OOSTATIC AND TRYPSIN MODULATING EFFECTS OF AEDES-TMOF PEPTIDE AND PEPTIDE-MIMICS ON THE MOSQUITO, *AEDES AEGYPTI* LINNAEUS AND THE STABLE FLY, *STOMOXYS CALCITRANS* LINNAEUS

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LOYCE MARTHA ALUNGAT OKEDI

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I dedicate my dissertation to my parents, Samuel and Esther and my children Brian Samuel and Esther Grace.

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Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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By

Loyce Martha Alungat Okedi

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Chairperson: D. A. Carlson
J. Nation, J. Hogsette and E. Greiner
Major Department: Entomology and Nematology

The decapeptide trypsin modulating oostatic factor (TMOF) is oostatic when introduced too early in a gonotrophic cycle of the female mosquito, *Aedes aegypti*. The possibility that Aedes-TMOF-derived synthetic peptides and peptide mimics may affect egg development through modulating trypsin biosynthesis was evaluated in the mosquito, *A. aegypti* and on the stable fly, *S. calcitrans*. Additionally, a search for an ovary-derived factor similar in properties to the Aedes-TMOF was conducted. Oostatic activity in Aedes-TMOF peptides and peptide mimics varied from being oostatic to toxic to no effect. Synthetic peptides A, A5, A7, B, and B2 were oostatic to mosquitoes, and A7 and A5 were slightly oostatic to stable flies.

Peptides A4 and A6 were toxic to mosquitoes and also to stable flies. Peptide mimics with pyrene-butyric acid and carboranylalanine amidated to the N-terminal of the TMOF-A were oostatic to mosquitoes. A direct relationship was observed between the degree of oostatic activity and trypsin-modulating properties of these compounds. A stable fly ovary-derived factor was also discovered that had oostatic and trypsin-modulating properties when introduced to mosquitoes. Oostatic activity with the Aedes-TMOF synthetic peptides was inconsistent in stable flies, trypsin modulation was not regulated as their trypsin-like enzyme levels remain more or less constant through 5-7 days, over the five blood meals required to mature an egg batch.

CHAPTER 1
GENERAL INTRODUCTION

Physiology of Blood Digestion in *Aedes aegypti*

Female mosquitoes, *Aedes aegypti*, require a blood meal from a vertebrate host to obtain nutrients required for oogenesis. Ingestion of a blood meal triggers numerous morphological and biochemical changes in the midgut that make it competent to digest a blood meal. Electron microscopic studies of the midgut of the mosquito, *A. aegypti* show that it has three types of cells, digestive cells (Hecker et al. 1971), endocrine cells (Brown et al. 1985) and regenerative cells (Hecker et al. 1971).

The midgut undergoes changes before and after ingestion of a blood meal whereby in unfed females, the digestive cells of the midgut contain no secretory granules (Hecker et al. 1971). In blood-fed mosquitoes, the surface of the rough endoplasmic reticulum greatly increases by loosening its tight whorls, a condition that exists in starved and sugar-fed mosquitoes (Bauer et al. 1977). The sectretory granules appear in increasing number 8 and 24 hours post-blood meal (hpbm) (Rudin & Hecker 1979). Free and membrane-bound

ribosomes increase up to 24 and 36 hpbm, reach a peak and decline by 96 hpbm (Gander et al. 1980). The increase in amount of endoplasmic reticulum after a blood meal is due to the production of trypsin-like enzymes (Rudin & Hecker 1979) and the peritrophic membrane (Graf et al. 1986).

This was further supported by the fact that there was a large increase in trypsin enzyme activity (Briegel & Lea 1985) which was absent in midguts from un-fed *A. aegypti*. Immediately following the blood meal, two trypsins of molecular weight 32 and 36kDa are present in the midgut. These early forms of trypsin are replaced a few hours later with a 30 kDa trypsin, the late and major proteolytic enzyme involved in blood meal digestion (Barillas-Mury et al. 1991; Graf & Briegel 1989). Tryptic activity and trypsin mRNA was only detectable 5 hpbm (Graf & Briegel, 1989).

Felix et al. (1993) established that trypsin synthesis occurred in two phases in female *A. aegypti*. The first phase of trypsin synthesis begins within a few hours of blood feeding when trypsin is synthesized (translated) directly from preformed mRNA. Subsequently, the peptides resulting from the initial phase of digestion, stimulate production of further mRNA from which additional trypsin is produced 8-10 hpbm. The second phase of trypsin synthesis starts about 8-10 hpbm, when trypsin is synthesized (transcribed) from

newly formed mRNA and utilizes amino acids derived directly from the current blood meal (Felix et al. 1993).

Food ingested by insects is mostly macromolecular, in form of polysaccharides, proteins and lipids. While small molecules can pass into tissues directly, larger molecules must be broken down into smaller components before absorption through the peritrophic membrane and midgut epithelium can occur. Proteins, the predominant constituent of blood are usually found with carbohydrate and lipid moieties that are released during digestion and are digested by carbohydrases and esterases. Blood protein digestion involves two steps and two classes of enzymes. Foremost, proteinases or endopeptidases cleave large proteins into large peptides and then peptidases or exopeptidases progressively shorten the large peptides by removal of one or two amino acids at a time all the way to individual amino acid fragments.

Endopeptidases hydrolyze peptide bonds at specific internal positions in the protein chain. The positions attacked are recognized by the nature of amino acids adjacent to the dipeptide bonds that are attacked. The principal endoproteases in the majority of insects are the serine proteases, trypsin and chymotrypsin. Trypsin and chymotrypsin belong to a family of endopeptidases that bear catalytically active serine and histidine residues at their

active center. Trypsin and chymotrypsin recognize and hydrolyze peptide bonds involving the carboxyl group of the amino acids arginine or lysine in protein molecules. Chymotrypsin is less specific as cleaving of bonds involve carboxy groups of tyrosine, phenyl alanine and tryptophan and bonds involving other amino acids more slowly.

Purification of trypsins extracted from *A. aegypti* 24 hpbm followed by SDS-PAGE at pH 8.4, yielded 3-6 active bands all with molecular masses in the range of 25-33 kDa. Analysis of amino acid sequences confirmed they belonged to the subclass of serine proteases and there was homology between mosquito trypsin and invertebrate and vertebrate trypsins. Hence the insect trypsin usually being referred to as trypsin-like enzymes (TLEs)(Gooding 1973, Briegel & Lea 1975, de Loof 1987)).

Borovsky (1986) proposed a system for blood digestion in mosquitoes and proposed the following theory: "A signal is sent to midgut epithelial cells to initiate trypsin synthesis. Trypsin is secreted into the ectoperitrophic space, traverses the pores in the peritrophic membrane and begins to digest the clot around its periphery. Peptides, amino acids and polypeptides smaller than 23,000 daltons diffuse out through the peritrophic membrane into the ectoperitrophic space. Polypeptides and peptides are then further digested into free amino acids and get transported

through the midgut epithelial cells into the hemolymph. The free amino acids are the used by the fat body to synthesize vitellogennin (egg yolk protein)". This theory is supported by studies on *A. aegypti* that show that 60% of total proteolytic activity occurs around the peritrophic area, and the rest of the activity is inside the midgut epithelium (Graf and Briegel 1982).

Egg Development in *Aedes aegypti*

The female reproductive system in most insects is composed of a pair of ovaries connected to a pair of lateral oviducts. The lateral oviducts join to form the median oviduct that opens posteriorly into a genital chamber, the uterus (Chapman 1998). Opening laterally into the genital chamber is a spermatheca, a sac for storing sperm, and frequently a pair of accessory glands.

Ovaries consist of ovariole compartments within which oocytes develop. Among the Diptera, the ovarioles open together into an expansion of the oviduct called the calyx. Each ovariole consists of a distal germarium in which oocytes stem from the oogonia and a more proximal vitellarium where yolk deposition into the oocytes takes place. The germarium and vitellarium reflect two phases of oocyte growth: the previtellogenic phase when the germarium

is regulated by the oocyte genome, and the trophic phase is when the larger vitellarium is regulated by genes outside the oocyte also involved in embryonic growth.

Throughout the holometabolous orders of insects, each oocyte leaves the germarium portion of the ovariole associated with one or more nurse cells. In higher Diptera the nurse cells and oocyte occupy the same follicle with the oocyte always occupying the proximal position. As oocyte development progresses, mRNAs, ribonucleoprotein, ribosomes and other cytoplasmic constituents are transferred from the trophocytes to the oocyte to add to the oocyte euplasm (nuclear material). Gradually the trophocytes collapse and the oocyte progresses in development up to metaphase of the first meiotic maturation division until ovulation as in *Drosophila* (Foe et al. 1993), or in *Culex* by sperm entry and syngamy (Jost 1971). Meiosis is not complete until after fertilization.

The oocyte euplasm makes up less than 10% of the total oocyte content. The remaining 90% or more is yolk, 40% of which is largely triacylglycerols and the rest is three to five proteins ranging in size from 44,000 to 51,000 kDa (Kawooya & Law 1988). The accumulation of yolk in the oocytes occurs in the vitellarium and results in a very rapid increase in the size of the oocyte. Cyclic yolk accumulation, which results in a gonotrophic cycle, is often

dependent on nutrition, which in mosquitoes and specifically *A. aegypti*, depends primarily on the intake of blood. The single batch of eggs produced after each blood meal and the number of eggs and frequency of oviposition all depend directly on the availability and size of the blood meal.

Newly emerged *A. aegypti* female mosquitoes become reproductively competent after a chain of events that commence with the release of juvenile hormones (JH) from the corpora allata. Juvenile hormones stimulate the growth of the primary follicles of newly emerged females to grow in size to a pre-vitellogenic stage (Lea 1963; Gwadz & Spielman 1975). In *A. aegypti*, JH levels are high during the first two days post-emergence, and thereafter gradually decline. This could be due to a feedback inhibition by the resting ovaries themselves (Feinsoid & Spielman 1980 a&b, Rossignol et al., 1981, Shapiro et al., 1986). Once the proximal follicle length reaches 50-75 μm, no further follicle growth is possible in the absence of a blood meal. Clements (1956) and Gillet (1956) suggested the presence of a brain-derived humoral factor responsible for mosquito egg development.

As the accumulation of yolk progresses within the oocyte, the surrounding follicular epithelium develops extensive intercellular spaces between follicle cells. The intercellular spaces permit direct access of the hemolymph to the oocyte surface. Selective uptake of vitellogenin by

endocytosis, from the hemolymph, occurs through the help of specific receptors on the plasma membrane of the oocyte (Raikhel & Dhadialla 1992, Ferenz 1993).

Follicle epithelium cells may also produce yolk proteins in smaller amounts that are directly contributed to the oocyte. The uptake of proteins from the hemolymph is regulated by juvenile hormone and produces changes in the surface of the oocyte (Yin et al. 1989). The fat body synthesizes yolk proteins, lipids and proenzymes that are involved in yolk metabolism during embryogenesis. Yolk transportation to the oocyte is also receptor-mediated and conducted by low-density recyclable lipophorins (Troy et al 1975, Gondim et al. 1989).

Hormonal Control of Insect Reproduction in *Aedes aegypti*

The trophic phase of ovarian development begins with the release of ovarian ecdysteroidogenic hormone (EH) formerly referred to as the "egg development neurosecretory hormone" that stimulates the ovaries to synthesize 20-hydroxyecdysone (Lea & Brown 1990). Midgut wall distention, from uptake of blood, stimulates the median neurosecretory cells of the brain to secrete ecdysteroidogenic hormone into the hemolymph (Meola & Lea 1972).

In *A. aegypti,* EH is released twice: immediately after

blood meal uptake and later, 8 hours post-blood meal. The release of juvenile hormone prepares primary ovarian follicles to become receptive to EH (Shapiro & Hagedorn 1982) and the fat body to become receptive to 20-hydroxyecdysone (Hagedorn 1977). Ecdysone produced in the ovaries stimulates the fat bodies to produce vitellogenin, the egg yolk protein precursors (Hagedorn et al. 1975). About 20 hours post-blood meal, secondary ovarian follicles separate from primary follicles. This probably results from a rise in the level of 20-hydroxyecdysone (Beckenmeyer & Lea 1980). The vitelline envelope synthesis, initiated and maintained by 20-hydroxyecdysone, reaches its peak in the hemolymph at this time (Raikhel & Lea 1991). The follicle epithelial cells begin to produce the proteins of both the vitelline envelope and chorion as yolk accumulation nears its end. Normally vitellogenin synthesis stops about 30 to 45 hours post-blood meal. The vitelline envelope is essentially a proteinaceous layer surrounding the oocyte, while the chorion is a proteinaceous complex that later also contributes to the darkening and hardening of the eggs.

Chorion secretion occurs in two phases resulting in the formation of two layers of the chorion, the endochorion and exochorion. Chorionic genes, 15a-1 and 15a-2, share 68% identity and appear to code for endochorionic proteins in *A. aegypti* (Lin et al. 1993). The proteins deduced from the

sequences of the 15-a genes shared a highly conserved hydrophobic sequence with the *Drosophila* proteins. Northern analysis of RNA extracted from vitelline membrane epithelia revealed a 650 base pair RNA transcript only in ovaries of blood-fed females. The RNA transcript was detectable at 5 hours, abundant at 20-30 hours and declined at 40-50 hours post-blood meal.

Furthermore, a tritiated transcript used to probe ovary sections frozen at 20 hours post-blood meal hybridized only to the ovarian follicle cells. This showed a more pronounced expression of the 15a-1 gene in follicles adjacent to the oocyte than in follicles adjacent to the nurse cells. This suggested that the local environment around and within the follicle cell modulated gene expression (Lin et al. 1993). Raikhel and Lea (1982 & 1991) suggests that chorionic proteins, too, are synthesized by follicle cells adjacent to the oocyte and not by those adjacent to the nurse cells.

While proximal follicles develop to maturity after uptake of the first blood meal, the secondary follicles separate from their germaria and grow to the previtellogenic resting stage (~50 μm). Treatment with 20-hydroxyecdysone can cause penultimate follicles to separate from germaria and then if females are blood-fed later, yolk deposition occurs simultaneously in the ultimate and penultimate follicles (Beckenmeyer & Lea 1980). Females that retained a

full complement of mature oocytes, and took a second blood meal, could not develop any of their penultimate eggs further beyond the resting stage. Borovsky (1988) and Borovsky et al. (1990, 1991a, 1996) put forward an explanation for termination of vitellogenin synthesis, involving negative feedback arising from an ovary-derived peptide secreted by the follicular epithelial cells in the mosquito, *A. aegypti*. The decapeptide sequence is tyrosine-aspartic acid-proline-alanine-proline$_{(6)}$-COOH (M_r 1047.6 Da). Injection of the peptide into normal blood-fed females inhibited blood meal digestion and resulted in retarded ovarian development. Therefore, the ovary-derived peptide was described as "trypsin modulating oostatic factor-A" (TMOF-A) secreted by ovaries 24 to 48 hours after a blood meal. It is released into the hemolymph and binds to midgut receptors where it blocks further production of trypsin-like enzymes and indirectly inhibits vitellogenesis in autogenous females (Borovsky et al. 1993).

That trypsin synthesis continued longer than normal in ovariectomized blood-fed females supports the fact that TMOFs have a functional role in the termination of trypsin synthesis. Lin et al. (1993) provide evidence that ovarian follicular epithelial cells of the mosquito, *A. aegypti* synthesize a chorionic protein encoded by gene 15a-2 with an open reading frame at the N-terminal coding for TMOF-B.

TMOF-B only differs from TMOF-A in the order of the first two amino acid residues (Borovsky et al. 1990a).

It is possible that, in evolution, peptide(s) synthesized and processed during ovarian development acquired a role in regulating the secretion of trypsin in hematophagous insects. A factor was found to inhibit blood-meal digestion and inevitably block ovarian development, if applied too early to young females.

Hormonal Control of Reproduction in *Stomoxys calcitrans*

Unlike nematoceran blood-feeders like mosquitoes and black flies (Diptera: Culicidae and Simuliidae respectively), blood-feeding Diptera in the sub-order Cyclorrapha, such as stable flies (Diptera: Muscidae) do not exhibit gonotrophic discordance i.e. producing one batch of eggs per full blood meal. Stable flies fed from 48 hours post-emergence need multiple blood meals to produce mature eggs with three to six blood meals required to mature the first batch of eggs (Kuzina 1942). Chia et al. (1982) reported that five blood meals are needed for the first egg batch and three to mature the second batch. Moobola & Cupp (1978) demonstrated that stable fly ovaries enter a juvenile hormone-mediated resting stage following eclosion.

Sutcliffe et al. (1993) suspected that the blood-feeding act results in the release of hormones that stimulate the resumption of ovarian development. Houseman & Morrison (1986) showed that in stable flies, the relationship between the fat body and egg development was uncertain because the fat body neither produces vitellogenin nor any female-specific protein during vitellogenesis. However, Sutcliffe et al., (1993) noted while fat body growth proceeded similarly with the first two blood meals in controls. However, fat body growth was halted in those females that were offered fewer than three blood meals during the period they were expected to have matured the first egg batch. Hence it was established that blood-feeding history in stable flies affected survival and more so, the reproductive process.

Besides gonotrophic discordance, stable flies also differ from mosquitoes in the way they manage their blood meal. Mosquitoes digest a single full-sized blood meal as a bolus with digestion proceeding over the entire surface of the meal. Digestion products of the bolus are made available immediately to mature an egg batch. Stable flies, on the other hand, mature an egg batch from several blood meals and digestion is organized into a production line that involves food storage, digestion and absorption happening at different positions along the length of the gut. Stable

flies are described as continuous-digesters as opposed to mosquitoes that are batch-digesters (Lehane et al. 1995). The batch-digesters undergo considerably more (and dramatic) changes in the levels of midgut digestive enzymes, which suggests more regulation control during the digestive cycle (Blakemore et al. 1995). Hence the trend of regulation control of enzyme processes that are involved in blood meal digestion, as described in the mosquito, *A. aegypti*, is less dramatic in the stable fly. While *A. aegypti* has an early trypsin mRNA transcript transcribed to form trypsin-like enzyme within the course of some 24 hours, *S. calcitrans* contain an inactivated trypsin zymogen that is converted to a fully active trypsin-like enzymes (Moffatt et al. 1995).

The Relation Between Digestive Enzymes and Egg Development

Because of the role of hematophagous arthropods as vectors, the digestive physiology of these arthropods has been intensely studied. Much of the attention has centered on demonstrating the presence of the enzymes by using an in-vitro assay or crude homogenates of some portion of the gut such as the midgut, or whole insects. Since proteins are the most obvious nutrient in blood, attention has been directed to studying proteinases in hematophagous arthropods including insects. The proteinases have been classified as

cathepsins if the optimum pH is acidic, or trypsin-like if the optimum pH of activity is alkaline.

Studies have also involved the state of the enzyme prior to and after ingestion of a blood meal; what initiates a rise in enzyme activity and what terminates the rise (Gooding 1966). In *A. aegypti*, there is a transitory decline in midgut proteinase activity immediately after blood feeding which Fisk & Shambaugh (1952) suspected to be due to substrate depletion of enzymes or inhibition by specific proteinase inhibitors in blood serum.

On the contrary, in *S. calcitrans,* blood feeding neither caused an immediate rise nor a transitory decline in midgut proteinase activity. A steady rise was noted in proteinase content in the midgut that was suggested to be due to activation of pre-existing material or de novo synthesis of the enzyme (Fisk 1950, Champlain & Fisk 1956). Lehane (1976, 1977) proposed that a secretagogue mechanism involving intake of a blood meal operates during the digestive process of hematophagous insects. Gooding (1969) used BApNA to determine levels of trypsin present in guts based on moles of BApNA hydrolyzed per minute per gut. He characterized and determined that synthesis of mRNA and protein occur after ingestion of a blood meal.

Rationale and Objectives

Initial studies on mosquito TMOFs centered on the effects of TMOFs on ovarian development and digestive enzyme activity in *A. aegypti*. In subsequent studies, attempts were made to modify the original TMOF peptide sequences and/or design substituents to create novel bioactive analogs (mimics). The small native decapeptides, TMOF-A and B, are highly water soluble and therefore unlikely to pass readily through the insect cuticle. The mimics though still hydrophilic compounds, are synthesized with the potential to remain unaffected by natural enzyme degrading systems that can sequester or remove TMOF circulating in the insect hemolymph. Design of hydrophobic side chains might confer prolonged activity of chemically modified peptides as with Pheromone Biosynthesis Activation Neuropeptide (PBAN).

The research objective is to establish the effects of these synthetic *Aedes*-TMOF peptides and peptide-mimics as oostatic and trypsin biosynthesis modulators for *A. aegypti* and *S. calcitrans*. Specific reversible substrates for trypsin-like enzymes such as BApNA was used to determine whether trypsin levels were affected in mosquitoes and stable flies treated with Aedes-TMOF peptide and peptide-mimics. Actual levels of trypsin-like enzymes present were

using an irreversible substrate such as tritiated DFP to determine enzyme levels by scintillation counting and PAGE gel fluorography to define isozyme patterns. Isozyme patterns were studied to observe discrepancies from the saline control treated insects.

