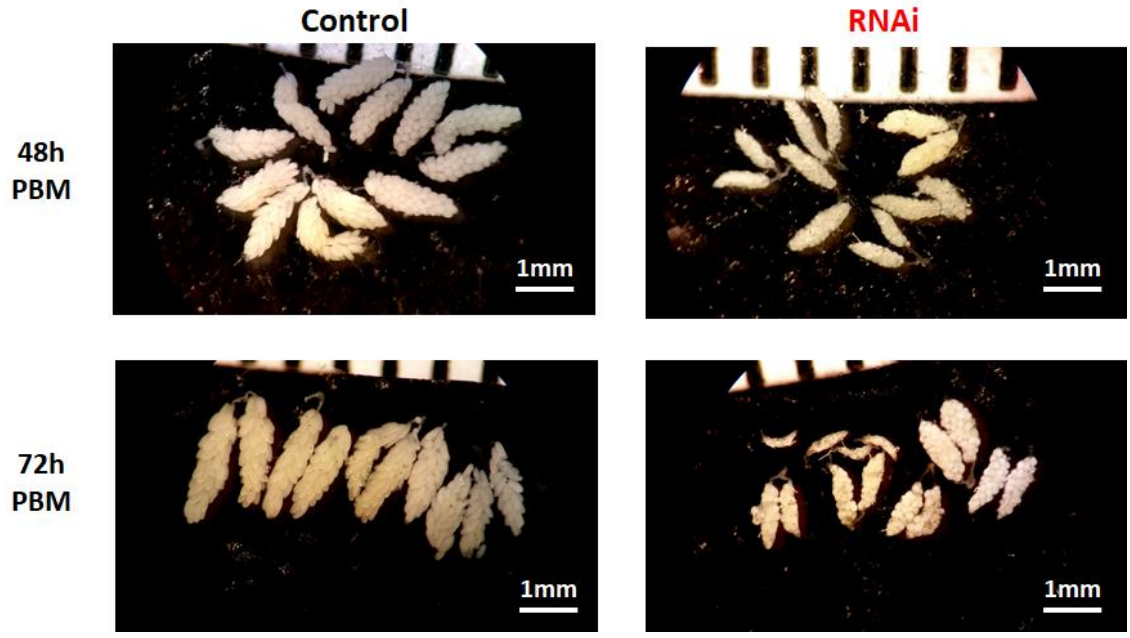

Fig. 17. Ovaries from RNAi-treated mosquitoes and control mosquitoes. Photographs of 5 – 7 pairs of ovaries of 48h and 72h PBM RNAi-treated mosquitoes and control mosquitoes (30x magnification).

