DAWN M. STANEK
Identification of a neuropeptide F in the yellow fever mosquito*, Aedes aegypti.*
(Under the direction of MARK R. BROWN)

A neuropeptide F (NPF) was isolated from an extract of adult *Aedes aegypti* (L.) with high performance liquid chromatography, and identified based on its immunoreactivity in the *Drosophila* NPF radioimmunoassay. Degenerate primers designed from the partial sequence of the purified peptide were used in the polymerase chain reaction to amplify products that contained the cDNA encoding *Aedes aegypti* NPF (*Aea*NPF). Similar to invertebrate NPF's and the neuropeptide Y family in vertebrates, the encoded prepropeptide was processed into an NPF of 36 residues with a carboxyl terminal amide. Immunocytochemistry and Northern analyses demonstrated NPF expression in the brain and midgut of larval and adult *A. aegypti*. The highest titers of NPF in the hemolymph of females were detected pre-blood meal and 24 hours post-blood meal. To determine if conservation of sequence was reflected in function, a bioassay was conducted with synthetic *Aea*NPF peptide to test its effects on trypsin levels after a blood meal. Identification and functional studies of regulatory peptides localized in the mosquito brain and midgut are crucial and may reveal opportunities to control mosquito populations and to make evolutionary comparisons to existing members of the invertebrate NPF family.

INDEX WORDS: Insect, Diptera, Midgut, Neuropeptide F

IDENTIFICATION OF NEUROPEPTIDE F IN THE YELLOW FEVER MOSQUITO, AEDES AEGYPTI

by

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DEDICATION

This work is dedicated to my parents John and Sybyl Stanek as well as my entire family for they have endlessly encouraged me to pursue my dreams.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

There are over 3,000 species of mosquitoes worldwide, with approximately 150 species occurring in North America [1]. Population management is a critical component of mosquito control programs. One of the most significant reasons for mosquito control is that some species transmit pathogens including malaria, viral encephalitis, and dog heartworm, which cause diseases in humans and domestic animals. Malaria, the best known of these diseases, affects more than 500 million people in 90 countries causing over a million deaths per year [World Health Organization fact sheet #94, www.who.int, 1998]. The yellow fever mosquito, *Aedes aegypti* (L.), transmits viruses that cause yellow fever, dengue, dog heartworm, and chicken malaria. Collectively, yellow fever and dengue result in tens of thousands of deaths each year and put hundreds of millions at risk [World Health Organization fact sheet # 100, www.who.int, 1999][World Health Organization fact sheet #117, www.who.int, 1998]. While a vaccine is available for yellow fever, none for dengue fever has been developed.

Not only do mosquitoes present a severe health risk, they are a nuisance to humans and domestic animals throughout the world. In developed nations, many control and abatement organizations investigate and implement different strategies for mosquito population reduction. Current mosquito control methods are aimed at limiting population size, and many organizations apply insecticides to minimize larval and adult populations. Resistance to insecticides and the decreasing number of available insecticides make investigation of physiological processes in the mosquito vital, perhaps even leading to new strategies for population control.

Insects offer ideal models to study regulated processes such as reproduction and digestion [2,3]. The mosquito, *A. aegypti* has a relatively short reproductive cycle,

beginning with the ingestion of a blood meal and ending approximately 72 hours later with egg deposition. Research is facilitated by the ease with which *A. aegypti* development can be synchronized through larval, pupal, and adult stages. Thus, hundreds to thousands of adult mosquitoes can be reared every seven to ten days. Eggs can also be collected and stored for months after oviposition and hatched when mosquitoes are necessary for research.

Regulation of digestion and reproduction in the mosquito is vital to its survival. Peptide hormones play an integral part in the regulation of physiological processes in all insects, and they form the most diverse class of chemical messengers in the metazoan nervous system [2,4,5]. When released, peptide hormones ranging in length from 5 to 200 amino acids, circulate throughout the insect body finding tissue specific receptors. These chemical messengers are packaged into secretory granules that are stored or released by cells directly into the circulatory system or the extracellular space [6]. The actions of peptides can be autocrine, paracrine, or endocrine, offering the cell intra- and extracellular communication capabilities.

Several mosquito peptides are found in the brain and midgut [7-9], thus demonstrating that these peptides have possible actions in both regions of the mosquito. This tissue distribution resembles that of the mammalian brain-gastro-entero-pancreatic system. The existence of such neuroendocrine and endocrine systems suggests that digestive processes in insects are highly ordered and similar to those in vertebrates [10].

The brain-midgut axis in the mosquito is composed of brain neuroendocrine cells and the midgut endocrine system. Here we will concentrate on the neuroendocrine cells found in the protocerebrum and the endocrine cells in the midgut. Clements [11] demonstrated that neuroendocrine cells found in the protocerebrum are bilaterally clustered in the medial and lateral domains of the brain. The medial neurosecretory group contains 12 cells on each side of the medial furrow in the posterior portion of the brain. Five neurosecretory cells in a group are located in the posterior lateral region, and two other groups are located bilaterally in the ventral regions of the anterior and

posterior portion of the brain [11]. Two glands are the main release sites for peptides produced in the neuroendocrine cells of the brain. These glands, the corpora cardiaca and the corpora allata, are connected to the cells of the protocerebrum by axon bundles.

The endocrine cells of the mosquito midgut are a second source for chemical messengers. The insect midgut contains hundreds of conically shaped endocrine cells with apical extensions capable of monitoring luminal contents and basolateral sites for release of hormonal signals to neighboring digestive cells and other tissues via the hemolymph. The entire gut is also extensively innervated by the stomatogastric nervous system. Peptides stored in midgut endocrine cells are presumed to be released, but this is rarely demonstrated [12].