In *A. aegypti* an early trypsin transcript would be seen to be converted (or not converted) to the late trypsin form by the pattern of the TLE isozymes. While newly emerged female mosquitoes need one blood meal to mature an egg batch, the female stable fly requires up to five sequential blood meals to mature the first egg-batch. Borovsky (1991b) observed that *A. aegypti* ovary-derived TMOF (Ae-TMOF) retarded trypsin biosynthesis and activity in several hematophagous insects, including stable flies, in which up to 48% inhibition of trypsin activity occurred. This suggests cross-bioactivity of *Aedes*-TMOF in hematophagous insects that have primarily trypsin-like enzymes (TLEs) for protein digestion. A second objective was to investigate the possible occurrence of *Aedes*-TMOF-like peptides in the ovaries of stable flies. If present, to determine whether it can modulate blood protein digestion and affect egg development in either the mosquito, *A. aegypti* or the stable fly, *S. calcitrans*.

CHAPTER 2
OOSTATIC ACTIVITY OF SYNTHETIC PEPTIDES AND PEPTIDE MIMICS ON THE MOSQUITO, *AEDES AEGYPTI*, LINNAEUS AND THE STABLE FLY, *STOMOXYS CALCITRANS*, LINNEAUS

Introduction

The existence of substances inhibiting egg follicle growth has been suspected since early studies on a number of insects including the cockroaches, *Blatella germanica* and *Blatta orientalis* (Iwavnov & Mescherskaya 1935), the eye gnat, *Hippelates collusor* (Adams and Mulla 1967) and the house fly, *Musca domestica* (Adams and Nelson 1968 and Kelly et al. 1984). In mosquitoes, Meola and Lea (1972) and Else and Judson (1972) separately observed that during oogenesis the ovary secretes a humoral factor that inhibits yolk deposition into less developed follicles. Such oostatic humoral factors were found to be peptide-like in the triatomine bug, *Rhodnius prolixus* (Davey & Kunster 1981) and the mosquito, *A. aegypti* (Borovsky 1982).

As a gonotrophic cycle progresses, humoral responses closely related to protein deposition into the oocytes invoke a feed back mechanism that results in the release of peptide fragments into the hemolymph. These are recognized

on the surfaces of midgut epithelial cells as signals to reduce or halt trypsin production altogether. Borovsky et al., (1991b) described an ovary-derived peptide originating from vitellogenic *A. aegypti* female mosquitoes with oostatic and trypsin modulating properties and named it a trypsin modulating oostatic factor [TMOF]. TMOF is secreted from the follicular epithelium of mature oocytes in ovaries of *A. aegypti* mosquitoes, then transported and bound to the midgut cells where it inhibits synthesis of trypsin-like enzymes. The peptide could be a by-product of yolk proteins cleaved off and liberated during the editing process of yolk protein precursors on the vitellin membrane. Amino acid sequences for mosquito-derived TMOF (Ae-TMOF) peptides are: NH_2-Tyrosine-Aspartic acid-Proline-Alanine-$(Proline)_6$-COOH for TMOF-A; NH_2-Aspartic acid-Tyrosine-Proline-Alanine-$(Proline)_6$-COOH for analog B and NH_2-Proline-Alanine-$(Proline)_6$-COOH for analog C (Borovsky & Carlson 1989, Carlson et al., 1993, 1994). Borovsky et al., (1991b) established that Ae-TMOF is not species specific in activity because it also modulates synthesis of trypsin-like enzymes and egg development in other mosquitoes like *Culex nigripalpus, Cx. quiquefasciatus, Anopheles quadrimaculatus* and *A. albimanus*. When injected into *S. calcitrans*, TMOF-A inhibited trypsin biosynthesis up to 46% at 4.8 nanomoles, a quantity that causes total inhibition of trypsin

biosynthesis in *A. aegypti* (Borovsky et al. 1991a). This treatment effectively deprives a treated female of the end products of blood protein digestion and hence limits availability of yolk protein precursors to be used by the fat body for yolk protein synthesis (Borovsky, t al. 1990). Recently, Bylemans et al. (1994, 1995) isolated two small peptides with folliculostatic and trypsin modulating activity in the gray flesh fly, *Neobelliera bullata*. One acts like TMOF, while the second factor, Neb-colloostatin or Neb-TMOF, inhibits yolk uptake by pre-vitellogenic oocytes and decreases the concentrations of vitellogenin present in the hemolymph. However, these TMOFs are structurally very different from Ae-TMOF-A and/or B.

Studies on mosquito TMOF first centered on its effects on ovarian development and midgut digestive processes in *A. aegypti* mosquitoes. Attempts have involved shortening, or modifying original peptide sequences and designing substituents to create novel analogs that were assayed for both oostatic and trypsin modulating activity. Small native peptides containing just ten amino acids as found in the Ae-TMOF are highly water soluble and therefore unlikely to readily pass through the insect cuticle. A potentially useful route for bioactive peptides involves structural modification or chemical derivativization of the TMOF-A sequence, H_2N-Tyrosine-Aspartic acid-Proline-Alanine-

(Proline)$_6$COOH (see Figure 2-1). Modifications to increase binding, potency and life span could involve blocking tyrosine OH via esterification with substituents to inhibit lysis; replacement of the internal proline with hydroxyproline or anthranilyl; and by exchanging, end-blocking and derivatizing various amino acids. The peptide-mimics can also be hydrophilic compounds, but synthesized with modifications that theoretically will remain unaffected by natural enzyme degrading systems that sequester or remove TMOF circulating in the insect hemolymph. Modifications could include addition of hydrophobic side chains at the N-terminal of TMOF-A, replacing the proline in the third position with anthraninyl in peptide A4; replacing the proline in the third position with hydroxyproline in A5; adding a phenoxyacetyl group to the first amino acid tyrosine in A6, replacing all the l-amino acid composition in TMOF-A with d-amino acids to form the retro-inverso analog of TMOF-A in A7; modification of TMOF-B by replacing the aspartic acid with a succinyl group at the N-terminal in B2. Other modifications could involve the presence of pyrene substituents to the peptide to form: or pyrene-butyric acid to form 1Pba-Y(OtBu)DPAPPPPPPNH$_2$ or Carboranylalanine to form CbAla-ARYDPAPPPPPPNH$_2$, 9-fluoreneacetic acid to form 9Fla-ARYDPAPPPPPPOH and 9Fla-ARYDPAPPPPPPNH$_2$. Such alterations could prolong the activity of chemically

modified peptides by providing means for transportation of bioactive substances across the insect cuticle.

Specific Objective

The research objective therefore is to establish the effects of the various synthetic TMOF peptides and peptide-mimics as egg development arrestants for biting flies such as the mosquito, *A. aegypti* and the stable fly, *S. calcitrans*. Hence, a bioassay was developed to evaluate the effect of Aedes-TMOF derived peptides and peptide-mimics on egg development in the study insects.

Materials and Methods

Experimental Insects

USDA Insectary reared *A. aegypti* (L) larvae were fed on liver powder/yeast (1:1) until they pupated. Maintenance conditions were 28°C and 80% relative humidity. The adults eclosed and were held in cages until they were 5 days old,

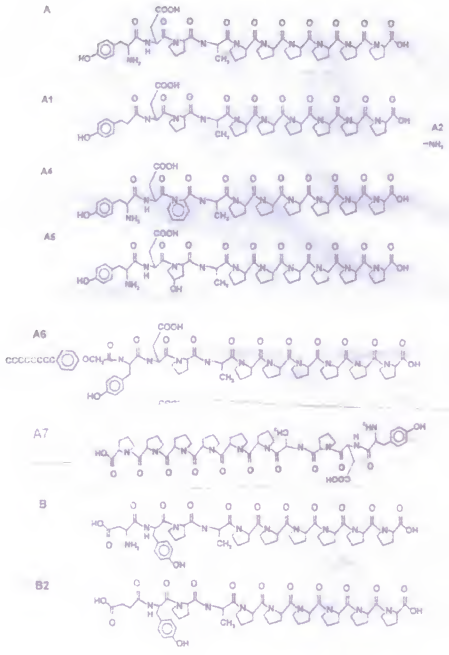


Figure 2-1. Structures of synthetic TMOF peptide and peptide-mimics used in the study.

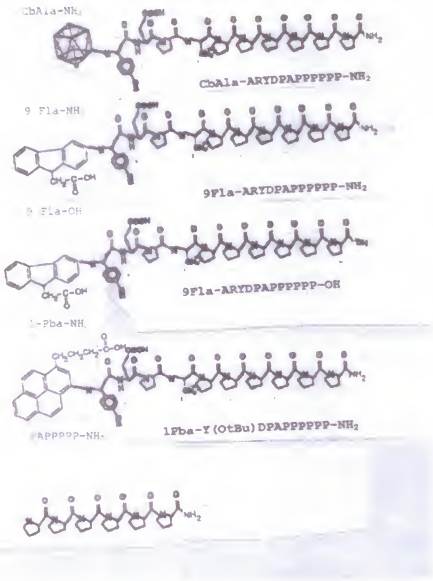


Figure 2-2. Structures of synthetic TMOF peptide-mimics with pyrene, 9-Fluoroacetic acid and caboranylalanine added to Aedes-TMOF.

and maintained on 10% w/v sugar water soaked cotton-wool pads. The adults were offered a guinea-pig blood meal and after three hours, the females that fully engorged were used in the bioassays with the various treatments. Insectary-reared stable fly adults, *S. calcitrans* (L), obtained from pupae from the USDA-CMAVE insectaries were maintained at 28°C and 80% relative humidity. A normal gonotrophic cycle development takes about three days in the mosquito, *A. aegypti* and seven to ten days in stable fly, *S. calcitrans* (see Table 2-1 and 2-2, respectively).

Measurement of Oostatic Activity

Dose-response treatments involved injecting peptides and peptide-mimics dissolved in Ringer's saline at various concentrations ranging from 1-40 µg per female. The structures of peptides used in the study are described in Figures 2-1 and 2-2.

The injection treatments were applied between the third and fourth pleura with glass needles pulled out of micro-capillary with a Pullman (Figure 2-3). A capillary tube is placed within a filament loop of the Pullman, screws of the rotor tightened and the switch set to heat the filament. As the filament became red hot, the capillary softens around the filament area and the pulling action on the two sides of

Table 2-1. Egg development stages in the mosquito, *A. aegypti* maintained at 27 °C and blood fed once (Christophers 1963).

Stage	Follicle Length (μm)	Characteristics of stage	Time post eclosion
1	50	no blood meal taken	0-3/5 days
2	200	blood meal ½ digested	24 hpbm
3	450	blood meal digested	52 hpbm
4	600-800	Oviposition, ready to take another blood meal	72-96 hpbm

Thereafter, oviposition every 72 hpbm.

Table 2-2. Egg development stages in the stable fly, *S. calcitrans* maintained at 24 °C and blood-fed daily (Moboola & Cupp 1978).

Stage	Follicle length (μm)	Characteristics of stage	Days post eclosion
N	70-160	Ovary with 16 undifferentiated cells tightly enclosed by tracheal system; no yolk	0
I	160-300	Oocyte occupying 20% of follicle Trace of yolk	3
II	300-680	Oocyte occupying 40% of follicle follicle elongated	4-5
III	680-820	Oocyte occupying >50% of follicle nurse cells reach maximum devt	5-6
IV	800-900	Oocyte occupying 70-90% of follicle Nurse cells degenerate	6-8
V	900-1200	Oocyte occupying 100% of follicle nurse cells in-apparent hatching pleat present chorion and surrounding entire follicle thickened	7-10

Thereafter, oviposition every 2-3 days.

the capillary pulls the capillary into a fine "needle-like" filament. The treated insects were examined at various hours post blood meal under a dissecting microscope to determine stages of egg development.

Dose Response Effect of Various Treatments on *A. aegypti*

Following initial treatments at 5 µg per female, the mosquitoes were injected with various doses of the peptides to determine dose-response effects. As mosquitoes became available, doses between 1 and 40 µg per insect were injected into each female and occasionally males. Evaluation of oostatic activity was done the same way as above with 5 µg treatments. Meanwhile some guts were also pulled and stored in 50 mM TRIS-HCl buffer, pH 7.9 or 8.4 for various trypsin assays described in Chapter 3.

The Effect of Treatments on the Stable Fly, *S. calcitrans*

Two-day old adults were offered a meal of bovine blood-soaked cotton-wool pads. Three hours later, they were used in the bioassays with various treatments either by injection or topical application. After seven days from day of treatment, both the saline treated control and those that had received various peptide and peptide-mimics were



Figure 2-3. Micro-capillary Pullman used to pull capillary tubes to make glass needles.

dissected and egg follicle length in ultimate follicles determined.

By this time, egg follicle length in control flies had reached 1200 µm and the eggs had or were being oviposited. The proportion that had retarded egg follicle development by day 7 was averaged and scored against the proportion that had normal development and the dead. Samples of gut were retained for trypsin analysis (see Chapter 3).

Results

The Effect of Ae-TMOF Peptides on *A. aegypti*

Female *A. aegypti* injected with 5 µg of oostatic hormone, TMOF-A, and peptides A5, A7 and A8 had ovaries with ultimate eggs developed about half the normal follicle length (263.5 ± 12.7; 184.2 ± 72.7; 250 ± 24.7 and 223.3 ± 56.3, respectively) compared to controls at 460 ± 28.1 µm at 48 hpbm. The trend at 72 hours, when controls had oviposited eggs of 673.5 ± 32 µm, revealed retarded development of egg follicles in the treated females with TMOF-A, A5, A7, A8 and B (Table 2-3), respectively. Yolk deposition into the ultimate oocytes was inhibited. However females injected

Table 2-3. Effect of TMOF peptides on egg development as determined by egg follicle length of the female mosquito, *Aedes aegypti*.

Treatment	Dose/ Female (µg)	Average follicle length in µm ± sd (n = no. measured) 48 hpbm	72 hpbm	96 hpbm
Control	0	460 ± 28.1 (20)	673.5 ± 32.0 (20)	
A	5	263.5 ± 12.7 (20)	341.5 ± 24.6 (20)	
A4	5	dead (20)	dead (10)	
A5	5	184.2 ± 72.7 (19)	430.8 ± 50.2 (18)	453.8 ± 51.9 (13)
A6	5	200 (3/20 survived)		
A7	5	250 ± 24.7 (20)	277.5 ± 23.6 (20)	
B2	5	223.3 ± 56.3 (15)	410.7 ± 21.3 (15)	
B	5	241.7 ± 39.2 (15)	435.7 ± 41.3 (15)	

Females blood fed, treated at 3 hpbm and dissected at 48, 72 and 96 hpbm.

with saline developed their eggs to > 650 µm which they oviposited at 72 hpbm (Table 2-3). Hence a delay in egg development to oviposition over several days resulted in some treated females (as with A5) which had oocytes measuring 453.8 ± 52.9 µm at 96 hours, 24 hours after control females had oviposited.

Figures 2-4 to 2-6 are light microscope photomicrographs of stages of blood-meal digestion and egg development at different times after the first blood meal. Figure 2-4 is the midgut of a normal blood-fed female mosquito, 3 hpbm. The blood is bright red in color with the peritrophic membrane just beginning to form around the blood. The oocytes are rather transparent, about 50 µm long, and have not developed beyond resting stage. By 48 hpbm, the blood has been digested and is brownish in color (Figure 2-5). The oocytes have increased in size to over 280 µm in length and are opaque in appearance.

Figures 2-6, and 2-9 to 2-14 shows ovaries of treated insects 30 hpbm, mostly with translucent oocytes and some evidence of still bright red blood in the gut, indicating occurrence of little protein digestion. Oostatic activity of treatments was confirmed if measurements of ultimate oocytes lengths at various hpbm were less than those in the controls.

Figure 2-6 shows ovaries of saline-treated blood-fed mosquitoes, 30 hpbm, in which eggs are clearly opaque and about 200 μm long. This constituted the standard oocyte size to which development in other treated insects was compared. Figure 2-7 shows recently oviposited eggs 72 hpbm, with maximum attainable egg follicle lengths of ~650 μm by which time eggs had been oviposited. Figures 2-8 and 2-9 show TMOF-A and B2 treatments with guts where the blood meal ingested 30 hours earlier was shrunken in size but still bright red in color, indicating little or no blood digestion. The reduction in size of the blood meal implied that other normal processes, such as peristalisis and diuresis, that accompany the digestion of a blood meal, were uninterrupted. Figure 2-11 shows rather translucent eggs in ovaries of A5 treated mosquitoes and egg follicle length was <150 μm indicating retarded growth compared to controls.

The Effect of Pyrene-Derived and other Aedes-TMOF Mimics on *A. aegypti*

Tables 2-4 and 2-5 show the effect of injecting blood-fed females with pyrene, carboraynl and 9-Fluroacetic acid derivatives of Ae-TMOF peptides. The results indicated oostatic activity in 1Pba(OtBu)DPAPPPPPP-NH_2 where 70% of treated insects were retarded with doses of 4 nM and 80% were retarded with doses of 2nM.

Table 2-4. Dose response of peptide-mimics injected into *A. aegypti* showing follicle lengths reached by the no. of treated females (µm).

Treatment	Dose/fem in nM	Ultimate oocytes follicle length <100 µm	100-200 µm	>200 µm	Dead
1pba(OtBu)-*	2	8	0	0	2
	4	7	0	0	3
PPPPPP-NH_2	2.3	1	9	0	0
	4.6	1	5	0	4
CbAla-*	7.0	4	2	0	4
	11.3	0	0	10	0
9Fla-*OH	4.7	0	0	10	0
	9.4	0	0	0	10
9Fla-*	2.0	0	7	3	0
	3.6	0	5	0	5
	7.3	0	0	10	0
Control	sal	0	0	10	0

NOTE: Females were injected 3 hpbm and examined 30 hpbm. (See Fig. 2-2 for formulae).
*=ARYDPAPPPPPP-NH_2

Table 2-5. Response to pyrene peptide mimics by *A. aegypti* showing follicle lengths reached by the number of treated females.

Treatment	Dose/ female (nM)	Ultimate oocyte length by number attaining stage <300 µm	300-499 µm	>500 µm	Dead
py1	40	0	0	0	20
**py1	40	0	0	0	20
py1	4	3	0	15	2
py3	40	0	0	20	0
py5	40	0	1	4	0
control	sal	0	0	20	0

NOTE: Females injected 3 hpbm and examined 72 hpbm.
(See figure 2-2 for formulae) ** topical application

CbAla-ARYDPAPPPPPP-NH_2 injection of 7.0 nM revealed 40% of insects with retarded egg development. 9Fla-ARYDPAPPPPPP-NH_2 showed moderate activity as 50% and 70% had egg sizes of less than 200 μm with 3.6 and 2.0 nM of 640[ϕ3] respectively; PPPPPP-NH_2 showed oostatic activity as 50% and 90% of insects had egg sizes below normal. Figure 2-3 shows responses with the pba derivatives, clearly indicating oostatic activity with 1Pba(OtBu)DPAPPPPPP-NH_2 and PPPPPP-NH_2 fragment. Overall mortality was observed particularly with 9Fla-ARYDPAPPPPPP-NH_2 at a dosage of 9.4 nM while at 4.7 nM the mimic was neither toxic nor oostatic.

Responses with pyrene mimics were noted only 72 hpbm whereby 15% treated with py1 (obtained one purification step before PPPPPP-NH_2) at 4nM had retarded growth indicated by under developed egg size. Py1 was toxic at 40 nM when applied by injection or topically. Py3 (obtained one purification step before 1Pba(OtBu)DPAPPPPPP-NH_2) and Py5 (obtained one purification step before 581-Pba[ϕ5]) at the dose of 40nM were neither oostatic nor toxic. Hence Py1 had oostatic activity at lower concentration but was toxic at higher concentrations. Figures 2-12 through 2-14 show ovaries of mosquitoes treated with pba TMOF peptide mimics 1Pba(OtBu)DPAPPPPPP-NH_2), 9Fla-DPAPPPPPP-NH_2, 9Fla-DPAPPPPPP-OH, and PPPPPP-NH_2 (Table 2-4).



Figure 2-4. ➡ Peritrophic membrane forming around a freshly ingested blood meal (1-2 hpbm).

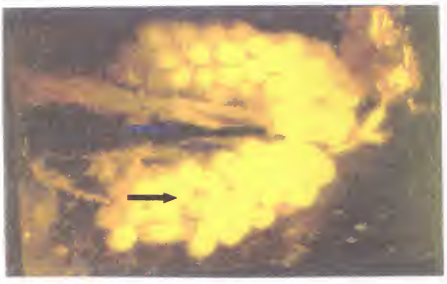


Figure 2-5. ➡ Digested blood meal and
➡ developing eggs ~280 µm (48 hpbm).