6. Other lipid-transport-related genes affected by apolipophorin silencing

6.1 Vitellogenin

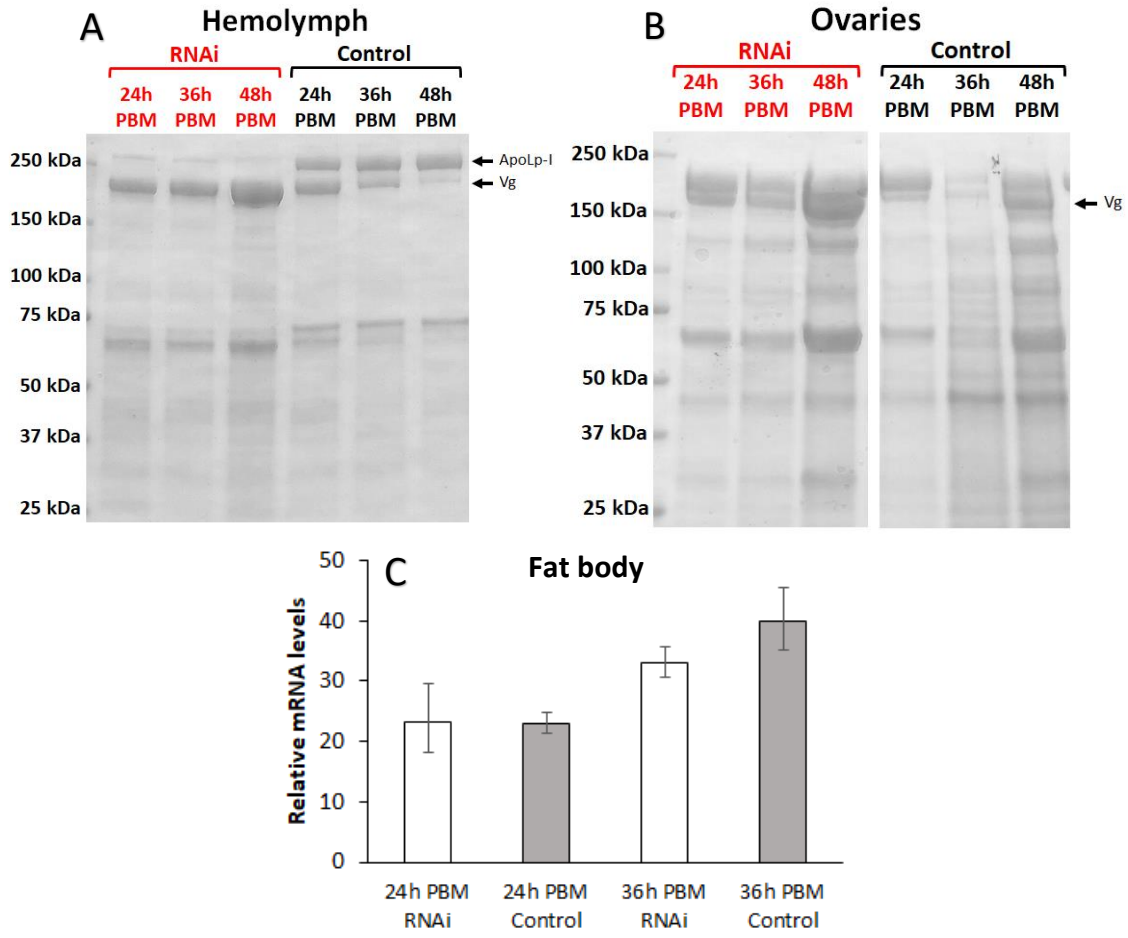

Fig. 18. Vitellogenin protein abundance and mRNA levels after apolipophorin silencing in RNAi-treated and control mosquitoes. A) Ponceau S. staining of membrane with hemolymph samples where apolipophorin-I (ApoLp-I) and vitellogenin (Vg) bands are visible. **B)** Ponceau S. staining of membrane with ovary samples where vitellogenin bands are visible. **C)** Vitellogenin mRNA levels were determined by qRT-PCR using ribosomal protein RpL8 mRNA as reference. Values for each time point are plotted as mean ± SD (n=3).

A surprising result of these experiments was given by the marked increase of vitellogenin that was observed in the hemolymph and ovaries of apolipophorin silenced insects at 48 hours PBM (Figure 18). As mentioned before, vitellogenin is another protein capable of transporting lipids but not as efficiently as lipophorin, as it only contributes to 5% of the total amount of lipids taken by the ovaries in normal conditions (Kawooya and Law, 1988). The increased levels of

vitellogenin observed could be the result of increased production of vitellogenin by fat body cells as a compensation mechanism of the insects, triggered by the incapability of transporting lipids due to lack of lipophorin. However, the upregulation of vitellogenin was not observed at transcript level in the fat body cells. Considering there is an excess of amino acids (due to the lack of lipophorin synthesis), this could activate the TOR pathway and selectively promote the translation of vitellogenin. This situation would resemble the regulation of vitellogenesis in ancestral species that lacked lipoproteins with the ability to load and carry larger amounts of lipids, such as lipophorin.