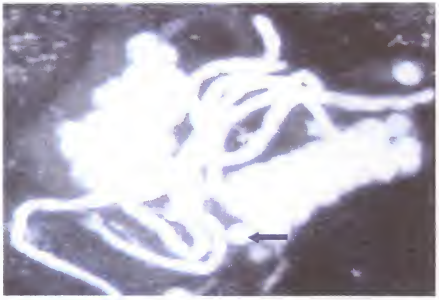


Figure 2-6: Developing eggs ~200 µm long (30 hpbm).



Figure 2-7: Oviposited eggs 72 hpbm (>650 µm long).

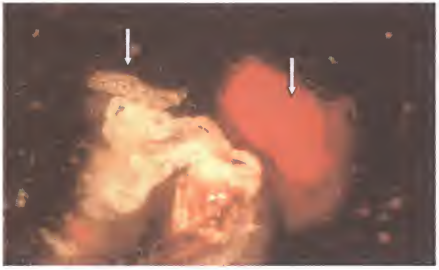


Figure 2-8: Appearance of ovaries and gut and of TMOF-A treated mosquito, 30hpbm. Eggs under-developed beyond 50 µm long and blood still bright red in color



Figure 2-9: Appearance of gut and ovaries of B2 treated mosquito, 30hpbm. Blood still red, eggs are not developed beyond 100 µm long.

Oostatic activity was evident since the egg size was below normal and the eggs were transparent indicating little yolk deposition into the oocytes at 30 hpbm. Figure 2-12 shows a few partly developed eggs (~200 µm) in a mass of under developed translucent eggs (<100 µm) in the ovary dissected from female mosquito treated with 1Pba(OtBu)DPAPPPPPP-NH_2.

A similar trend of the presence of some portion of a rather translucent ovary with opaque oocyte plaques is evident in Figure 2-12. These imply the presence of a few eggs developed to ~200 µm within a mass of underdeveloped eggs with treatment, 9Fla-ARYDPAPPPPPP-OH.

Figure 2-14 a translucent mass of tiny underdeveloped eggs <150 µm long with more obvious opaque appearance with treatment, PPPPPP-OH. The activity with the mimics, 1Pba(OtBu)DPAPPPPPP-NH_2, PPPPPP-NH_2, 9Fla-ARYDPAPPPPPP-OH, 9Fla-ARYDPAPPPPPP-NH_2. CbAla-ARYDPAPPPPPP-OH and even A5, B2 revealed that the ovaries appeared to have some resources to distribute into the yolk granules of the eggs but there may not have been enough to achieve completely normal egg development in the ultimate egg follicles in the ovaries.

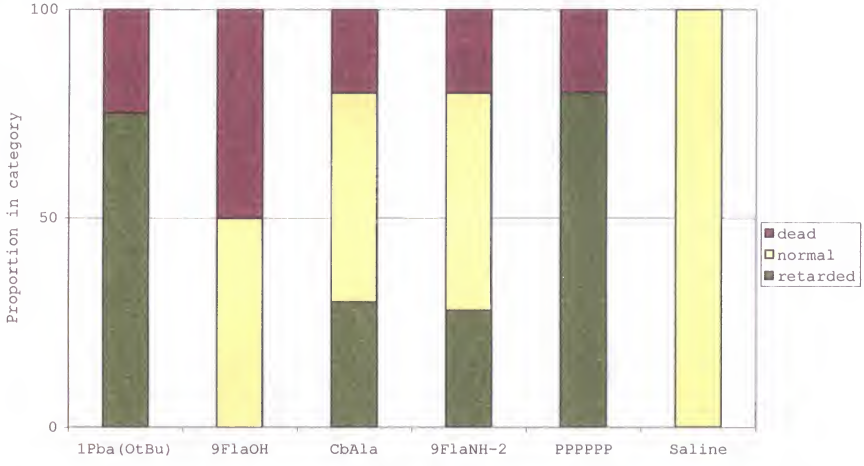


Figure 2-10. Survival of and growth inibition effects of pyrene, carboranylalanine and 9-Fluoreneacetic acid derived Aedes-TMOF amide mimics on *A. aegypti*.

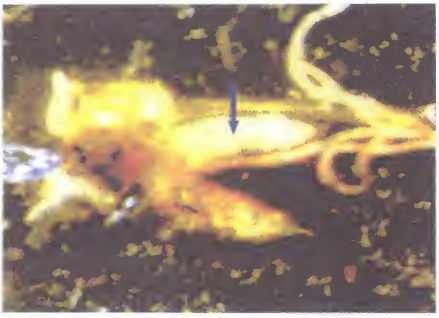


Figure 2-11: Appearance of gut and ovaries of A5 treated mosquito, 30hpbm. Eggs are not developed beyond 150 µm long.



Figure 2-12: Appearance of gut and ovaries of 1Pba(OtBu)DPAPPPPPP-OH treated mosquito, 30hpbm. Eggs translucent & <150 µm long.

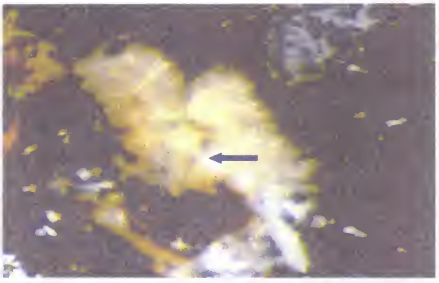


Figure 2-13: Appearance of gut and ovaries of 9Fla-ARYDPAPPPPPP-OH treated mosquito, 30hpbm. Eggs translucent but few developed beyond to 200 μm long. Appear fuzzy in this picture.

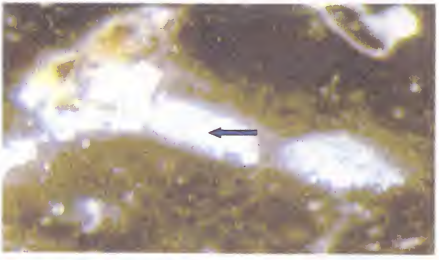


Figure 2-14: Appearance of gut and ovaries of PPPPPP-NH_2 treated mosquito, 30hpbm. Eggs translucent & < 150 μm long.

Dose Response Effect of Various Treatments on *A. aegypti*

Table 2-6 and Figures 2-15 to 2-16 show results of exposing the females to serial doses of the various peptides and peptide-mimics. Besides TMOF-A and B that had oostatic activity, A5, and B2 were over 70% of the females had underdeveloped eggs at 10 µg and A7 at 5 µg per insect.

Increasing the dosages to >10 µg per insect usually resulted in increased mortality, as with B2. Decreasing doses led to instances where a few eggs could be found developing at an apparently normal pace scattered within a mass of underdeveloped and rather transparent eggs. In such cases, it was not easy to establish oostatic activity with mimics shown in Figure 2-8 through 2-14. However guts of treated insects that had responded to 10 µg were pulled for trypsin assays. A positive correlation was seen between TMOF-A and B and peptides A5, A7 and B2 for oostatic properties, while toxicity was evident in peptide-mimics A4 and A6. Figure 2-15 and 2-16 illustrate oostatic activity and toxic properties of the various compounds. Data in Figure 2-15 and 2-16 indicate that uniform doses of the compounds could not yield uniform results. However dosage/mortality curves could not be easily derived under the conditions of these tests at the dosage rates tested.

The Effect of Treatments on the Stable Fly, *S. calcitrans*

As usual the oostatic effects were more definite with TMOF-A, with eggs retarded in 60% insects, eggs developing normally in 32.5% and 10% of the insects dead. TMOF-B retarded eggs in 31.1% of the insects, eggs developed normally in 48.0% of the insects and 10.4% of the insects died.

Peptide-mimic A4 resulted in 5.8% of the insects with eggs retarded, 24.5% of the insects with eggs developing normally and 62.5% of the insects died. A6 had similar trends with 5.0% of the insects with eggs retarded, 19.4% of the insects with eggs developing normally and 70.8% of the insects died. A4 (with anthraninyl) and A6 (with phenoxyacetyl) appeared more toxic than oostatic to the stable flies. A5 caused 41.8% of the insects to have eggs retarded, and 10.5% of the insects with eggs normally developed, and 28.7% insects died. B2 had 30.8% of insects with eggs retarded, 60% of insects with eggs developing normally, and 17.5% of the insects died. These two peptides, A5 (with hydroxyproline) and B2 (with succynyl) appeared oostatic and less toxic to stable fly females. The dead flies (by day 7) survived the initial injection and appeared un-damaged by injection 24 hours after treatment.

Table 2-6. Dose-response effects of Ae-TMOF peptides and mimics on egg development in *A. aegypti* showing follicle lengths reached by the number of treated females (µm).

Treatment	Dose/fem (µg)	Ultimate egg follicle length reached (number attaining stage) <100 µm	100-200 µm	>200 µm	Dead
Control	saline	0	0	20	0
A	5	13	2	5	0
A	10	20	0	0	0
A	15	20	0	0	0
A4	4.8	0	0	0	20
A4	9.6	0	0	0	20
A4	19.3	0	0	0	20
A5	2.5	2	0	6	0
A5	5	8	4	3	5
A5	10	13	4	2	1
A5	20	5	0	0	15
A6	2.5	0	0	0	20
A6	4	4	0	2	14
A6	5	0	0	0	20
A6	8	2	0	2	16
A7	1	0	0	10	10
A7	5	13	2	2	3
B2	10	7	1	2	10
B2	21	0	0	0	20
B2	42	10	1	2	7
B	5	0	0	20	0
B	10	8	2	0	0
B	20	3	0	5	12

Females fed blood, treated 3 hpbm and dissected 30 hpbm.

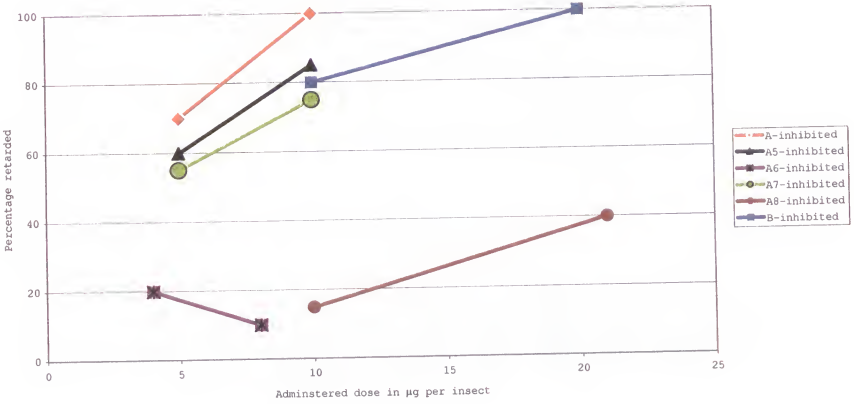


Figure 2-15. Growth inhibition of *A.aegypti oocytes* following injection with Aedes-TMOF peptides and analogs.

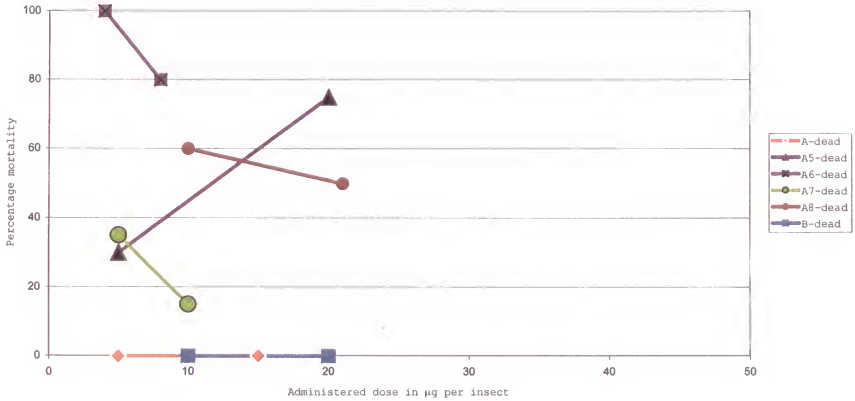


Figure 2-16. Survival of *A.aegypti oocytes* following injection with Aedes-TMOF peptides and analogs.

in Table 2-8 and show that A4 has oostatic properties when applied topically. Egg follicles were retarded in 56.2% and 72.2% of the insects when A4 was applied to back and legs of stable flies, respectively. A6 retarded egg growth in 31.4% and 0% when applied to back and legs respectively. Retardation was obvious with applications to the back as 85.7% of the insects had retarded egg development from peptide TMOF-A and 68% with TMOF-B. It was not possible to keep all of the peptide treatment on the insects because the peptides were dissolved in DMSO and the treatment droplets tended to fall off; thus there was little control on how much peptide was taken up by legs. The DMSO solvent used in this study was not in itself toxic to the stable flies as treated flies continued to develop normally in all processes to seven days.

Discussion

Small peptides in the Ae-TMOF family are highly water-soluble and therefore less likely to readily pass through the insect cuticle. The developed mimics are hydrophilic compounds designed and synthesized with a potential to remain unaffected by the insect's body system, that can sequester or remove TMOF from the insect. Most modifications

Table 2-7. Effects of injected Ae-TMOF peptides and mimics on the stable fly, *S. calcitrans* showing follicle length category reached and the percentage of treated females.

Peptide 5ug	Ultimate egg length reached (% ± SD attaining stage by day 7) <800µm	>800µm	Dead
A	60 ± 27.3	32.5 ± 3.5	10 ± 14.1
A4	5.8 ± 2.7	24.5 ± 17.8	62.5 ± 43.6
A5	41.8 ± 17.6	10.5 ± 0.7	28.7 ± 12.8
A6	5.0 ± 2.0	19.4 ± 13.7	70.8 ± 31.8
A7	28.1 ± 10.8	32.6 ± 5.6	20.5 ± 9.9
B	31.1 ± 13.5	48.0 ± 3.4	10.4 ± 1.3
B2	30.8 ± 17.0	60 ± 0	17.5 ± 12.9
CONTROL	0	100	0

Stable flies fed blood, treated 3-6 hpbm, dissected on day 7

Table 2-8. Effects of topical treatments of Ae-TMOF peptides and peptide mimics on egg development in *S. calcitrans* showing follicle length category and the % of treated females.

Peptide	Treatment	Ultimate egg follicle length (% attaining stage by day 7)		
5ug	Site	<800µm	>800µm	Dead
A	back	85.7	14.3	0
A	leg	25.6	74.4	0
A4	back	56.2	43.8	0
A4	leg	72.2	27.8	0
A6	back	31.8	68.2	0
A6	leg	0	100	0
A7	back	70	30	0
A7	leg	43.8	56.3	0
B	back	68	32	0
B	leg	4.5	95.5	0
CONTROL	back	0	100	0
	Leg	0	100	0

Stable flies fed blood, treated 3-6 hpbm, dissected on day 7

in the peptides were at the N-terminus of the original TMOF-A. Addition of a phenoxyacetyl group to the tyrosine in the chain of A6 and the replacement of aspartic acid in position 2 with an anthraninyl group in A4 proved more toxic than oostatic to the insects.

However, the replacement of a third position proline with the hydroxyproline group in A5 and the addition of a succinyl group to aspartic acid at the N-terminus of the TMOF-B in B2 sequence all seemed to retain oostatic properties. A7, the TMOF retroinverso analog proved oostatic to mosquitoes with no difference between its effect and TMOF-A and B.

The carboranylalanine and pyrenebutyric acid mimics of TMOF and the hexaproline fragment proved oostatic to mosquitoes. The hexaproline fragment affected egg development mosquito insect with normal development and 90% were less than normal. It is possible the insects also are affected by polyproline fragments floating in the hemolymph. This is interesting since many polyproline fragments are found in the 15-a gene of yolk proteins (Lin et al., 1993 and Marten, 1996). The effect of adding a pyrene-butyric acid to the TMOF peptide was that the derivative, 1pba(OtBu)DPAPPPPPP-NH_2, was oostatic at low but toxic at higher dosages to mosquitoes. The fluoroneacetic acid mimics, 9Fla-ARYDPAPPPPPP-OH and 9Fla-ARYDPAPPPPPP-NH_2 had

no oostatic activity on mosquitoes. 9Fla-ARYDPAPPPPPP-OH however appeared to be toxic at lower concentrations. The fact that a more dilute sample was more toxic could be the reverse as addition of saline as a diluent may have released more quantities of the compounds bound to the walls of the container, thus effectively increasing the concentration.

Increased bioactivity shown with CbAla and 1Pba derived peptide-mimics could well be due to enhanced or prolonged binding of the peptide-mimics to the trypsin biosynthesis receptors located in the midgut. Alternatively, increased bioactivity could have been due to the inability of the insect body system to degrade and sequester circulating peptide mimic in the hemolymph. Hence the mode of action which is unknown could be then due to the insect's gut continuing to receive a signal to negatively affect or inhibit trypsin production process. It is also possible that the treated insects were stressed, producing little TLEs to manage the blood meal. This would lead to scarce yolk protein resources absorbed into the hemolymph, from the midgut, and hence such insects could only develop a few eggs probably by re-distributing available yolk protein precursors. This was seen with TMOF-A at 5 µg where 25% of the insects appeared to have normal ultimate oocyte development (Table 2-6).

The scenario was also seen with TMOF-B at 5 µg; A5 at 2.5, 5 and 10 µg; A7 at 5 µg; and also B2 at both 10 and 42 µg. This could have led to a few to many normal looking (opaque and normal size) eggs among smaller (translucent) ones. In such cases, it would be difficult to determine real oostatic activity of the compound in question.

CHAPTER 3
TRYPSIN MODULATING PROPERTIES OF SYNTHETIC PEPTIDES AND PEPTIDE-MIMICS ON THE MOSQUITO, *AEDES AEGYPTI*, LINNAEUS AND THE STABLE FLY, *STOMOXYS CALCITRANS* LINNAEUS

Introduction

An adult female mosquito requires a blood meal to obtain protein nutrients for egg-yolk protein synthesis (vitellogenesis). The uptake of a blood meal stimulates the midgut cells to synthesize and secrete various protein-digesting enzymes such as trypsin, chymotrypsin, and amino and carboxy-peptidases that make the midgut competent to digest a blood meal. In *A. aegypti*, post-feeding induction of trypsin activity is separable into early and late phases (Felix et al. 1991), and each phase is characterized by the presence of a specific group of trypsin isozymes (Graf & Briegel 1989). The early phase begins immediately after feeding (Rudin & Hecker 1979) and involves the translation of mRNA that is already present in the midgut epithelial cells before feeding (Felix et al. 1991). Translation of early trypsin mRNA occurs only after ingestion of a blood or a protein meal (Noriega et al. 1996a, b). The early trypsin mRNA levels decrease rapidly during the first 24

hours following feeding and rise again at the end of blood digestion, about 60 hours after feeding (Noriega et al. 1996b, 1997). Early trypsin plays an essential role in the activation of late trypsin transcription, resulting in the major endoprotease involved in blood meal digestion. Late trypsin isozyme levels peak about 24 hours after uptake of a blood meal and most of the blood meal will have been digested at this time (Graf & Briegel 1985). Midgut protease levels enable efficient utilization of blood, because blood digestion has a direct correlation with vitellogenesis as digested blood proteins supply the amino acids for yolk production. However, any negative feedback response to the midgut can affect the turnover rate of early trypsin transcript into late trypsin, leading to reduced trypsin availability. This leads to retarded blood digestion in the gut and hence, reduced availability of amino acids as precursors for egg protein synthesis (Borovsky et al. 1989). In mosquitoes, Meola and Lea (1972) and Else and Judson (1972) observed that during oogenesis the ovary secreted a humoral factor that inhibited yolk deposition in less developed follicles.

A factor named TMOF was eventually isolated, purified, identified and synthesized from *A. aegypti* ovaries. The mosquito derived TMOF (Ae-TMOF) amino acid sequences for

TMOF-A and TMOF-B are provided in figure 2-1 (Borovsky & Carlson 1989, Carlson et al. 1993, 1994). Borovsky et al. (1990) noted that the Ae-TMOF is a non species specific modulator of trypsin synthesis and egg development in various mosquito species (e.g., *Culex nigripalpus, Cx. quiquefasciatus, Anopheles quadrimaculatus* and *A. albimanus)*. TMOF-A also inhibited trypsin biosynthesis in the stable fly, *Stomoxys calcitrans*, up to 46% at 4.8 nM, a quantity that causes total inhibition of egg development in *A. aegypti* (Borovsky et al. 1991).

Recently, Bylemans et al. (1994, 1995) isolated two small peptides with folliculostatic and trypsin modulating activity from the gray flesh fly *Neobelliera (Sarcophaga) bullata*. The first factor is TMOF-like, while the second one, Neb-colloostatin, inhibits yolk uptake into oocytes and decreases vitellogenin concentrations in the hemolymph.

Trypsin belongs to a family of serine proteases, protein digesting enzymes that have a serine amino acid residue at the active site. Mammalian alpha-chymotrypsin has three peptide chains interconnected by two disulfide bonds. The most critical charges that play a role in enzyme activity of these proteases are serine 195, histidine 57 and aspartic acid 102. Serine at position 195 is a strong nucleophile and plays an active role in the reactivity of

both trypsin and chymotrypsin. These serine-bearing proteases hydrolyze peptide bonds by attacking the carbonyl group of the peptide bond through the oxygen atom of the hydroxyl group of serine 195. The active site is later regenerated by addition of water resulting in proton removal and ionic attack of the carbonyl carbon of the acyl group (Figure 3-1). Trypsin-like enzyme activity has been reported in most insects examined with exceptions in the order Hemiptera, and the Cucujiformia in the order Coleoptera (Terra et al. 1996). Protease activity is frequently assayed by its esterase activity on specific synthetic (chromogenic) substrates. Trypsin and trypsin-like enzymes preferentially cleave protein chains on the carboxyl side of peptide bonds bearing either of two basic L-amino acids, lysine and arginine.

Trypsin activity can be assayed spectrophotometrically with a soluble chromogenic substrate such as alpha N-benzoyl-DL-arginine-*p*-nitroanilide (BApNA). BApNA bears the amino acid arginine on the carboxyl end which trypsin and trypsin-like enzymes recognize with specifity. The colorimetric assays spectrophotometrically follow the degradation of BApNA to yield *p*-nitroanilide, a distinct yellow product, exclusively diagnostic of the presence of

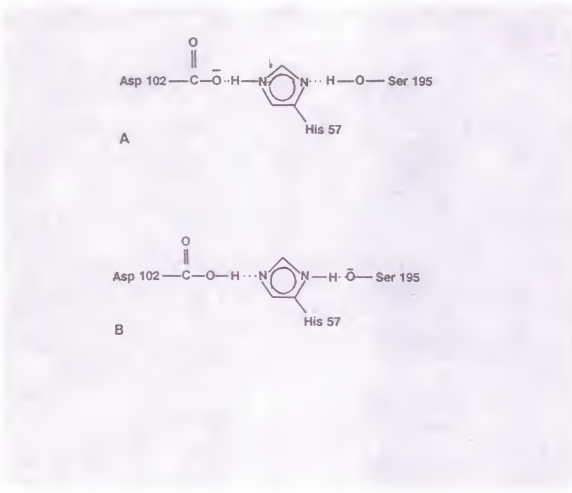


Figure 3-1. Charge relay system for a chymotrypsin molecule. A = normal charge distribution; B = Conversion of the molecule into powerful nucleophile by the removal of one proton by aspartate 102 from serine 195 via histidine 57 results in the conversion of serine 195 into a powerful nucleophile (Stryer 1995).

trypsin and trypsin-like enzymes. Actual trypsin levels can be established by using [^{3}H] diiso-propylfluorophosphate (^{3}H DFP), a substrate that can selectively and irreversibly acylate the serine residue at the trypsin active site, to form [^{3}H] diiso-propylphosphate (DIP) derivatives (see Figure 3-2) (Graf and Briegel 1985; Borovsky and Schlein 1988). The ^{3}H DIP derivatives can be visualized on native PAGE gels with fluorography.

Specific Objective

The objective of this work was to determine whether promising peptides and peptide-mimics affect protein digestion in the gut of *A. aegypt*i and *S. calcitrans* by influencing levels of trypsin.

Materials and Methods

Experimental Insects

USDA insectary-reared *A. aegypti* larvae were fed on liver powder/yeast (1:1) until they pupated. Maintenance

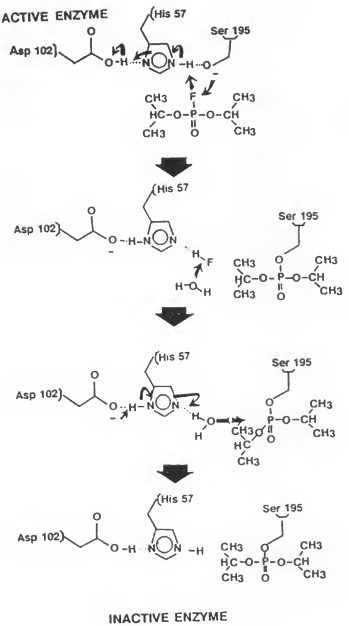


Figure 3-2. Irreversible deactivation of trypsin-like enzymes by DFP.

conditions were 28°C and 80% relative humidity. The adults eclosed and were held in cages until they were 5 day old, and maintained on 10%w/v sugar water soaked cotton pads. The treated female mosquitoes were examined 30 hours post blood meal by dissecting them with the aid of a dissecting microscope to establish the stages of egg development attained. Midguts were pulled out for trypsin assays with BApNA and tritiated DFP.

Insectary reared stable fly *S. calcitrans* adults obtained as pupae from USDA-CMAVE insectaries were maintained at 28°C and 80% relative humidity. Newly emerged adults were fed on bovine blood soaked into cotton pads. Adults were injected with selected treatments. The treated stable flies were examined several days after a blood meal, *ad lib*, by dissection under a dissecting microscope to establish stages of egg development attained. The alimentary canal was pulled and placed in buffer for the trypsin assays as with mosquitoes.

Preparation of Insect Midgut for BApNA Assays

Female mosquitoes engorged in 30 minutes to one hour when exposed to a guinea-pig. Three hours later fully fed adults were easy to separate by appearance of the swollen, reddish abdomen for injection or for topical treatments.

The guts were dissected and placed in 0.1 ml of 50 mM TRIS-HCl buffer with 0.1 M $CaCl_2$, pH 7.9 per abdomen 30 hours post blood meal (hpbm) for later trypsin assays.

Insectary reared stable fly adults engorged in 1-3 hours following exposure to bovine blood soaked cotton-wool pads. They were injected with selected treatments and examined several days later, after dissecting to establish stages of egg development. The guts were dissected and placed in 0.1 ml of 50 mM TRIS-HCl buffer with 0.1 M $CaCl_2$, pH 7.9 per abdomen and homogenized in 100 µl of buffer. A portion of the gut homogenate was diluted 5x for protein estimation with Bradford's reagent (Bradford 1976). Midguts were then stored at -20°C. The procedure was repeated until 1-5 samples were available to run the BApNA assay, whereupon they were thawed to room temperature and homogenized with a glass homogenizer. The homogenates were centrifuged at 4°C at 14,000g for 20 minutes and supernates used for protein estimation and subsequent trypsin activity assays.

Protein Estimation Assays

The Bio-Rad protein assay is based on the changing of the absorbance maximum of an acid solution of Coomassie

Brilliant Blue G-250 shifting from 465 nm to 595 nm when binding to a protein occurs. The Coomassie Blue dye reagent was prepared by dissolving 0.25 g of Brilliant Blue G (Aldrich) in 125 ml of 95% ethanol to which was added 250 ml of 85% O-phosphoric acid. The solution was shaken well, left to stand for 15 minutes and made to to 500 ml with water after 30 minutes and again after three hours. The stock solution was then filtered through two pieces of Whatman #1 filter paper and the filtrate collected and stored. Reagent concentration was obtained by diluting the stock solution five times.

Spectrophotometric Determination of Protein

Three ml of reagent was measured into three tubes and 97.5 ml of TRIS-HCl buffer with 0.1 M $CaCl_2$, pH 8.5 was added to each tube. Crude homogenate (100 µl) was added and measurements of absorbance taken after 2 minutes on a spectrophotometer at 595 nm. Three readings were averaged and based on the standard curve with bovine serum albumin, and calculations of protein concentrations in mg/ml were made with every sample (Bradford 1976). The calculations were extrapolated to determine mg of protein per midgut required for calculations of nm of BApNA per gut.

Preparation of BApNA Calibration Curves

A stock solution of 1 mg/ml of porcine pancreatic trypsin type IX (Sigma) was prepared in 0.1M HCl and stored at -20°C to ensure stability. BApNA (43.5 mg) was dissolved in 1 ml of DMSO and made to 1 nM BApNA with 50mM TRIS-HCl buffer with 0.1 M $CaCl_2$, pH 8.5. To determine whether treatments with the experimental peptides affected trypsin production, non-treated guts were also included in the assays as controls for experimentally treated samples.

BApNA was prepared by incubating 0.1M BApNA in 50 mM TRIS-HCl buffer with 0.1 M $CaCl_2$, pH 8.5, with gut sample digestive enzymes and measuring rate of hydrolysis of BApNA to p-nitroaniline (pNA). The quantity of enzyme that hydrolyzes one nmole of BApNA per minute was determined by measuring absorbency at 410 nm of 1 ml of the reaction mixture. The values obtained from the gradient, defining differences in absorbance values reflecting the rate of p-nitroaniline formation per minute over two minutes, were used to calculate nmoles of BApNA degraded per minute per insect midgut. Standard curves were obtained with porcine pancreatic trypsin IX (Sigma) incubated with BApNA (Figure 3-3).

Preparation of Insect Midguts for DFP Assays

Following eclosion, adults were held in cages until they had reached the appropriate stages after which they were allowed to feed on a guinea-pig for one hour. Three hours later, those that had fully engorged received treatments under cold anesthesia. The bioassay treatments involved injecting insects between the third and fourth abdominal pleura with a peptide or peptide-mimic solution in Ringer's saline with a micro-capillary pulled glass needle. After examining the mosquitoes under dissecting microscope for stages of egg development attained, the guts were dissected and transferred into 0.1 ml per gut of 50 mM TRIS-HCl buffer, pH 8.5 with 0.1 M $CaCl_2$. The samples were homogenized and centrifuged at 4°C at 14,000g for 20 minutes. The supernatants were stored at -20°C until use and the procedure was repeated when additional samples were available.

Determination of [1,3-^{3}H] DIP-TLE Products

[1,3-^{3}H] DIP-trypsin-like derivatives were prepared by incubating above supernates, with/without tosyl-L-phenylchloro ketone (TPCK) inhibitor, with 1 µl of [1,3-^{3}H]

DFP (5 microCuries (μCi)) per μl, specific activity 35 μCi /millimole (mM), New England Nuclear) for 24 hours at 4°C. Likewise different dilutions (0-5 μg) of porcine pancreas trypsin and controls containing 100 μl of TRIS-HCl buffer, 0.1 M $CaCl_2$, pH 7.9, were also incubated with [1,3-^{3}H] DFP as above. Supernatants were incubated with [1,3-^{3}H] DFP in the presence or absence of TPCK. TPCK inhibits chymotrypsin activity. Five μl of 10mM TPCK per 50 μl of midgut homogenate inhibits up to 95% of [1,3-^{3}H] DIP-chymotrypsin derivatives from forming (Borovsky and Schlein 1988). TPCK binds specifically with chymotrypsin and allows the formation and hence estimation of only the [1,3-^{3}H] DIP-trypsin-like products. Aliquots (5-50 μl) of the different dilutions (0-5 μg) were adsorbed onto filter paper (4 X 4 cm). The papers were then washed for 15 minutes in 200 ml of 10% Trichloroacetic acid (TCA), replaced twice with 5% TCA for 15 minutes and followed by a 15 minute wash in absolute alcohol. The papers were dried at 50°C and added to tubes containing 3 ml of scintillation fluid (Liquiflor) and radioactivity was counted in a liquid scintillation counter. The maximum range of radioactivity expected is around 610,000 counts per minute (cpm) of [1,3-^{3}H] DIP-trypsin-like derivatives. Calibration curves with

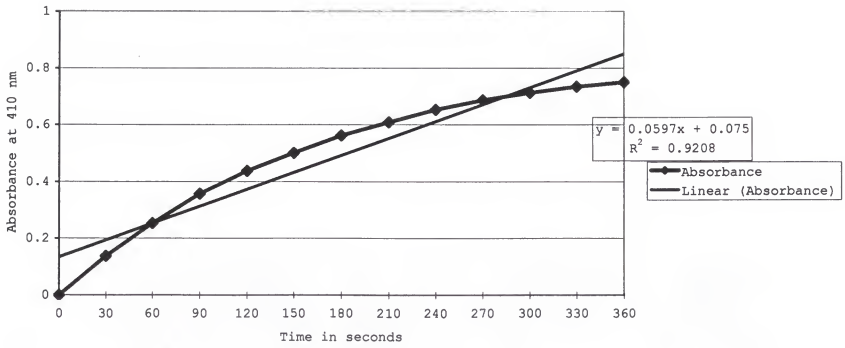


Figure 3-3. Spectra for standard trypsin degradation of BApNA by measurement of p-nitroanilide.

trypsin were constructed by plotting radioactivity in disintegrations per minute (dpm) of [1,3-^{3}H] DIP derivatives auto-corrected from cpm by the counter. The procedure was standardized against trypsin concentrations in µg/0.1 ml and concentrations of trypsin-like enzymes.

Calibration of [1,3-^{3}H] DIP-trypsin-like Derivatives

Several [1,3-^{3}H] DIP-trypsin-like derivatives were quantified by scintillation counts and visualized on PAGE gels after treatment with Ae-TMOF, peptides and peptide mimics dissolved in Ringer's saline. To establish a linear relationship between the conversion of [1,3-^{3}H] DFP to [1,3-^{3}H] DIP derivatives, blanks/standards, control and treated midgut homogenates were incubated in 0.1 ml of 50mM TRIS-HCl buffer, pH 7.9 containing 10mM $CaCl_2$. Incubations were done for 24 hours at 4 ^{o}C in the presence of 5 µCi of [1,3-^{3}H] DFP and radioactivity (dpm) of [1,3-^{3}H] DIP derivatives determined. With blanks, determinations were made of the number of washes and for which concentrations of TCA were needed to be above background noise, compared to controls (Table 3-1). This assay proved that TCA washes at various

concentrations were effective at removing any un-reacted [1,3-^{3}H] DFP substrate radioactivity on filter papers. As all counts were below 100 dpm, it was decided to proceed with washes of 10% TCA replaced twice with 5% TCA washes, for 10 minutes in every case as in Borovsky & Schlein (1987). The standards set up with porcine pancreatic trypsin revealed that [1,3-^{3}H] DIP-TLE products remained bound to the filter paper after several washes as 58,045 dpm were in the range expected with 0.1 mg /ml of trypsin. Scintillation counts of 33,968 dpm for the reference and 229,994 dpm with 1.2 µl of [1,3-^{3}H] DFP incubated alone, proved that the counter was actually detecting tritiated incubates (Table 3-1).

Figure 3-4 shows a linear relationship between the quantities of trypsin in incubates and dpm counts of tritiated DIP-TLE derivatives (r^2=0.9993). The straight lines obtained were used to quantitatively determine the amounts of trypsin like enzymes in insects treated with the various treatments described in Chapter 2.

Polyacrylamide Gel Electrophoresis (PAGE) and Fluorography

Polyacrylamide Gel Electrophoresis (PAGE) of [1,3-^{3}H] DIP-trypsin-like derivatives of midgut homogenates were run in a modified Laemli system (Laemli 1970) as described by Borovsky & Schlein (1988). A slab gel (1.5mm thick, 15 cm long), containing a stacking gel of 3% (w/v) (3 cm) polyacrylamide and 0.125 M TRIS-HCl, pH 6.8 and a separating gel of 10% (w/v) (12 cm) polyacrylamide and 0.375 M TRIS-HCl, pH 8.8 was used during electrophoresis. In each gel several lanes were run simultaneously. PAGE was run at 25 volts for 2 hour and at 35 volts for 1 hour. Gels were initially stained in 0.1% (w/v) Coomassie brilliant blue R-250 for 15 minutes. They were then de-stained in a mixture of acetic acid, methanol and water (10% glacial acetic acid: 25% methanol) for one hour, washed in 10% glacial acetic acid: 10% methanol for one hour and destained overnight (5% glacial acetic acid: 7% methanol). The gels were then incubated with dimethylsulfoxide (DMSO) (80 ml) for one hour, the solution was replaced with 22% 2,5-diphenyl oxazole (PPO) in DMSO and incubated for one hour. After the incubations, gels were rinsed with distilled water and kept in water for 30 minutes to facilitate full precipitation of

Table 3-1. Trend of production of [1,3- ^{3}H] DIP-TLE derivatives in the assays.

	Trypsin levels [1,3-^{3}H] DIP derivatives (dpm)
Blank	43.11
TCA washes, 10:10:5	54.06
TCA washes, 10:10:10	62.50
TCA washes, 5:5:5	55.94
TCA washes, 10:10:5:5	49.64
TCA washes, 10:10:10:5	47.65
TCA washes, 10:10:10:10	53.71
Reference	33,968.00
1.2 µl [1,3-^{3}H] DFP	229,994
0.0 mg porcine pancreatic trypsin	678.75
0.1 mg	58,045.6
1.0 mg	121,046

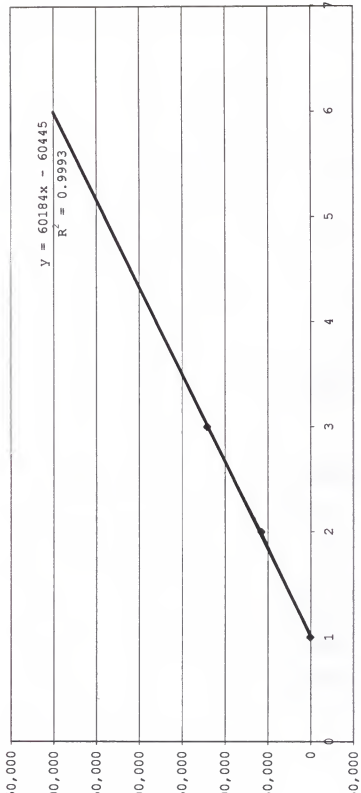


Figure 3-4. Trend of radioactivity retained in standard trypsin incubations.

PPO. The gels were dried at 60°C for several days under vacuum on a filter paper in a gel drier and dry gels were exposed to x-ray films at -70°C for 45-60 days and then developed in a dark room.

Results

The Effect of Treatments on Midgut Blood Digestion

Injections of oostatic hormone into blood-fed female *A. aegypti* resulted in inhibition of oostatic activity (Table 2-3) and modulation of trypsin-like enzymes in the midgut of treated females at 30 hpbm. Persistently high protein levels in the midgut, above 200 mg/ml, confirmed that blood was actually retained in their guts during the whole treatment course. An indication of which component of the total protein was trypsin was done through observing an activity gradient from the rate of hydrolysis of BApNA with crude gut homogenates (Table 3-2). Trypsin activity recorded in guts of the control and saline control insects was 4.92 and 3.76 nmoles of BApNA/minute/gut, respectively. Treated insects had trypsin activity of 2.87 for TMOF-A, 2.04 for A5, and 2.00 for TMOF-B. The other peptides had

Table 3-2. Effect of TMOF peptides on trypsin activity in female *A. aegypti*.

Females Fed on pig	Treatment in µg or nm	Trypsin activity* (nmol BApNA/min/gut	Protein (µg/ml)	n
Colony control		4.92	289.82	(10)
Saline control		3.76	269.57	(10)
A	10 µg	2.87	278.08	(10)
A5	10 µg	2.04	501.67	(10)
A6	10 µg	0.26	349.24	(3)
A7	10 µg	1.63	332.16	(10)
B2	10 µg	1.31	407.10	(10)
B	10 µg	2.00	332.32	(10)
Py1	0.4 nm	0.76	329.01	(3)
9Fla-NH_2	3.6 nm	2.46	245.60	(10)
9Fla-OH	9.4 nm	1.09	272.05	(7)
$P_{(6)}$	4.6 nm	1.36	294.37	(10)
CblAla-NH_2	7.0 nm	0.16	323.30	(6)
1Pba(OtBu)-NH_2	4.0 nm	0.19	375.79	(7)

* = Trypsin activity at 30 hpbm (when ovarian follicle lengths were measured.

activity levels below 2.0, e.g., 0.26 for females that survived the toxic A6 treatment, 1.63 for A7, and 1.31 for B2. The pyrenebutyric acid-derivatives of TMOF-A (pba) variously affected trypsin activity as activity was 2.46 for 9Fla-NH_2, 0.76 for py1 collected one step before 1Pba(OtBu)-NH_2, 1.09 for 9Fla-OH, 1.36 for $P_{(6)}$, 0.16 for CblAla-NH_2 and 0.19 for 1Pba(OtBu)-NH_2. In the previous chapter, various light photo-micrographs revealed a bright red color of blood, persistent for 24 hpbm, which strongly suggested the presence of undigested blood in the gut of treated insects, and even beyond one to two days post treatment. However, the gut of control mosquitoes had blood digestion ongoing and the blood had turned a characteristic darkish brown color, indicating protein-degradation by proteolytic enzymes.

Trypsin activity recorded in the gut of the control and saline control stable flies was 3.82 and 2.24 nmoles of BApNA/minute/gut, respectively (Table 3-3). Treated insects had trypsin activity of 2.76 for TMOF-B, 3.72 for B2, and 1.255 for a stable fly ovary-derived peptide described in Chapter 4.