6.2 Lipid transfer particle (LTP)

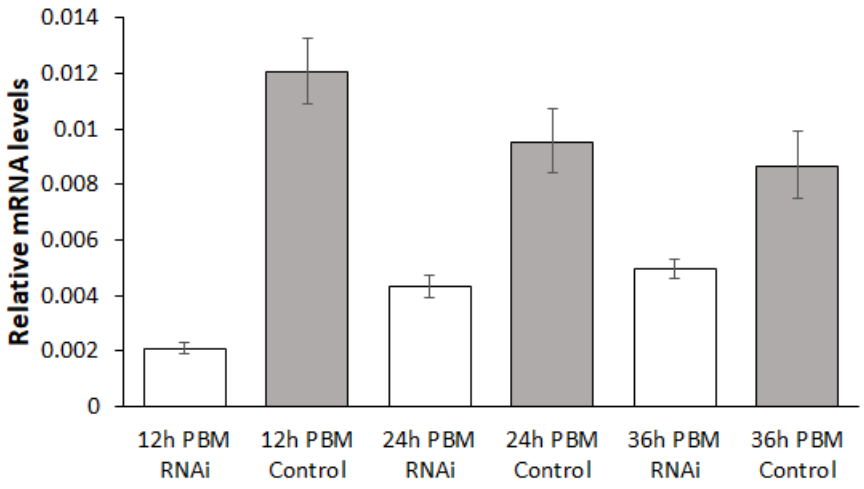

Fig. 19. LTP mRNA levels in fat body of RNAi-treated (RNAi) and control mosquitoes post blood meal (PBM). Lipid transfer particle (LTP) mRNA levels were determined by qRT-PCR using ribosomal protein RpL8 mRNA as reference. Values for each time point are plotted as mean ± SD (n=3).

Lipid transfer particle (LTP) mRNA levels were affected by apolipophorin gene silencing. The overall effect observed was downregulation of the transcript during the first 36 hours after blood feeding in RNAi-treated mosquitoes compared to the control. LTP has been shown to facilitate distribution of lipids among lipophorin particles in *M. sexta* (Ryan et al., 1986). It has also been related to the lipid transfer from midgut to lipophorin and from lipophorin to ovaries, and to the

formation of LDLp from HDLp with AKH signal. The relationship between apolipophorin gene, LTP gene and their regulation in *Aedes aegytpi* is not well understood yet. Our results suggest that LTP expression could respond to the increasing synthesis of apolipophorin after blood feeding, and when apolipophorin synthesis is silenced, there is no signal to trigger its production. More studies are needed to elucidate the actual functions of LTP regarding lipid metabolism and transport during vitellogenesis in this particular species.

7. Conclusions

- There is a clear correlation between blood feeding and the upregulation of apolipophorin mRNA levels in fat body cells that can only be observed during the first 24h PBM, in response to the requirement of a massive lipid mobilization to the ovaries during vitellogenesis. Protein levels in fat body are higher up to 48h PBM.

- Apolipophorin gene upregulation in the fat body precedes lipophorin secretion to the hemolymph and its uptake by the ovaries, confirming the fat body as the main site of synthesis.

- Lipid content in ovaries correlates with their development.

- Double stranded RNA can be effectively used as an inducer of RNA interference for apolipophorin gene silencing in *Aedes aegypti* mosquitoes.

- The overall effect of apolipophorin gene silencing is preventing lipid mobilization to the ovaries during vitellogenesis, producing lipid accumulation in the fat body and lipid shortage in hemolymph and ovaries.

- Lack of lipids alters proper ovary development, producing smaller-size and underdeveloped ovaries.

- Apolipophorin gene silencing produces upregulation in vitellogenin protein without affecting its mRNA levels.

- Apolipophorin gene silencing appears to induce downregulation of LTP mRNA levels in fat body cells.

8. Future directions

The present study clearly showed that lipophorin plays an essential role in the transport of lipids to ovaries. However, it also gave rise to many important questions regarding lipid transport and the function of other genes related to lipid mobilization in *Aedes aegypti*. Lipid delivery from lipophorin to the ovaries may take place by more than one mechanism. Further studies are needed to clarify the actual contributions of possible and alternative mechanisms. The function of other key molecules such as lipophorin receptors (both in ovaries and fat body), vitellogenin and lipid transfer particles still need to be elucidated. Regulation processes for all these genes and their interaction with each other could give important and useful information that could be used someday to design a better alternative to control mosquito population.

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VITA

Claudio Arafat Galvez Sagastume

Candidate for the Degree of

Master of Science

Thesis: APOLIPOPHORIN GENE SILENCING BY RNAi INHIBITS LIPID TRANSPORT TO THE OVARIES DURING VITELLOGENESIS IN *Aedes aegypti*

Major Field: Biochemistry and Molecular Biology

Biographical:

Education:

Completed the requirements for the Master of Science in Biochemistry and Molecular Biology at Oklahoma State University, Stillwater, Oklahoma in July, 2018.

Completed the requirements for the Bachelor of Science in Biochemistry at Universidad de San Carlos de Guatemala, Guatemala in 2011.

Experience:

2012 to present: Professor of the Biochemistry Department, College of Chemistry Sciences and Pharmacy. Universidad de San Carlos de Guatemala, Guatemala.

2013 to 2016: Professor of the School of Medical Sciences and Health, Universidad Mariano Gálvez de Guatemala, Guatemala.

Professional Memberships:

2017 to present: Member of The Honor Society of Phi Kappa Phi