Table 3-3. Effect of TMOF peptides on trypsin activity in female *S. calcitrans*.

Females Fed on pig	Treatment in µg	Trypsin activity* (nmol BApNA/min/gut	Protein (µg/ml)	n
Colony control		3.82	1089.86	(10)
Saline control		2.25	1435.39	(10)
B	10 µg	2.79	816.06	(10)
B2	10 µg	3.79	554.02	(10)
SFOV*	8.5 µg	1.26	1154.39	((10)

Trypsin activity at 30 hpbm (when ovarian follicle lengths were measured).
* = HPLC portion collected at 9-10 minutes.

Comparison of Total Serine Proteases Produced in *A. aegypti* Females Treated With Ae-TMOF Peptides and Peptide Mimics

The production of total tritiated DIP-TLE products varied greatly in treated mosquitoes. The smallest amount of serine proteases was secreted by *A. aegypti* females that were un-fed 3 hours later and gave counts of 3,046 dpm. This was in comparison to 4,922, 39,432 and 93,850 dpm in blood-fed controls 3 hpbm injected with saline, 30hpbm injected with saline and 30hpbm touched (but not injected), respectively (Table 3-1 and Figures 3-5 and 3-6). In all cases with treatments dissolved in saline, and dpm counts were lower than saline controls at 39,4322 dpm (Figures 3-6 & 7). The counts from various treatments were A, 10 µg yielded 20,181 dpm; B, 10 µg yielded 10,402 dpm; B2, 10 µg yielded 7,044 dpm; A5, 10 µg yielded 3,902 dpm while A7, 10 µg yielded 16,325 dpm. Peptide treatments affected protease levels available in the guts of treated insects, and the results were that little or no digestion of blood occurred, with reduction in the end products available to the insect for egg development. Trypsin production was correlated with retarded egg development. In addition to an effect on normal trypsin production, a chymotrypsin inhibitor (TPCK)

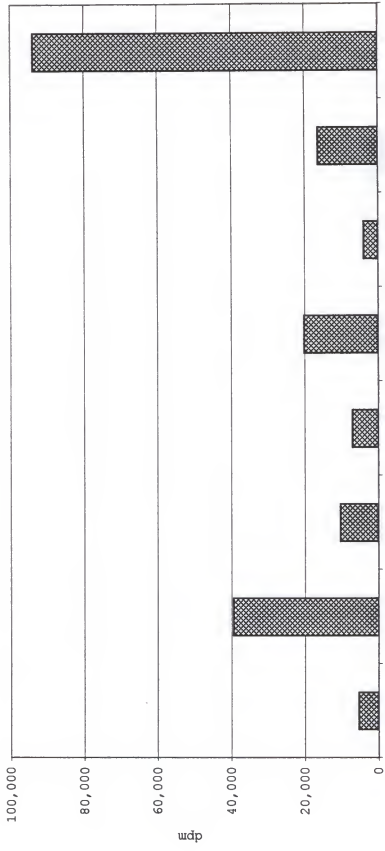


Figure 3-5. Trend of radioactivity retained in incubations involving *A. aegypti* females treated with various peptides. 10 = 10 µg per female.

was incorporated in some of the incubations and comparisons made to ascertain how much retardation would be achieved with and without chymotrypsin products included. In all cases the addition of TPCK into the incubates led to decreased amounts of [1,3- ^{3}H] DIP-TLE products in the female *A. aegypti* mosquito.

Table 3-4 and Figure 3-7 show the trend of production of [1,3- ^{3}H] DIP-TLE derivatives in the presence or absence of TPCK. The results revealed dpm of 3,046 (blood-fed at 3 hours), 4,922 (blood fed), 39,432 (injected with saline at 30 hours) and 93,850 in saline only treated controls that were un-fed at 3 hours. Hence the saline injection alone affected protease enzyme production in the female mosquito because just pricking the insect cuticle results in some trauma. Consequently, the saline control at 30 hours (39,432 dpm) was taken as the control for the experiment in all cases. There was, however, less pronounced retardation with this control value as opposed to adopting the 93,850 dpm count (Table 3-4). Based on the 39,432 dpm count as control, reduction in production of tritiated DIP-TLE derivatives was up to 51% for A, 41% for A7, 64% for B, 57% for B2 and 100% for A5. The trend with pba-mimics was 40% with 1Pba(OtBu)-NH_2, 42% with 9Fla-OH, and 111% with 9Fla-NH_2 topically applied. Adding TPCK revealed that not all

protease activity in the gut of these female mosquitoes was trypsin-like. There was reduced dpm counts with TPCK included in the incubations. Counts as low as 32% were scored in saline only controls at 3 hours as opposed to 13,186 dpm (100%) in saline only controls at 30 hours. With the 13,186 dpm count taken as control in gut samples incubated with TPCK, the counts were 26% for A, 61 % for A7, 41% for B, 70% for B2 and 23% for A5. The trend with pba-mimics was up to 37% with 1Pba(OtBu)-NH_2, 20% with 9Fla-OH, and 122% with 9Fla-NH_2 topically applied.

Polyacrylamide Gel Electrophoresis and Fluorography of Trypsin and Chymotrypsin-like Enzymes.

Various aliquots of incubates were separated on PAGE gels and visualized by fluorography with X-ray films incubated for 45-60 days. Several isoenzyme bands were detected in treated adult females of *A. aegypti* (Figures 3-7 to 3-9). Figure 3-7 reveals that nulliparous females never exposed to a blood meal had almost no bands of tritiated DIP-TLEs (lane B from 3,046 dpm) as compared to blood fed controls only 3 hours later in which three bands were evident (lane A from 5,462 dpm). Thirty hours post blood meal, up to six bands of trypsin isozymes were

Table 3-4. Effect of TMOF peptides on production of [1,3- ^{3}H] DIP-TLE derivatives in *A. aegypti* with and without TPCK.

Treatment	Time (hpbm)	Levels of [1,3-^{3}H] DIP-TLE derivatives (dpm) no TPCK	%	TPCK	%
Saline un-fed	3	3,046	8	-	
Saline	3	4,922	12	2,807	21
Saline control	30	39,432	100	13,186	100
A, 10µg	30	20,181	51	13,279	101
B, 10µg	30	25,281	64	5,468	41
A5, 10µg	30	39,606	100	3,025	23
A7, 10µg	30	16,325	41	8,011	61
B2, 10µg	30	22,417	57	9,262	70
1Pba(OtBu)-NH_2, 4 nm	30	15,968	40	4,869	37
9Fla-OH, 4.6 nm	30	16,445	42	2,628	20
CbAla-NH_2, wash	30	-	-	3,805	29
9Fla-NH_2, 7 nm top	30	43,666	111	16,042	120
Un-injected	30	93,850	237	-	

Blood fed taken as 0 hpbm; injected at 3 hpbm and dissected at 30 hpbm.

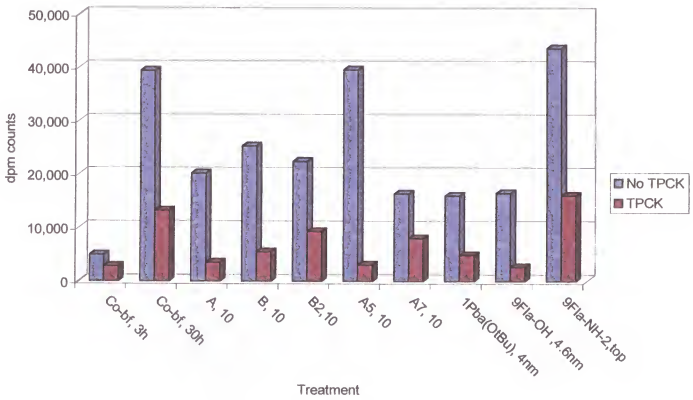


Figure 3-6. Effect of Ae-TMOF peptides and peptide-mimics on [1,3 ^{3}H] DIP-trypsin (and chymotrypsin-like) derivatives in the mosquito, A. aegypti (with or without TPCK).

visible in saline treated females (lane D from 39,432 dpm). Guts from *A. aegypti* females treated with peptides B, B2, A, A7 and B were placed in lane E from 10,402 dpm, lane F from 7,044 dpm, lane G from 20,181 dpm, lane H from 16,325 dpm and lane I from 16,541 dpm counts respectively. The upper bands clearly present in lane D of the control appeared less pronounced in incubated guts (lanes E through I) from the treated mosquitoes. Apparently, the bands present in lane A with 3 hpbm saline control gut incubates were the only prominent bands seen in lanes E through I. This indicated little change in TLE profiles from 3 hpbm when the mosquitoes received treatment. It is possible that there was retarded conversion of early trypsin mRNA needed to trigger transcription of late trypsin prominently represented as the third dark band in the controls 30 hpbm. Figure 3-8 further shows that two clear bands were present in 3 hpbm blood-fed females (lane I) compared to 4 bands in the controls 30 hpbm (lane H from 13,186 dpm). Saline controls with less dpm of tritiated DIP-TLE still continued to reveal 4 distinct bands irrespective of low dpm counts. Other lanes (A, B, C, F and G) with treatments showed less of the bands found in the saline control (lane I) 30 hpbm. Lane A showing results from treatment with 10 µg of B had

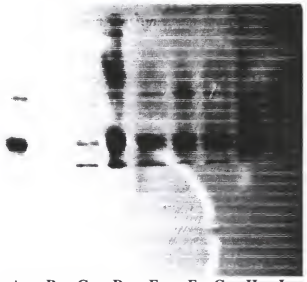


Figure 3-7. PAGE Fluorography of [1,3-^{3}H] DIP-trypsin-chymotrypsin like isozymes of females of *A. aegypti*. The X-ray film was exposed to the gel for 60 days at -70°C. Each lane represents gut aliquots from one midgut equivalent.

(A)	Saline control	3hpbm	(5,462 dpm);
(B)	Saline control, un-fed 3h later		(3,046 dpm);
(C)	Saline control	3hpbm	(6,541 dpm);
(D)	Saline control		(39,432 dpm);
(E)	B, 10µg		(10,402 dpm);
(F)	B2, 10µg		(7,044 dpm);
(G)	A, 10µg		(20,181 dpm);
(H)	A7, 10µg		(16,325 dpm);
(I)	B, 10µg		(16,541 dpm).

All trypsin levels at 30 hpbm, unless stated otherwise.

2 distinct bands that were seen also in lanes F and G with guts from 10 µg treatment with A5. Lane B from mosquitoes treated with 1Pba(OtBu)-NH_2 showed 2 diminished bands with little tritiated DIP-TLE products. Lane D shows 2 clear and two unclear bands present in guts from 9Fla-NH_2 treatment with 87,038 dpm. There was no retardation of tritiated DIP-TLE products in this case.

Figure 3-9 is an over-exposed plate kept in the cold for 70 days before developing, but otherwise similar to Figure 3-10. The gel however was prepared with less quantities (12 µl) of aliquots per lane as opposed to 30 µl per lane in the rest of the gels. It shows that saline treated controls (lane M) had several bands at 3 hpbm that were not present in lane L 30 hpbm. Not all the same bands continued to persist in treated females 30 hpbm (lane L). Lane D is a blank lane. In the treated insects some isozymes which disappeared in the controls 30 hpbm continued to persist while also some isozymes that appeared in the 30 hpbm saline control showed up. But in all cases (except for the topical treatments), the dpm counts in this aliquots from treated insects continued to be lower than 39,850 taken as the control dpm count at 30 hpbm. Lanes G and H had treatments where 1Pba(OtBu)-NH_2 and 9Fla-NH_2 mimics were applied topically.

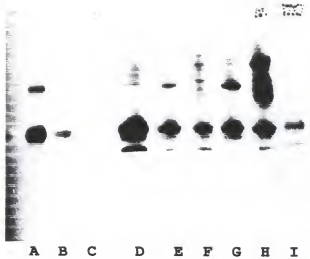


Figure 3-8. PAGE Fluorography of [1,3- ^{3}H]DIP-trypsin-chymotrypsin like isozymes of females of *A. aegypti*. The X-ray film was exposed to the gel for 45 days at -70°C. Each lane represents gut aliquots from one midgut equivalent.

(A) B, 10µg (25,851 dpm);
(B) 1Pba(OtBu)-NH_2, 4nmol (15,968 dpm);
(C) 9Fla-OH, 4.6nmol (16,445 dpm);
(D) 9Fla-NH_2, 2.0 nmol (87,038 dpm);
(E) 1Pba(OtBu)-NH_2, 4nmol. top, (136,126 dpm);
(F) A5, 10µg (31,249 dpm);
(G) A5, 10µg (56,950 dpm);
(H) Control, (13,186 dpm);
(I) Control, 3 hpbm (4,922 dpm).

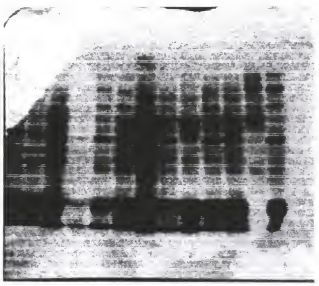
All trypsin levels at 30 hpbm, unless stated otherwise.

The dpm counts were 136,126 and 87,038 for the topical applications with 1Pba(OtBu)-NH_2 and 9Fla-NH_2, respectively, compared to injections with the same mimics that showed 15,968 and 25,851 dpm, respectively. Lane G showed much activity since probably the insect continued to synthesize its TLEs unaffected by the topical treatment.

Figure 3-10 shows incubations of some guts done with TPCK, an inhibitor of the production or expression of [1,3-^{3}H]DIP-chymotrypsin-like enzyme products in the incubates. The dpm counts were much lower in all cases than saline controls at 3 and 30 hpbm, which gave 2,807 and 18,786 dpm, respectively. Dpm counts in treatments were 5,468; 13,279; 6,485; 8,011; 6,226 and 7,044 dpm for guts dissected out of females treated with B; A; A5; A7; 9Fla-OH and B2, respectively. The bands of tritiated DIP-TLE products were similar in lanes B and C and somewhat similar to lane D with saline treatments at 3 hpbm; B, 10 µg; and A, 10 µg, respectively. This showed that retarding production of chymotrypsin-like products leads to evidence that trypsin-like products present in controls were absent in guts from treatments B and A. Lane E with A5, 10 µg treatment was similar to controls at 30 hpbm. However, treatment A7, and 9Fla-OH had upper profile bands absent revealing that the second isozyme present in controls 30 hpbm was diminished.

In all lanes B through H a middle isozyme band present in control lane A of 30 hpbm was absent. This revealed that three isozyme bands are affected differently by the treatments. They probably all have to be present in proportion to enable normal trypsin activity.

Figure 3-11 gives data on incubates from stable fly females and males. Control un-fed female had 6,969 dpm compared to un-fed male with 5,332 dpm. Three later dpm counts were 4,486 and 5,360 for female and males, respectively. Dpm counts did not change much after feeding as females had 7,892 and 5,345 dpm 24 and 120 hpbm given blood ad lib, while guts from a female treated with A7 at 5 µg had 4,138 dpm. Male dpm counts were 6,345 at 24 hpbm. The data showed a different trend of effect on protease availability in guts of the stable fly, as [1,3- ^{3}H]DIP-TLE product levels appeared stable or uniform in females from emergence to 120 hpbm. This supports the fact that stable fly TLEs are produced and present in the gut all the time and no dramatic episodes occur since they are not batch feeders.



A B C D E F G H I J K L M

Figure 3-9. PAGE Fluorography of [1,3- ^{3}H]DIP-trypsin-chymotrypsin like isozymes of females of *A. aegypti*. The X-ray film was exposed to the gel for 60 days at -70°C. Each lane represents gut aliquots from one midgut equivalent.

(A) B2,	10µg	(22,417	dpm);
(B) B2,	10µg	(21,159	dpm);
(C) Saline		(13,186	dpm);
(D) Blank;			
(E) A5,	10µg	(39,606	dpm);
(F) A5,	10µg,	(14,046	dpm);
(G) 1Pba(OtBu)-NH_2	4nmol. top	(136,126	dpm);
(H) 9Fla-NH_2,	4nmol. Top.	(87,038	dpm);
(I) 9Fla-OH,	4.6nmol	(16,445	dpm);
(J) 1Pba(OtBu)-NH_2,	4nmol	(15,968	dpm);
(K) 9Fla-NH_2,	4nm	(25,851	dpm);
(L) Saline		(13,186	dpm);
(M) Saline	3hpbm	(5,462	dpm);

All trypsin levels at 30 hpbm, unless stated otherwise.

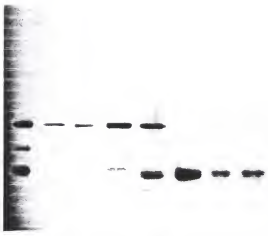


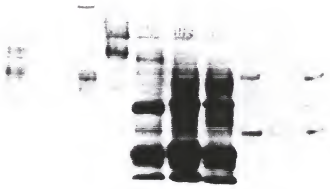
Figure 3-10. PAGE Fluorography of [1,3- ^{3}H]DIP-trypsin-like isozymes of females of *A. aegypti*. The X-ray film was exposed to the gel for 60 days at -70°C. Each lane represents gut aliquots from one midgut equivalent inhibited with TPCK.

(A)	Saline,		(18,786 dpm);
(B)	Saline, 3hpbm		(2,807 dpm);
(C)	B,	10µg	(5,468 dpm);
(D)	A,	10µg	(13,279 dpm);
(E)	A5,	10µg	(6,485 dpm);
(F)	A7,	10µg	(8,011 dpm);
(G)	9Fla-OH,	4.6nm	(6,226 dpm);
(H)	B2,	10µg	(7,044 dpm)

All trypsin levels at 30 hpbm, unless stated otherwise

Discussion

Gooding et al. (1973) reported that following uptake of a blood meal de novo synthesis of trypsin-like enzymes was initiated in the midgut of female mosquitoes. Briegel & Lea (1975) suggested that the stimulation was due to globular proteins present in the blood meal. These enzymes provide for orderly digestion of blood that is necessary for successful egg development. The overall process of ovary and egg development is regulated by a complex series of hormonal interactions. The mechanism whereby Aedes-TMOF oostatic hormone and the derived peptide-mimics cause inhibition of proteolytic enzyme activity was followed by concentrations that would cause over 50% oostatic activity. A direct relationship between oostatic activity and lack of synthesis of proteolytic enzymes was explored by directly injecting oostatic hormone into female *A. aegypti* (Borovsky 1988). About 75% of protease activity in the midgut of female *A. aegypti* is trypsin-like (Briegel & Lea 1975). Saline control TLE activity was lower than that from sham colony control guts. Based on the saline control activity at 3.76 nmol BApNA per minute per gut, the lowest activity



A B C D E F G H I J

Figure 3-11. PAGE Fluorography of [1,3- ^{3}H]DIP-trypsin-chymotrypsin like isozymes of females of *S. calcitrans*. The X-ray film was exposed to the gel for 60 days at -70°C. Each lane represents gut aliquots from one midgut equivalent.

(A) Control Un-fed female (6,969 dpm);
(B) Control un-fed male, (5,332 dpm);
(C) Control un-fed female, 3h later (4,486 dpm);
(D) Control un-fed male, 3h later (5,360 dpm)
(E) Vitellogenic control 60 h later (5,345 dpm);
(F) Control 24 hpbm female (7,892 dpm);
(G) Control 24 hpbm female (5,313 dpm);
(H) Control 24 hpbm male (6,345 dpm);
(I) Saline control 24 hpbm male (8,244 dpm);
(J) A7, 5 µg, 24 hpbm (4,138 dpm).

All trypsin levels at 30 hpbm, unless stated otherwise

was observed with A6, CblAla-NH_2 and 9Fla-OH, hexaproline fragment, 1Pba(OtBu)-NH_2 and PY1 collected one purification step before 1Pba(OtBu)-NH_2, with less than 1 nmol BApNA degraded. Moderate activity was observed with TMOF-A, A5, A7, TMOF-B, B2, and 9Fla-NH_2. TLE activity could not be assessed with toxic samples that generally proved lethal to the insects between 3 and 30 hpbm for mosquitoes as in A4 and A6.

However, Graf & Briegel (1985) and Borovsky & Schlein (1988) reported that several [1,3-^{3}H] DIP-TLE isozymes are synthesized in the midgut of *A. aegypti,* sand flies, *P. papatasi*, house flies, *M. domestica*, stable flies, *S. calcitrans* and even lice, *Pediculus humanus.* Female mosquitoes injected with oostatic hormone synthesized 87% less of the [1,3-^{3}H] DIP-TLEs i.e. 13,625: 99,167 cpm (Borovsky et al. 1989). In these studies saline control readings of 93,850 dpm were obtained with fresh sham operated females, while saline controls placed in storage for months gave readings of 39,432 dpm. When compared to the 39,432 dpm reading, 50% reduction in TLEs levels was obtained (79% if the 93,432 dpm count is taken as standard). Percentage retardations below 50% were attained with A7 at 10 µg and 1Pba(OtBu)-NH_2. Retarded TLE levels were also obtained in insects that survived the toxic 9Fla-

OH mimic. It is possible that toxic peptide-mimics cause the stressed (dying) insects to not perform physiological functions normally. Samples were shared between assays with BApNA and tritiated DFP such that in cases with A4 and A6, samples were not available for DFP assays. The two assays show reduced activity and levels of enzymes, especially so with the toxic peptides and moderate TLE activity and levels with oostatic peptides and peptide-mimics. The fact that there were reduced levels of tritiated DIP-TLE products in all cases revealed that not all products in this mosquitoes were trypsin-like. TPCK inhibits production of chymotrypsin-like enzymes. Therefore even with retarded TLE activity in treated mosquitoes, chymotrypsin digests some amount of blood meal protein to avail the insect with some amino acids for yolk protein synthesis. This could also be another reason why some peptide and peptide-mimic treatments were only partially oostatic even if little TLE were being produced.

Isozyme profiles in saline treated controls supported earlier studies by Noriega et al. (1996a & b) and Khalkok et al. (1993) that the trypsin profiles in *A. aegypti* change following uptake of a blood meal from an early trypsin to late trypsin form in about 24-36 hours. With bands present in 3 hpbm guts, it was interesting to see how

they continued to persist in treated mosquito guts and the absence of the late trypsin prominent band seen in Figure 3-7 kept at -70°C for 60 days. This indicated that the conversion of early to late trypsin was being negatively affected by the treatments. Figure 3-9 also exposed at -70°C for 60 days showed some mosaics whereby both the early and late trypsin bands appeared. This revealed a situations whereby partial oostatic activity was recorded and the incubates were from a homogenized group sample. Incubations with TPCK, a chymotrypsin inhibitor revealed, less banding at 3 hpbm and in all treatments at 30 hpbm. This also showed that the 3 hpbm band was present in TMOF-A, TMOF-B treated insects and the band present in 30 hpbm saline treated insects was clearly absent. This indicated inhibition of trypsin-like enzymes in the midgut of mosquitoes that were treated with TMOF-A and B. The peptide and peptide mimics treatments revealed interesting mosaics whereby some early trypsin bands and also the late trypsin band appeared slightly with A5 with a light late trypsin band and A7, B2 and 9Fla-OH with light early trypsin bands. This again showed that the normal process of conversion of early to late trypsin was inhibited with TMOF-A and B, and slightly affected with the peptide mimics.

The trend of banding was not followed in stable flies in which dpm counts did not reveal dramatic differences between un-fed and saline treated controls from day zero to 60 hours provided blood available ad lib. The banding pattern of the isozymes showed remarkable tritiated DIP-TLE products with females at 24 and 60 from first blood meal. There were more bands formed in the 24 post first blood meal lanes when compared to the other lanes. This supported the idea that early in the adult life, mature trypsin results from cleavage of 2 kDa fragment of about 18 amino acids to produce a mature trypsin isozymes of 22,900 and 31,600 kDa. Stable flies have translational and not transcriptional control of trypsin biosynthesis (Moffatt & Lehane 1990). While trypsin is stored as an inactive pro-enzyme, it is secreted into the gut lumen in a fully active form.

CHAPTER 4
EVIDENCE OF OOSTATIC AND TRYPSIN MODULATING ACTIVITY IN EXTRACTS FROM OVARIES OF THE STABLE FLY, *STOMOXYS CALCITRANS*

Introduction

Since the report on the existence of an ovarian development inhibiting hormone present in the corpora lutea of ootheca-bearing cockroaches, *Blatella germanica* and *Blatta orientalis,* research has been geared towards the elucidation of mechanisms regulating oogenesis and egg production. Since then research has uncovered oostatic hormones in a variety of insects with a majority of them of ovarian origin (Iwavnov and Mescherskaya 1935). In Diptera, Adams et al., (1968) first demonstrated the existence of oostatic hormone(s) in the house fly, *Musca domestica* in when complete inhibition of a second gonotropic cycle occurred, mature eggs from the first ovarian cycle were retained.

Furthermore, extracts from mature ovaries injected into newly emerged females inhibited progress into the vitellogenic phase of ovarian maturation. Meola and Lea (1972) demonstrated a similar mechanism in mosquitoes,

involving an ovary produced humoral factor inhibiting yolk deposition in less developed egg follicles.

In *A. aegypti,* Borovsky (1985) observed that ovary-derived TMOF (Aedes-TMOF), injected into newly emerged females disrupts orderly blood digestion essential for successful egg development. Trypsin biosynthesis in several hematophagous insects, including stable flies, was retarded up to 48%. This suggested cross-reactivity of Aedes-TMOF among hematophagous insects that use trypsin-like enzymes for protein digestion. To date, three inhibitors of ovarian development from Diptera were characterized: These are Aedes-TMOF from *A. aegypti* (Borovsky et al., 1990, 1993) and Neb-TMOF and Neb-Collostatin from *Neobellaria bullata* (Bylemans et al., 1994). It is possible that all three oostatins are cleaved products of yolk proteins. Cross reactivity of Aedes-TMOF across hematophagous insects that utilize trypsin-like-enzymes for protein digestion and the occurrence of such factors in both mosquitoes and flesh flies led to speculation that TMOF-like peptides occur in the ovaries of stable flies too. Such an ovary-derived hormone may function to modulate blood digestion and can therefore be assessed for bioactivity in the mosquito, *A. aegypti* and the stable fly, *S. calcitrans*.

While *A. aegypti* needs one blood meal to mature an egg batch, female stable flies require up to five sequential

blood meals to mature the first egg batch. Therefore I extracted, characterized, and evaluated an extract from vitellogenic ovaries of stable flies for a fly ovary derived factor (SFOV) with similar properties to Aedes- and Neb-TMOF. Assays for oostatic and trypsin modulating properties were conducted on *A. aegypti* and *S. calcitrans*. Characterization of SFOV was done following the procedure used for Aedes-TMOF (Borovsky et al., 1990). The evaluations for bioactivity were performed as described with Aedes-TMOF peptides and peptide-mimics on *A. aegypti* and *S. calcitrans*.

Materials and Methods

Stable Fly Rearing

Stable flies reared in the fly rearing facilities at the USDA-CMAVE insectaries were used for this study. Maintenance conditions were as described in Chapter 3. Newly emerged adults at various ages provided ovaries for the search for a stable fly oostatic factor.

Preparation of the Stable Fly Ovary-Derived Factor (SFOV).

SFOV was isolated from vitellogenic ovaries five days after female stable flies were fed blood ad lib. The ovaries were dissected out and placed in distilled water containing 1 mM phenyl methyl sulfonyl fluoride (PMSF) acidified to pH 4.5 with 1 M hydrochloric acid. Fifty ovary equivalents were homogenized and the homogenate was centrifuged for 20 minutes at 20,000g in an Eppendorf centrifuge at 4 °C and the supernatant harvested. The supernatant was heat treated at 50 °C in a water bath for 10 minutes and recentrifuged in the cold for 20 minutes. The fresh supernatant was collected, frozen and lyophilized on a Virtis lyophilizer.

The freeze-dried sample was reconstituted in 0.1% trifluoroacetic acid (TFA) in HPLC water and placed on Sep-Pak (35 cc) and eluted with increasing concentrations (10, 20, 30, 40, 50, 80%) of acetonitrile / 0.1% TFA (Borovsky, et al., 1990). The fractions were dried on a Speed-Vac at 40°C, lyophilized and reconstituted in saline for bioassays for both ovarian development and trypsin biosynthesis and activity.

Characterization of the SFOV.

A portion of the lyophilized 10% acetonitrile in 0.1% TFA fraction from Sep-Pak separation of vitellogenic ovaries

was reconstituted with 0.1% TFA and further separated with Hewlett Packard High Pressure Liquid Chromatography (HPLC) on a reverse-phase C_{18} HPLC column. The HPLC parameters were as follows: two minutes pre-run with 100% water in 0.1% TFA, followed with a gradient run of 0 to 80% acetonitrile in 0.1% TFA over 30 minutes (Figure 4-1) at ~800 millibars. Samples evaluated came from newly emerged females, 2-day old females, vitellogenic females, and 5-day old males.

The methods of chapters 2 and 3 were adopted for evaluation of oostatic and trypsin modulating activity, respectively.

Results

Preparation of the Stable Fly Ovary-Derived Factor (SFOV).

The preparation process for the ovary-derived peptides placed strong emphasis on looking for a safe procedure that would not damage conformation and active site(s) of a probable SFOV. It was expected that an SFOV would be similar to Aedes-TMOF which is a deca-peptide with its active site for recognizing and interacting with the midgut receptors

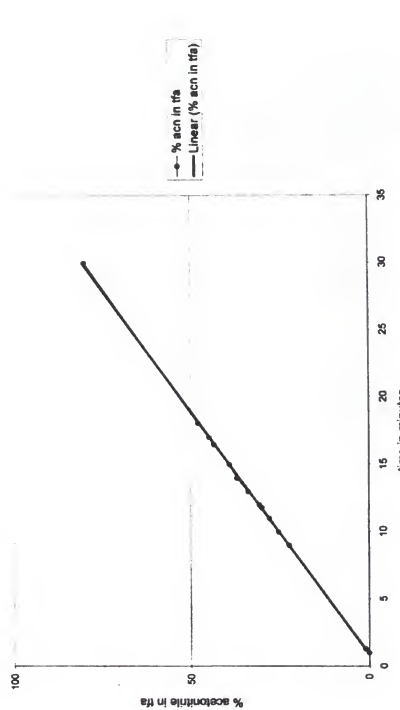


Figure 4-1. Reverse HPLC gradient used in the preparative runs

responsible trypsin production center around the amino acid tyrosine found at the N-terminal.

Evaluation of Oostatic Activity and Trypsin Properties

Ovaries from 200-1000 females in a batch provided a starting tissue sample. Heat-treatment at 50 °C for exactly 10 minutes enabled larger peptides and yolk proteins that might be toxic to precipitate. Lyophilizing allowed removal of solvent from the sample without use of heat, which denatures proteins and peptides creating distortions in configuration of compounds and possibly affecting their bioactivity. The 0.1% TFA was the preferred solvent for HPLC runs both with Sep-Pak and regular chromatography on the Hewlett Packard Chromatograph.

Characterization of the SFOV

Data summarized in Figure 4-1 were obtained by taking readings of % acetonitrile in 0.1% TFA against time as the gradient was running. High peaks were consistently found highest at around 10 minutes, for five sets of samples were collected from 1997 to 1999. At times 0-4, 4-6, 6-9, 9-12, 12-15 minutes into the run, collection of fractions 1, 2, 3,

4, and 5 were made up to twenty times, respectively. The acetonitrile in the collected fractions was removed from samples by using a Speed-vac and the remaining liquid frozen and lyophilized to remove the remaining liquid. A portion was reconstituted in Ringer's saline, and injected into 5-day old mosquitoes at varying doses, and remaining portions that were bioactive were submitted to ICBR for Matrix Assisted Laser Desorption Ionization-Time of Flight-Mass Spectroscopy (MALDI-TOF-MS).

Evaluation of SFOV Oostatic Activity

Bioactivity of the lyophilized Sep-Pak fractions reconstituted with 1x Ringer's saline from 10, 20, 30, to 40% acetonitrile varied when tested against *A. aegypti*. Dried weights obtained for the fractions were 8.5, 160, 80, 20, 13, 13, and 84 mg for fractions at 10, 20, 30, 40, 50, 60, and 80% acetonitrile in 0.1% TFA respectively. The dried fractions were reconstituted in 100 µl start solution and dilutions made to appropriate concentrations so that around 10 µg was injected into each female treated. Usually mortality resulted at higher concentrations with no survivors at 20 and 40 µg doses (not shown). Females that survived at the dose of 10 µg per female revealed oostatic

activity with the 10% acetonitrile fraction as egg follicle length was no more than 88.5 μm ± 19.41 (21/0) at 30 hpbm. This was in comparison to follicle length measurements for the 20, 30, 40, 50% and saline control fractions of 166 μm (12/8), 111 μm (9/12), 163 μm (15/4), 100 μm (1/19) and >200 μm (18/2) respectively, at 30 hpbm (Table 4-1).

The 10% acetonitrile in 0.1% TFA Sep-Pak fractions were lyophilized and the dried sample reconstituted in 0.1% TFA. It was injected into the HPLC (Hewlett Packard) on reverse-phase C_{18} HPLC to attain further separation. The fractions were collected based on Fig 4-2a as a guide and portions were collected at: 1) 0-4, 2) 4-6, 3) 6-9, 4) 9-10, 5) 10-12, and 6) 12-25 minutes and categorized as portions 1, 2, 3, 4, 5 and 6, respectively. Individual portions 1, 2, 3, 4, and 5 were re-analyzed under the same conditions (as above) on the reverse-phase C_{18} HPLC and gave consistently similar peaks with retention times similar to peaks that appeared in the original 10% ACN fraction with portions 4 and 5 (Figures 4-2b-d). The collected portions 1, 2, 3, 4 and 5 were bioassayed on 5-day old *A. aegypti* females, 3 hpbm and oostatic activity scored at 30 hpbm. The bioassays showed that portion 4 tested at 6.1 μg / female had oostatic activity and egg follicle length was 83.33 ± 12.19 μm (15/5), compared to 200 ± 28.1 μm (18/2) for controls (see

Table 4-1). Portion 5 at 6.7 µg / female was toxic, with egg follicle length of 75 µm (1/9) compared to 200 ± 28.1 µm (90%) for controls.

Evaluation of Trypsin Modulating Activity

Injections of SFOV Sep-Pak fractions and HPLC portions into blood-fed female *A. aegypti* resulted in inhibition of trypsin-like enzyme (TLE) activity in the midgut of treated females at 30 hpbm (Table 4-2) with fraction 10% acetonitrile in 0.1% TFA and specifically portion 4 collected off the HPLC at 9-10 minutes. The kinetics of trypsin-like enzymes were 2.376 nmoles BApNA/minute/gut with SFOV HPLC portion 4, 4.916 nmoles with colony control, and 3.764 nmoles with saline injected control females.
The protein content measurement (total blood protein and enzyme protein) in all samples analyzed was above 250 mg/ml, which indicated that the insects were alive during the period of treatment. Analysis of excreta from treated insects revealed some trypsin activity in the excreta but very low protein content which in some cases could not be calculated (results not shown).

Table 4-1. A comparison of the effect of peak fractions of SFOV on ovarian development in *A. aegypti* showing follicle length achieved and number attaining length or number dead (%)

% ACN in 0.1% TFA	Dose per female (µg)	Follicle length at 30 hpbm (µm)	(%)	Mortality (%)
Control		200 ± 28.1	(18)	(10)
50% ACN	6.5	100	(1)	(95)
40% ACN	10	163.33 ± 39.94	(15)	(20)
30% ACN	8	111.11 ± 22.05	(9)	(60)
20% ACN	8	166.67 ± 32.57	(12)	(40)
10% ACN	8.5	88.46 ± 19.41	(21)	(0)
4 of 10% ACN*	6.1	83.33 ± 12.19	(15)	(25)
5 of 10% ACN*	6.7	75	(1)	(95)

Follicle length at 30 hpbm.
* = portions 4 and 5 obtained 9-10 and 10-12 minutes into the HPLC run of the 10% acetonitrile Sep-Pak fraction.

Figure 4-2a. Reverse phase C_{18} HPLC Chromatogram of 6-day old, vitellogenic, *S. calcitrans* females (10% ACN/0.1% TFA Sep-Pak fraction 0-30 mins)

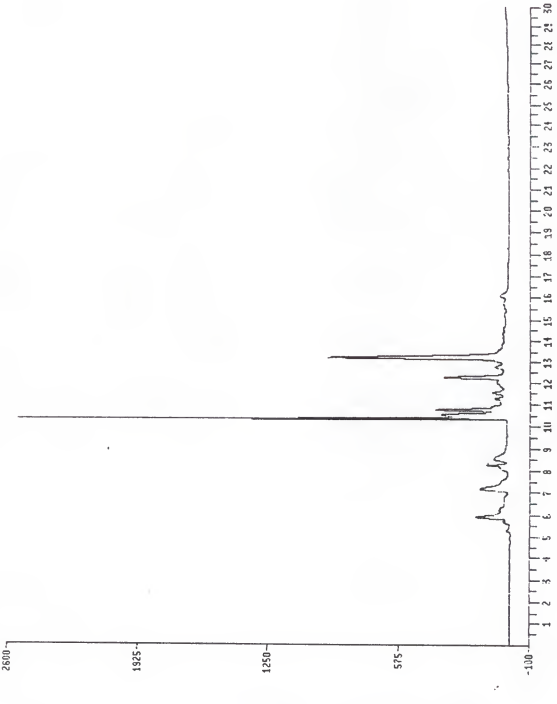


Figure 4-2b. Reverse phase C_{18} HPLC Chromatogram of 6-day old, vitellogenic, *S. calcitrans* females (10% ACN/0.1% TFA Sep-Pak portion 4-6 mins)

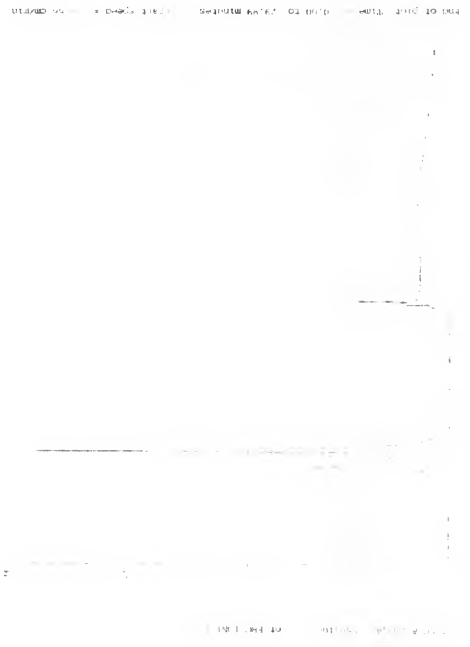


Figure 4-2c. Reverse phase C_{18} HPLC Chromatogram of 6-day old, vitellogenic, *S. calcitrans* females (10% ACN/0.1% TFA Sep-Pak portion 9-10 mins)

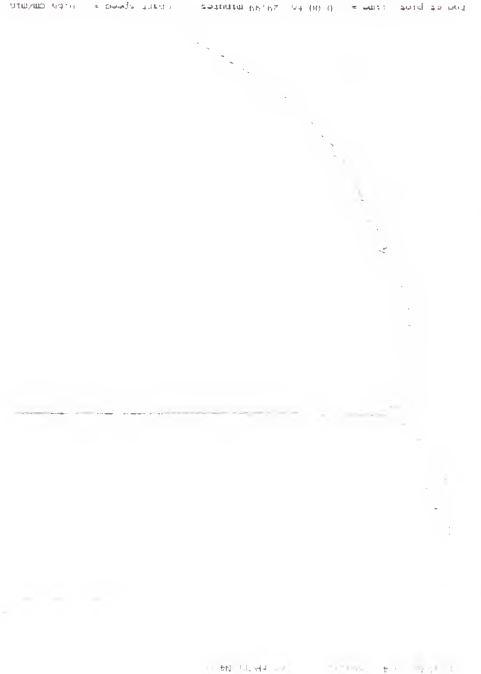


Figure 4-2d. Reverse phase C_{18} HPLC Chromatogram of 6-day old, vitellogenic, *S. calcitrans* females (10% ACN/0.1% TFA Sep-Pak portion 10-12 mins)

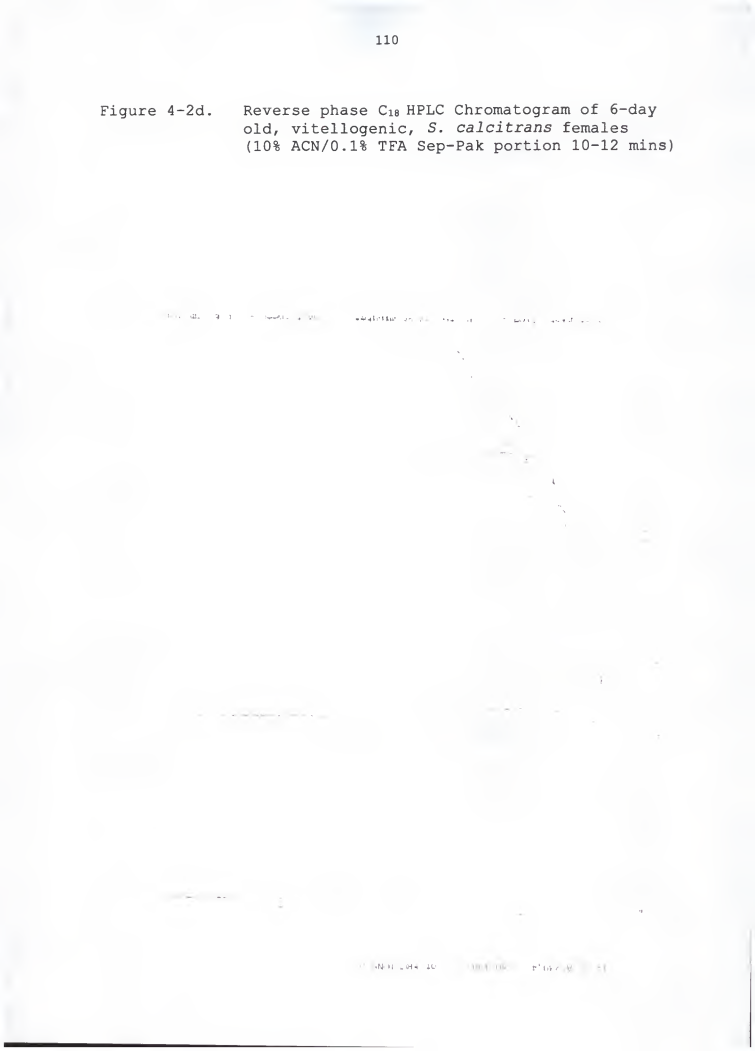


Table 4-2. A comparison of the effect of SFOV on trypsin activity in female *A. aegypti*.

Females Fed on pig	Trypsin activity (nmol BApNA/min/gut)	Protein (mg/ml)
Control	4.916	289.824
Saline control	3.764	269.567
A	2.872	278.080
A5 10ug/female	2.043	501.667
A6 10ug/female	0.264	349.239
A7	1.632	332.160
A8 10ug/female	1.309	407.102
B 10ug/female	2.002	332.320
SFOV (HPLC portion)	2.376	254.27

Trypsin activity and levels at 30 hours post blood meal.

Matrix Assisted Laser Desorption Ionization-Time of Flight-Mass Spectra (MALDI-TOF-MS) of the SFOV Sample.

MALDI-TOF analysis of 10% SFOV Sep-Pak fractions from newly emerged, two-day old, vitellogenic females and older males revealed the presence of more peptide compounds in the sample from vitellogenic females. MALDI-TOF-MS spectra showed the presence of a candidate peptide at *m/z* 908.8 in the bioactive fraction (Figure 4-3a). This peptide was not seen in spectra of younger females or males of the same age as vitellogenic females. Other potential peptides were seen at *m/z* ~ 2,935 in both young and vitellogenic females, and others ranging from 3,603 to 6,944 were present only in females with vitellogenic ovaries (Figure 4-3a & b).

Amino acid analysis of HPLC portion 4, originally from the 10% Acetonitrile in 0.1% TFA fraction, revealed a peptide of the following amino acids: Aspartic acid, Asparigine, Serine, Glutamine, Threonine, Glycine, Glutatamic acid, Histidine, Alanine, Arginine, Tyrosine, Proline, Methionine, Valine, Phenyl alanine, Isoleucine, Lysine and Leucine. The proportions of Proline, Arginine and Lysine were highest.

Figure 4-3a. Mass spectra of HPLC fraction from 10% ACN in 0.1% TFA from vitellogenic ovaries of *S. calcitrans* (L) offered blood for four days.

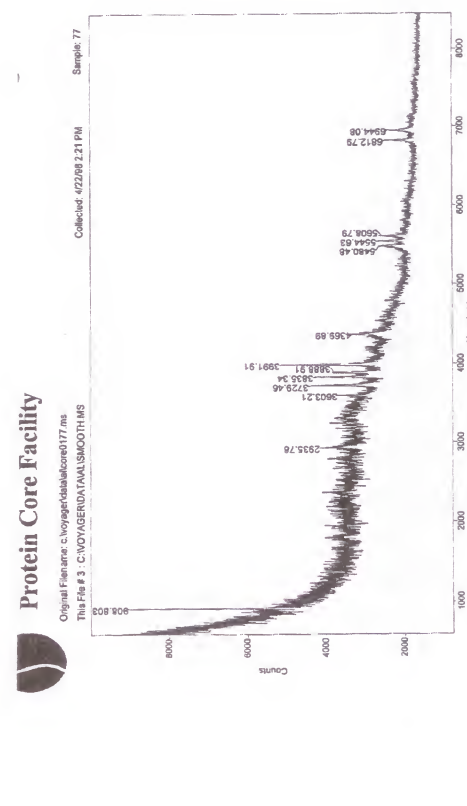


Figure 4-3b. Mass spectra of HPLC fraction from 10% ACN in 0.1% TFA from ovaries of 3-day old *S. calcitrans* (L) offered blood for two days.

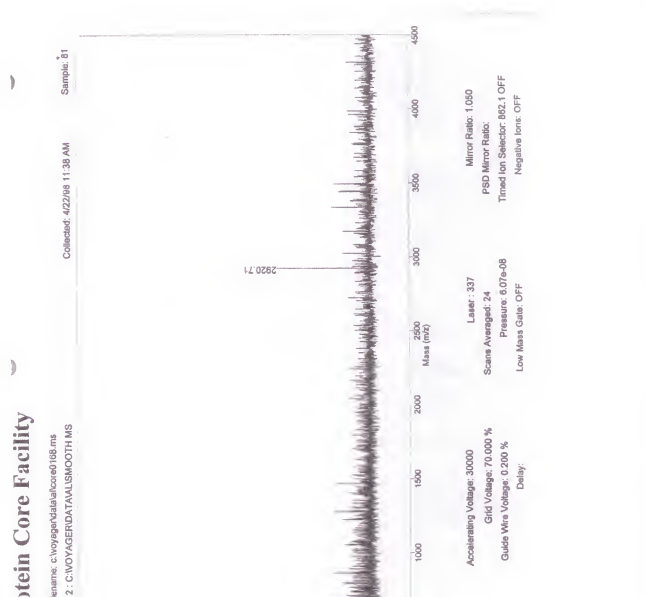
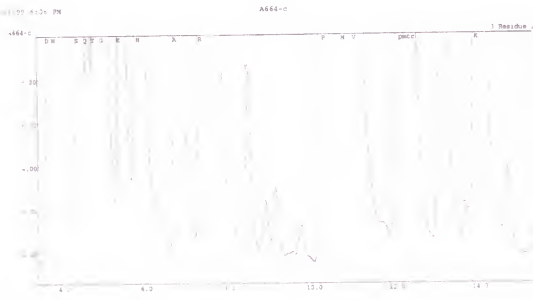


Figure 4-4. Amino acid analysis of SFOV portion 4 of the 10% ACN in 0.1% TFA fraction.



Residue 1 Interpolated Baseline

Peak No	RT	Peak ID	Type	Height	Pmol Ht
1	3.80	Asp	B	120540	297.68
2	3.96	Asn	C	548855	428.50
3	4.54	Ser	C	540999	1013.11
4	4.74	Gln	C	125248	213.72
5	4.93	Thr	BC	290534	395.50
6	5.15	Gly	BC	577565	647.24
7	5.56	Glu	C	390359	564.50
8	5.9	His	C	154321	296.76
9	6.92	Ala	C	537412	792.06
10	7.53	Arg	C	1688897	9148.82
11	7.88			17579	1740.53
12	9.25			11645	0.00
13	9.52	Tyr	C	72066	75.78
14	9.2			21623	0.00
15	9.27			26497	0.00
16	9.67			1305	0.00
17	9.90			12625	497.25
18	10.50	Pro	C	3572145	9346.02
19	10.98	Met	C	238895	261.88
20	11.25	Val	C	857729	937.82
21	11.86			12752	0.00
22	12.18	pmcc	B	272527	647.64
23	12.57			180576	0.00
24	13.10			469633	0.00
25	13.89	Ile	C	19990	26.55
26	14.19	Lys	C	1224377	1121.05
27	14.48			13089	0.00
28	15.43			9752	0.00
29	15.62			1163	0.00
30	16.36			422	0.00
31	16.60			5543	0.00
32	17.22			1423	0.00
33	17.60			414	56.77
34	17.90			10524	559.79

Discussion

The ability of oostatic extracts to inhibit vitellogenesis has now been established in several species of Diptera including *M. domestica* (Adams et al. 1968), *Aedes atropalpus, D. melanogaster* (Kelly et al. 1984), *A. aegypti* (Borovsky 1985), and recently *N. bullata* (Bylemans et al. 1994). The existence of oostatic hormone was observed first in aqueous ethanol extracts of ovaries from *M. domestica*. The extracts inhibited ovarian development but were relatively toxic. Semi-purified extracts induced 50% inhibition of ovarian development at 25 µg (Adams et al. 1968, 1970), while heat-treated aqueous extracts were oostatic at a dose of only 11.2 µg protein.

Tobe (1980) cautioned that a general mechanism of action of such oostatins was difficult to establish since it would probably act on different levels of the endocrine hierarchy controlling reproduction in insects. Mechanisms may inhibit release of neurosecretory material from the median neurosecretory cells e.g. EDNH (Adams 1981); act directly on the as ovary inhibitors JH-induced follicle cell patency as in *R. prolixus* (Davey & Huebner 1974) or may inhibit ovarian ecdysteroid synthesis in *A. atropalpus* (Kelly et al. 1984).

The extraction procedure that was used to obtain Aedes-TMOF was adopted for the present search for the stable fly derived oostatic hormone. Oostatic hormone activity was first noticed with ovary extracts that were prepared from five-day old vitellogenic females. Vitellogenic ovary sample was compared to samples prepared the same way obtained from newly emerged, three-day old females exposed to two blood meals ad lib or even 5-day old male abdomens. Stable flies use the first two blood meals for general adult development and it is the last three blood meals that are directly used for vitellogenic growth Moobola & Cupp (1978). Oostatic activity of extracts appears low during pre-vitellogenesis and increases during vitellogenic stages. Cross-reactivity between Ae-TMOF and NEB-TMOF has also been reported (Bylemans et al. 1994). These data suggest that oostatins are widely distributed in insects that exhibit cyclic ovarian maturation, especially as *Musca* oostatins were recognized by Ae-TMOF antibody (Borovsky et al. 1994a). However, Neb-collostatin has no sequence similarity with Ae-TMOF but appear to bear the same mode of action. Oostatic hormone may therefore be a family of small peptides synthesized and secreted in different tissues to inhibit the initiation of second and subsequent cycles of oocyte development while the already mature and developing first cycle oocytes are still present.

Dissecting ovaries from females was done with at least 200-1000 females to enable describing the sample in terms of ovary equivalents later. Placing ovary tissue into 1 mM PMSF at pH 4.5 helped to preserve the bioactivity of any peptides when held under in acidic conditions. Heat treatment at no higher than 50 °C enabled larger peptides, yolk proteins and toxins to precipitate and not interfere with sample preparation later. From then onwards freezing temperatures were preferred as well as acid conditions to retain bioactivity.

Lyophilizing enabled drying of the sample without use of harsh heat which would have denatured the peptides, leading to distorted conformations and loss of bioactivity. TMOF-like peptides modulate trypsin bioactivity by recognizing a midgut receptor and interacting with it. Hence the emphasis on use of a procedure unlikely to damage an active site(s) suspected to reside on Aedes-TMOF is on the amino acid tyrosine found at the N-terminal of TMOF (Borovsky et al. 1994b).

Lyophilized samples were reconstituted in 0.1% TFA, which is acidic in nature, to preserve the peptides in a bioactive condition. TFA also promotes adsorption of peptides on the front of the C_{18} reverse phase columns both in the Sep-Pak and RP-HPLC columns. Adsorption to the column front is the basis of RP-HPLC that enables eluents run

through the column at an increasing gradient of polar to non-polar solvents to separate compounds by their polarity. As the gradient progresses, and depending on its interaction with both the column and the peptides, the peptides are separated. Both the Sep-Pak and RP-HPLC use the same type of C_{18} reverse phase column packing. The Sep-Pak columns enabled rapid separation of the sample into fractions that were checked for bioactivity.

After confirming that the 10% fraction was bioactive, a portion of the sample was reconstituted in 0.1% TFA for HPLC, and later for MALDI-TOF-MS spectra. Preparative HPLC of samples collected sequentially and processed at separate times between 1997 and 1999 continued to yield the same characteristic peaks seen in Figure 4-2a. Furthermore, the individual portions were collected on the RP-HPLC at different times by using an increasing acetonitrile concentration gradient over 30 minutes. All the portions collected at 0-4, 4-6, 6-9, 9-10 and 10-12 minutes continued to yield peaks at times very similar to the original 10% acetonitrile /0.1% Sep-Pak sample from which they were collected. This provided the confidence that HPLC was an excellent tool for separation of the peptide fractions. The individual portions were injected into *A. aegypti* females and fraction 4 with a near-perfect single peak was found to be oostatic at 30 hpbm. The females were analyzed for

evidence of egg development retardation and also gut bioactivity. Females injected with fraction 4 had reduced trypsin-like activity and produced less trypsin over the 30 hours until dissection. MALDI-TOF-MS of portion 4 included a candidate material, possibly a peptide, that could be responsible for biological activity. Females injected with portion 4 had little gut gut trypsin-like activity as it probably produced very little trypsin over the 30 hours until they were dissected. MALDI-TOF of 10% acetonitrile in 0.1% TFA showed a posible novel peptide responsible for biological activity seen here.

CHAPTER 5
SUMMARY AND CONCLUSIONS

Because of the role of hematophagous arthropods as vectors, the reproduction of these arthropods has attracted much attention. Adult hematophagous insects require a blood meal for egg production and midgut proteases play a major role in the digestion of the meal. Digestive enzymes in hematophagous insect vectors constitute one of the early barriers for any parasite ingested with a blood meal. Since proteins are the most obvious nutrient in blood, much effort has been placed on studying proteinases in hematophagous arthropods.

The present study was designed to investigate the effect of different chemically formulated peptides and mimics derived from Aedes-TMOF on egg development and trypsin like enzymes were affected. Compounds with Aedes-TMOF activity could be possible oostatins for hematophagous insects. In *A. aegypti*, there is a transitory decline in midgut proteinase activity immediately after blood feeding which Fisk & Shambaugh (1952) suspected to be due to substrate depletion of enzymes or inhibition by specific proteinase inhibitors in blood serum. Khalkok et al. (1993)

confirmed that there was a transtition from an early trypsin to late trypsin. This study on various Aedes-TMOF peptide and peptide mimics revealed that the conversion of early to late trypsin can be delayed by certain treatments. The fact that autogenous *Aedes atropalpus* with mature or nearly mature eggs are incapable of normal digestion of a blood meal (Hudson 1970) and that decapitation of *A. aegypti* after feeding depresses proteinase levels suggested the possibility of hormonal involvement in digestion especially when females are gravid with eggs. The appearance of hormonal signals early in a gonotropic cycle leads to information being transmitted to the midgut to depress its level of TLE production. While *A. aegypti* has naturally occurring TMOF coming into play to moderate trypsin production, this study proved that altering the original natural peptide could achieve an oostatic effect or a toxic effect. Gooding (1973) used BApNA to evaluate levels of trypsin present in guts based on µmol BApNA hydrolyzed per minute per gut and was able to characterize and determine that in *A. aegypti* both mRNA and trypsin protein synthesis take place after ingestion of a blood meal. Isoenzyme profiles of tritiated DIP-TLE products in saline treated controls supported earlier studies by Noriega et al., 1996a

& b) that an early trypsin mRNA transcript appears and is prominent within 3 hours from uptake of a blood meal. It then disappears and is replaced by the mature late trypsin form that actively digests a blood meal by 24-36 hpbm. BApNA was also used in this studies to determine whether trypsin activity profiles were affected in mosquitoes and stable flies treated with Aedes-TMOF peptide and peptide-mimics. Injections with all the treatments affected trypsin activity. BApNA as a substrate was very useful because the rate of degradation to p-NA directly depended on how much active trypsin was available in the gut homogenate. Lower degradation rates implied that less enzyme was available to degrade (cleave) the substrate and release the colorimetric p-NA. In addition, more support to the amount of available active TLE was obtained by irreversibly binding available TLEs to tritiated DFP. Actual levels determined by scintillation counting revealed that peptide and peptide-mimics treatments were also affecting trypsin levels. This case was not seen with topically applied peptides. Acetone used to dissolve them may not have been the best solvent for peptide-mimics. It was therefore not likely that much mimic could have stayed on the insect cuticle long enough to penetrate the cuticle. Levels of actual TLEs present were established by irreversibly binding TLEs to tritiated DFP

and determine by scintillation counting and isoenzyme profile patterning detected by fluorography of the tritiated DIP-TLE products.

Egg development closely follows blood digestion as primary egg follicles begin to grow in size beyond the resting stage after uptake of a blood meal. The primary follicles of newly emerged females are very small (below 50 µm long) and cannot be differentiated from the surrounding nurse cells. In treated mosquitoes with peptide and peptide-mimic, the primary follicle growth is not so synchronized and does not occur in a normal pace when compared to saline treated controls that had reached over 200 µm over the 30 hpbm period. This was correlated to the fact that with most peptide treatments, the early trypsin profiles continued to be seen long after this early transcript had disappeared from saline control guts. In some cases, there were both early and late trypsin transcripts present at 30 hpbm, which implied that some late trypsin biosynthesis was going on. However, in both situations, the persistence of an early trypsin transcript must have created a deficit in late trypsin supply to digest the blood to provide yolk protein precursors.

In *S. calcitrans,* blood feeding neither causes an immediate rise nor a transitory decline in general midgut proteinase activity. A steady rise of TLE activity was instead noted in proteinase content in the midgut. This may be due to activation of pre-existing material or de novo synthesis of the enzyme (Champlain & Fisk 1956). It was not conclusive whether TLE production was actually being affected by the treatments. It was concluded that Aedes-TMOF peptides were not active oostatins for stable flies. In the course of five blood meals required for maturing an egg batch, it appeared that the insect could recover and compensate for any retarded availability of blood meal-derived yolk protein precursors.

The search for natural TMOFs in stable flies revealed an ovary derived oostatic factor (SFOV) from vitellogenic ovaries. SFOV was oostatic and trypsin modulating in *A. aegypti* and had trypsin modulating properties but no observable oostatic activity on the stable fly. It is possible that stable flies possess proline- specific peptidases in their hemolymph that could actively sequester, destroy and thereby regulate any excess or unnatural peptides circulating in the hemolymph (Martensen et al., 1998). While Neb-TMOF (sequence NPTNLH) that was hydrolyzed

by this peptidase had only one proline in its sequence, a high proline content was observed in the amino acid composition of the SFOV portion 4 obtained at 9-10 minutes in the RP-HPLC run.

REFERENCES

Adams, T. S. & M. S. Mulla. 1967. Reproductive biology of *Hippelates collusor* (Diptera: Chloropidae) Gametogenesis. Ann. Entomol. Soc. Amer. 60: 1177-1182.

Adams, T. S. & D. R. Nelson. 1968. Bioassay of crude extracts for the factor that prevents second matings to female *Musca domestica*. Ann. Entomol. Soc. Amer. 61: 112-116.

Adams, T. S., J. D. Johnson, C. L. Fatland & G. Olstad. 1970. Preparation of a semi-purified extract of the oostatic hormone and its effect on egg maturation in the housefly, *Musca domestica*. Ann. Entomol. Soc. Amer. 63: 1565-1569.

Adams, T. S. 1981. The role of voarian hormones in maintaining in maintaining cyclical egg production in insects. In: Advances in Invertebrate Reproduction. Clark, W. H. Jr., Adams, T. S., eds. Elsevier/ North Holland, Amsterdam, pp 109-125 (1981).

Barillas-Mury, C., R. Graf, H. H. Hagedorn & M. A. Wells. 1991. cDNA and deduced amino acid sequence of a blood meal induced trypsin from the mosquito *Aedes aegypti*. Insect Biochem. 21: 825-31.

Bauer, P. W. Rudin & H. Hecker. 1977. Ultrasturtural changes in midgut cells of female *A. aegytpi* L. (Insects, Diptera) after starvation or sugar diet. Cell Tissue Res. 177: 215-219.

Beckenmeyer, E. F. & A. O. Lea. 1980. Induction of follicle separation in the mosquito by physiological amounts of ecdysone. Science 209: 819-821.

Blakemore, D., S. Williams & M. J. Lehane. 1995. Protein stimulation of trypsin secretion from the opaque midgut cells of *Stomoxys calcitrans*. Comp. Biochem. Physiol. 110B. #2: 301-307.

Borovsky, D. 1982. Release of egg development neurosecretory hormone in *Aedes aegypti* and *A. taeniorhynchus* is

induced by an ovarian factor. J. Insect Physiol. 28: 311-316.

Borovsky, D. 1985a. Isolation and characterization of a highly purified mosquito oostatic hormone. Arch. Insect Biochem. Physiol. 2: 333-349.

Borovsky, D. 1985b. Characterization of proteolytic enzymes from the midgut and excreta of the biting fly, S. *calcitrans* Arch. Insect Biochem. Physiol. 2: 145-159.

Borovsky, D. 1986. Proteolytic enzymes and blood digestion in the mosquito, *Culex nigripalpus*. Arch. Insect Biochem. Physiol. 3: 147-160.

Borovsky, D. 1988. Oostatic hormone inhibits biosynthesis of midgut proteolytic enzymes and egg development in mosquitoes. Arch. Insect Biochem. Physiol. 7: 187-210.

Borovsky, D. & D. A. Carlson. 1989. The role of oostatic hormone in the regulation of midgut serine proteases. In A. B. Borkevec & E. P. Masler, eds., Insect Neurophysiology. Humana Press. pp 251-254.

Borovsky, D., D. A. Carlson & D. F. Hunt. 1991a. Mosquito oostatic hormone - A trypsin modulating oostatic factor in Insect Neurophysiology eds. J. J. Menn, T. J. Kelly & E. D. Master. Am. Chem. Soc. Symp. ser. 453. pp 133-142. Washington, DC: American Chemical Society.

Borovsky, D., D. A. Carlson. P. R. Griffin, J. Shabanowitz & D. F. Hunt. 1991b. Mosquito oostatic hormone: a novel decapeptide modulating trypsin like enzyme biosynthesis in the midgut. FASEB J. 4: 3015-3020.

Borovsky, D., D. A. Carlson, P. R. Griffin, J. Shabanowitz & D. F. Hunt. 1993. Mass spectrophotometry and characterization of *A. aegypti* TMOF and its analogs. Insect Biochem. Molec. Biol. 23: 703-712.

Borovsky, D., I. Jansen, V. Broeck, R. Huybrechts, P. Verhaert, H. L. deBondt, D. Bylemans & A. de Loof. 1996. Molecular sequencing and modeling of *Neobelleria bullata* trypsin: evidence of translational control by Neb-TMOF. Eur. J. Biochem. 237-287.

Borovsky, D., F. Mahmood & D. A. Carlson. 1990. Mosquito oostatic hormone, and its potential as a unique adulticide and larvicide. J. Florida Anti-Mosquito Assoc. 60: 66-70.

Borovsky, D., C. A. Powell, J. K. Nayar, J. E. Blalock & T. K. Hayes. 1994b. Characterization and localization of midgut receptors of TMOF using a complementary peptide and immunocytometry. FASEB J. 8: 305-355.

Borovsky, D. and Y. Schlein. 1988. Quantitative determination of trypsin and trypsin-like enzymes in insects. Arch. Insect Biochem. Physiol. 8: 249-60.

Borovsky, D., Q. Song, M. Ma & D. A. Carlson. 1994a. Biosynthesis, secretion and inmmnoctochemistry of TMOF of *A. aegypti*. Arch Insect Biochem. Physiol. 11: 27-38.

Bradford, M. M. 1976. A rapid and sensitive method for the determination of protein utilizing the principles of protein binding. Anal. Biochem. 72: 428-254.

Breigel, H. & A. O. Lea. 1975. Relationship between protein and proteolytic activity in the midgut of mosquitoes. J. Insect Physiol 21: 1597-1604.

Bylemans, D., D. Borovsky, D. F. Hunt, J. Shabanowitz, L. Grauwels & A. De Loof. 1994. Sequencing and characterization of trypsin modulating oostatic factor from ovaries of the gray flesh fly *Neobellieria (Sarcophaga) bullata*. Regul. Pept. 50: 61-72.

Bylemans, D., P. Proost, B. Samin, D. Borovsky, L. Grauwels, R. Huybrechts, J. Van Damme, J. Van Beemen & A. De Loof. 1995. Neb-colloostatin, a second folliculostatin of the gray flesh fly *Neobellieria bullata*. Eur. J. Biochem. 228: 45-49.

Carlson, D. A., C. M. Krueger, D. Borovsky, A.F. Cockburn & A.M. Rossi. 1993. Expression of trypsin modulating oostatic factor gene in *E. coli*. Proceedings of the Third conference on Host-Regulated Developmental Mechanisms in Vector Arthropods,.Vero Beach, FL, USA. Feb 8-11, 1993. D. Borovsky & A. Spielman., eds. Florida Medical Entomology Laboratory. pp 26-31.

Carlson, D. A, C.M. Krueger, G. Hehman, D. Borovsky, A. M. Rossi, A. F. Cockburn, J. Shabanowitz & D. F. Hunt. 1994. Cloning and partial sequencing of the trypsin modulating oostatic factor gene from mosquito genomic libraries and expression of the TMOF gene in *E. coli.* Perspectives in Comp. Endocrinol. pp. 626-630.

Champlain, R. A. & F. W. Fisk. 1956. The digestive enzymes of the stable fly *S. calcitrans* (L). Ohio J. Sci. 56: 52-62.

Chapman, R. F. 1998. The Insects: Structure and function. 4th ed. Cambridge: Cambridge University Press.

Chia, L. S., J. A. Baxter & P. E. Morrison. 1982. Quantitative relationship between ingested blood and follicular growth in the stable fly *S. calcitrans.* Can. J. Zool. 60: 1917-1921.

Clements, A. N. 1956. Hormonal control of ovary development in mosquitoes. J. Exp. Biol. 33: 211-223.

Clements, A. N. 1996. The biology of mosquitoes. Vol. 1. development, nutrition and reproduction. London: Chapman & Hall.

Davey, K. J. & E. Hubner. 1973. The response of follicle cells of Rhodnius prolixus to juvenile hormone and antigonadotropin in vitro. Can. J. Zool. 52: 1407-1412.

Davey, K. J. & J. E. Kunster. 1981. Hormonal control of vitellogenin uptake in *Rhodnius prolixus* Stal. Am. Zool. 21: 701-705.

De Loof, A. 1987. The impact of the discovery of vertebrate-like steroids and peptide hormone-like substances in Insects. Entomol. Exp. et App. 45: 105-113.

Else, J. G. & C. L. Judson. 1972. Enforced egg retention and effect on vitellogenesis in the mosquito *Aedes aegypti* (Diptera: Culicidae) Can. Entomol. 105: 599-603.

Feinsod, F. M. & A. Spielman. 1980a. Nutrient mediated JH hormone secretion on the mosquito. J. Insect Physiol. 26: 113-117.

Feinsod, F. M. & A. Spielman. 1980b. Independently regulated JH activity and vitellogenesis in mosquitoes. J. Insect Physiol. 26: 829-832.

Felix, C. R. B. Betschart, P. F. Billingsley & T. A. Freyvogel. 1991. Post feeding induction of trypsin in the midgut is separable into two cellular phases. Insect Biochem. 21: 197-203.

Ferenz, H. J. 1993. Yolk protein accumulation in *Locusta migratoria* (R. & F.) (Orthoptera: Acrididae) oocytes. Internat. J. Insect Morphol. & Embryol. 22: 295-314.

Fisk, F. W. 1950. Proteolytic digestion in adult *A. aegypti* L. Ann. Entomol. Soc. Am. 43: 555-572.

Fisk, F. W. & G. F. Shambaugh. 1952. Protease activity in adult Aedes aegypti mosquitoes as related to feeding. Oho J. Sci. 52: 80-88.

Foe, V. E., G. M. Odell & B. A. Edgar. 1993. Mitosis and morphogenesis in *Drosophila* embryo: point and counterpoint. In the development of *D. melanogaster*. Vol. 1, ed. M. Bate & A. M. Arias, pp. 149-300. New York: Cold Spring Harbor Laboratory Press.

Gander, E. S., C. Schoennenberger & T. A. Freyvogel. 1980. Ribosome and ribosome-function in the midgut of A. aegypti. Insect Biochem. 10: 441-447.

Gillett, J. D. 1956. Initiation and promotion of ovarain development in the mosquito Aedes (Stegomyia) aegypti (Linnaeus). Ann. Trop. Med. Parasitol. 50: 375-380.

Gondim, K. C., P. L. Oliveria & H. Masuda. 1989. Lipophorin and oogenesis in *Rhodnius prolixus*: transfer of phospholipids. J. Insect Physiol. 35: 19-27.

Gooding, R. H. 1966. In vitro properties of proteinases in the midgut of adult *A. aegypti* (L) and *Culex fatigans* (Wiedemann). Comp. Biochem. Physiol. 17: 115-127.

Gooding, R. H. 1969. Studies on proteinases of some blood sucking insects. Proc. Entomol. Soc. Ontario. 100: 139-145.

Gooding, R. H., A. C. Cheung, & B. M. Rolseth. 1973. The digestive processes of hematophagous insects. III. Inhibition of trypsin by honey and the possible functions of eosophageal diverticula of mosquitoes (Diptera). Can. Entomol. 105: 433-436.

Graf, R. & H. Briegel. 1982. Comparison between aminopeptidase and trypsin activity in blood-fed females of *A. aegypti* Rev. Sisse Zool. 89: 845-850.

Graf, R. & H. Briegel. 1985. Isolation of trypsin isoenzymes from the mosquito *A. aegypti*. Insect Biochem 15(5): 611-618.

Graf, R. & H. Briegel. 1989. The synthetic pathway of trypsin in the mosquito, A. aegypti L. (Diptera: Culicidae) and in vitro stimulation in isolated midguts. Insect Biochem. 18: 463-470.

Graf, R., A. S. Raikhel, M. R. Brown, A. O. Lea & H. Briegel. 1986. Mosquito trypsin: immuno-cytochemical localization in the gut of blood fed *A. aegypti*. Cell Tiss. Res. 245: 19-27.

Gwadz, R. W. & A. Spielman. 1975. Corpus allatum control of ovarian development in *A. aegypti*. J. Insect Physiol. 19: 1441-1448.

Hagedorn, H. H. & A. M. Fallon. 1973. Ovarian control of vitellogenin synthesis by the fat body in *A. aegypti*. Nature 244: 103-105.

Hagedorn, H. H., J. D. O'Connor & M. S. Fuchs. 1975. The ovary as a source of α-ecdysone in an adult insect. Proc. Nat. Acad. Sci. USA 72: 3255-3259.

Hagedorn, H. H., S. Turner, E. A. Hagedorn. 1977. Post-emergence growth of ovarain follicles of *A. aegypti*. J. Insect Physiol. 23: 203-206.

Hecker, H., T. A. Freyvogel, H. Briegel & R. Steiger. 1971. The ultra structure of midgut epithelium in female *A. aegypti* (L.) (Insecta, Diptera) imagines. Acta tropica 28: 80-84.

Hori, K. & K. Kuramochi. 1983. Localization of trypsin like enzyme and aminopeptidase in the midgut of the adult horn fly *H. irritans* (L) and the stable fly *S. calcitrans* and the effect of anterior midgut homogenates on proteinases. Res. Bull. Obuhiro Univ. 13: 223-227.

Houseman, J. G. & P. E. Morrison. 1986. Absence of female specific protein in hemolymph of stable fly, *S. calcitrans* (L.). Arch. Insect Biochem. Physiol. 3: 205-213.

Hudson, A. 1970. Factors affecting egg maturation and ovipositon by autogenous *A. atropalpus* (Diptera: Culicidae) Can. Entomol. 102: 939-948.

Iwavnov, P. P. & K. A. Mescherskaya. 1935. Die physiologischen Besonderheiten der geschlechtlich unreifen Insektenovarien und die zyklischen Veranderungen ihrer Eigenschaften. Zool JB (Physiol) 55: 281-348.

Jost, E. 1971. Meiosis in male *Culex pipiens* and *Aedes albopictus* and fertilization in the *Culex pipiens*-complex. Can. J. Gen. Cytol. 13: 237-50.

Kang , S.H. & M. S. Fuchs. 1976. Isolation of a chymotrypsin inhibitor from the fruit fly, *Drosophila melanogaster*. Insect Biochem. 4: 1.

Kawooya, J. K. & J. H. Law. 1988. Role of lipophorin in lipid transport to the insect egg. J. Biol. Chem. 263: 8748-8753.

Kelly, T. J., M. J. Birnbaum, C. W. Woods & A. B. Borkovec. 1984. Effects of house fly oostatic hormone on ovarian development and neurosecretory hormone action in *Aedes atropalpus*. J. Exp. Zool. 229: 491-496.

Khalkok, S. E., L. M. Tabak, D. E. Prosse, W. Brook, A. E. R. Downe & B. N. White. 1993. Isolation, sequencing and charaterization of two cDNA clones coding for trypsin-like enzymes from the midgut of *A. aegypti*. Insect Mol. Biol. 2: 71-79.

Kuzina, O. S. 1942. Gonotrophic relationship in *Stomoxys calcitrans* L and *Hematobia irritans* L. Med. Parasitol.

Bolenzi 11: 70-78. cited in Sutcliffe, J. F. et al., 1993. Effect of blood feeding regimes on mortality and female reproduction in a laboratory colony of stable flies, *S. calcitrans*. Med. Vet. Entomol. 7: 111-116.

Lea, A. O. 1963. Some relationships between environment, corpora allata, and egg maturation in aedine mosquitoes.J. Insect Physiol. 9: 793-809.

Lea, A. O. 1982. Artifactual stimulation of vitellogenesis in *A. aegypti* by 20-hydroxyecdysone. J. Insect Physiol. 28: 173-176.

Lea, A. O. & M. R. Brown. 1990. Neuropeptides of mosquitoes. In Molecular Insect Science, H. H. Hagedorn, J. G. Hildebrand, M. G. Kidwell & J. H. Law eds., New York Plenum Press, pp 181-188.

Leamli, U. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 277: 680-685.

Lehane, M. J. 1976. Digestive enzyme secretion in *S. calcitrans*. Cell Tiss. Res. 170: 275-287.

Lehane, M. J. 1977. An hypothesis of the mechanism controlling proteolytic digestive enzyme production levels in *S. calcitrans*. J. Insect Physiol. 23: 713-715.

Lehane, M. J. 1991. Biology of blood sucking insects. London, Harper Colins Academic.

Lehane, M. J., D. Blakemore, S. Williams & M. R. Moffatt. 1995. Mini Review. Regulation of digestive enzyme levels in insects. Comp. Biochem. Physiol. 110B # 2: 285-289.

Lin, Y., M. T. Hamblin, M. J. Edwards, C. Barillas-Mury, M. R. Kanost, D. C. Knipple, M. F. Wolfner & H. H. Hagedorn. 1993. Structure, expression and hormonal control of genes from the mosquito *A. aegypti*, which encodes proteins similar to the vitelline membrane proteins of *Drosophila melanogaster*. Devel. Biol. 155: 558-568.

Marten, E. J. 1996. The vitelline membranes of *A. aegypti* and *Drosophila melanogaster*. Invert. Reproduction & Devel. 30: 255-264.

Martensen, I., J. Koolman & R. Mentlein. 1998. Proline specific peptidase from the blue blow fly, *Calliphora vicina* hydrolyses in vitro the ecdysiostatic peptide trypsin modulating oostatic factor (Neb-TMOF). Arch. Insect Biochem & Physiol. 37: 146-157.

Meola, R. & A. O. Lea. 1972. Humoral inhibition of egg development in mosquitoes. J. Med. Entomol. 9: 99-103.

Moobola, S. M. & E. W. Cupp. 1978. Ovarian development in the stable fly, *S. calcitrans* in relation to diet and juvenile hormone control. Physiol. Entomol. 3: 317-321.

Moffatt, M. R. & M. J. Lehane. 1990. Trypsin is stored as an inactive zymogen in the midgut of *S. calcitrans*. Insect Biochem. 20: 719-723.

Noriega, F. G., J. E. Pennington, C. Barillas-Mury, X. Y. Wang & M. A. Wells. 1996a. Early trypsin, an *Aedes aegypti* female specific protease, is post-transcriptionally regulated by the blood meal. Insect Molec. Biol. 5: 25-29.

Noriega, F. G., X. Y. Wang, J. E. Pennington, C. Barillas-Mury & M. A. Wells. 1996b. Early trypsin, a female specific midgut protease in *A. aegypti*: isolation, amino terminal sequence determination, and cloning and sequencing of the gene. Insect Biochem. Molec. Biol. 26: 119-126.

Noriega, F. G., D. K. Shah & M. A. Wells. Juvenile hormone controls early trypsin gene transcription in the midgut of A. aegypti. Insect Mol. Biolo. 6 (1): 63-66.

Pennington, J. E., & F. G. Noriega. 1995. The expression of early trypsin in *A. aegypti*. J Cell Biochem. 21A (Suppl): 211.

Raikhel, A. S. & T. S. Dhadialla. 1992. Accumulation of yolk proteins in insect oocytes. Ann. Review Entomol. 37: 217-251.

Raikhel, A. S. & A. O. Lea. 1982. Abnormal vitelline envelope induced by unphysiological doses of ecdysterone in *Aedes aegypti*. Physiol. Entomol. 7: 55-64.

Raikhel, A. S. & A. O. Lea. 1991. Control of follicular epithelium development and vitelline envelope formation in the mosquito; role of juvenile hormone and 20-hydroecdysone. Tissue & Cell 23: 577-591.

Rossignol, P. A., F. M. Feinsod & A. Spielman. 1981. Inhibitory regulation of corpus allatum activity in mosquitoes. J. Insect Physiol. 27: 651-654.

Rudin, W. & H. Hecker. 1979. Functional morphology of the midgut of *A. aegypti* L. (Insecta, Diptera) during blood digestion. Cell Tissue 200: 193-203.

Shapiro, A. B. & H. H. Hagedorn. 1982. Juvenile hormone and the development of ovarian responsiveness to a brain hormone in the mosquito, *Aedes aegypti* Gen Comp. Endocrinol. 46: 176-183.

Shapiro, A. B., G. D. Wheelock & H. H. Hagedorn. 1986. Juvenile hormone and juvenile hormone esterase in adult females of the mosquito, *Aedes aegypti*. J. Insect Physiol. 32: 867-877.

Stryer, L. 1995. Biochemistry 4th Ed. W. H. New York: Freeman & Company.

Sutcliffe, J. F., C. D'Cambre & A. E. R. Downe. 1993. Effects of two blood feeding regimens on mortality and female reproduction in a laboratory colony of stable flies, *S. calcitrans*. Med. Vet. Entomol. 7: 111-116.

Terra W. R., C. Ferreira, B. P. Jordao and R. J. Dillon. 1996. Digestive enzymes VI. In Biology of the Insect midgut. M. J. Lehane and P. F. Billingsley, eds. London: Chapman & Hall.

Troy, S., W. A. Anderson & A. Spielman. 1975. Lipid content of maturing ovaries of *A. aegypti* mosquitoes. Comp.. Biochem. Physiol 50B, 457-461.

Yang, Y. J. & D. M. Davies. 1972. Mosquito chymotrypsin inhibitor in tissues of adult *A. aegypti*. Comp. Biochem. Physiol. 43: 137.

Yin, C. M., B. X. Lou, M. F. Li & J. G. Stoffolano. 1989. Precocene II treatment inhibits terminal oocyte development but not vitellogenin synthesis and release in the black blow fly, *Phormia regina* Meigen. J. Insect Physiol. 35: 465-474.

BIOGRAPHICAL SKETCH

Loyce Okedi was born on 27 October 1959 in Ngora, Uganda. She grew up in Soroti town where she attended Soroti Primary School up to 1971. She then proceeded to attend Gayaza High School, Kampala, where she attained both the East African Certificate of Education in 1975 and the Advanced East African Certificate of Education in 1977. She received her Bachelor of Science degree (Honors) in zoology and botany from Makerere University in 1981.

In 1982 she joined the Uganda Trypanosomiasis and Malaria Research Organisation and worked in the malaria section. She was awarded a research training grant from the by WHO/TDR Special Programme for Research and Training in Tropical Diseases and enrolled for a master's degree at the University of Nairobi, Kenya. She obtained a Master of Science degree in medical entomology in 1990 under the supervision of Professor C. P. M. Khamala. Her research was on resistance to synthetic insecticides by malaria vectors of *Anopheles gambiae* larvae from rice schemes in Kenya and advocated for bacterial larvicides (*Bacillus thuringiensis* and *B. sphaericus*).

On returning to Uganda Trypanosomiasis Research Organisation at Tororo (now Livestock Health Research

Institute) she joined the trypanosomiasis program and worked on the tsetse and trypanosomiasis epidemiology project on Lake Victoria Islands in Uganda prior to enrolling at the University of Florida, Gainesville.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

David A. Carlson, Chair
Associate Professor of
Entomology and Nematology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Jerome Hogsette
Assistant Professor of
Entomology and Nematology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

James L. Nation,
Professor of Entomology and
Nematology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Ellis Greiner
Professor of Veterinary
Medicine

This dissertation was submitted to the Graduate Faculty of the College of Agricultural and Life Sciences and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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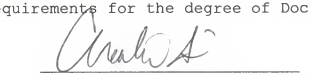

Dean, College of
Agricultural and Life Sciences

Dean, Graduate School