Identification and functional studies of insect regulatory peptides are crucial, not only as a means of insect pest control, but for the purpose of evolutionary comparison to other invertebrate and vertebrate peptides. The last decade of neuropeptide research has witnessed an explosion of sequence and structural information about peptides [12]. As a consequence, the function and mode of action for these peptides have begun to be investigated. The Phe-Met-Arg-Phe-NH_2 (FMRFa) related peptide (FaRP) family consists of peptides that contain a penultimate Arg and an amidated hydrophobic Tyr or Phe residue at their carboxyl (C)-terminus. Peptides related to the FaRP family have been identified in the central nervous system of both invertebrates [2] and vertebrates [13]. Functions identified for this diverse group of peptides include modulation of heartbeat [14], blood pressure [15], contraction of intestinal and exoskeletal muscle [16], synaptic transmission [17,18], ionic channel function [19], and ovarian maturation [12].

This explosion of sequence and structural information has not come without a problem, as many newly isolated peptides have been incorrectly categorized. A group of short peptides that have a terminal Arg-Phe NH_2 (RFa) in common show cross reactivity with FMRFa antiserum. Many peptides have been isolated based on their affinity to FMRFa antiserum in radioimmunoassays, and subsequently categorized as members of the FaRP family. Upon analyzing the amino (N)-terminal extensions of these peptides it

becomes evident that the similarity is limited to only the RFa C-terminus. Studies of the amino acid sequence, gene organization, and function of these newly isolated peptides should be conducted so that they may be correctly categorized. One such example of a misnamed peptide is the putative short NPF isolated from the Colorado potato beetle *Leptinotarsa decemlineata* [20]. Upon peptide sequence comparison to members of the invertebrate NPF family it is evident that this peptide is not an NPF and should not be characterized as such.

Another family of peptides with a similar C-terminus is the vertebrate neuropeptide Y family, which is composed of neuropeptide Y (NPY)/ peptide YY (PYY), and pancreatic polypeptide (PP). Members of the NPY family typically contain 36 amino acids, and are amidated at their C-terminus. They are distinguishable from RFa peptides due to their longer length. Neuropeptide Y has important roles in the regulation of food intake, circadian rhythms, and other physiological processes in mammals [21-23]. As well NPY is a potent stimulant of food ingestion, causing an up to 20-fold increase in food uptake in rats [24]. Peptide YY and PP have been shown to regulate enzyme secretion and motility [25]. The conserved function of the NPY family is the inhibition of cAMP production in mammalian cells that express NPY receptors, which are known to be G protein-coupled receptors [23,26].

The invertebrate ortholog of the NPY family is neuropeptide F (NPF), and can be identified by an amidated Phe residue at its C-terminus. These peptides are commonly 36 to 40 amino acids in length and contain a C-terminus amide. Until recently, little was known about NPF and its role in feeding behavior among invertebrates. Available information was limited to mollusks and plathyhelmiths [27,28]. A mutation in an NPF receptor in the nematode, *Caenorhabditis elegans,* has been shown to affect feeding behavior and digestion, but to date the ligand for this receptor has not been identified. The first NPF from an arthropod was isolated from the fruit fly *Drosophila melanogaster* [29], an insect of the order Diptera and suborder Brachycera [1]. This NPF (*Dm*NPF) is localized in neuroendocrine cells of the brain and midgut endocrine cells. Although the

cellular distribution of this peptide would implicate a role in feeding behavior and digestion, no biological function has yet been established [29,30]. Evidence suggests, however, that *Dm*NPF plays a role in regulating sensory stimulation to the central nervous system due to the presence of food [31].

Energy and basic nutrients, including amino acids, simple sugars, and fatty acids, are provided by the ingestion of food. The diet of mosquitoes consists mainly of water and nectar, but females of many species also require a protein rich blood meal [11]. Among anautogeneous mosquitoes like *A. aegypti*, blood meal digestion is intricately linked with reproduction. During the first three to five days, the female mosquito prepares for a reproductive cycle, which is triggered by a blood meal. The protein supplied in the blood meal is necessary for yolk protein production and ultimately the maturation of eggs. This process, known as vitellogenesis, depends upon the interaction of juvenile hormone, 20-hydroxyecdysone, ovarian factors, and neuropeptides. In turn, each of these components are released or regulated by a blood meal [32]. In response, a shift is induced from the previtellogenic phase to the vitellogenic phase of yolk deposition. Proteins are the predominant constituents of blood, and to aid in their digestion, the midgut secretes a number of proteolytic enzymes, including trypsin. Trypsin plays a major role in the digestion of a blood meal in the mosquito *A. aegypti* and is considered the most prevalent endopeptidase in the adult female midgut [33-35].

Pancreatic polypeptide, a member of the NPY family, plays a role in inhibiting pancreatic enzyme secretion [36] and stimulating feeding behavior in rats [37,38]. Notably, a PP-like peptide has been localized in the nervous system of the flies *Calliphora erythrocephala* and *Calliphora vomitoria* [39-41]. Due to previous localization of PP-like peptides in the mosquitoes *A. aegypti* [42] and *Anopheles stephensi*, [43], and the role that PP plays in enzyme secretion [36], it was proposed that *Aedes aegypti* NPF (*Aea*NPF) has a function in blood meal digestion.

PURPOSE OF STUDY

The physiology of blood meal digestion and reproduction in *A. aegypti* has been well characterized, but many mechanisms remain unclear. The ability to devise new control methods to limit mosquito populations is crucial due to the severe health risk that mosquitoes present to humans and domestic animals. Increasing basic knowledge of the endocrine control and the peptides involved in blood meal digestion and reproduction in the mosquito may ultimately lead to new methods to control mosquitoes and possibly other insect pests. Therefore it was my interest to isolate and characterize NPF from the mosquito *A. aegypti,* and investigate its relation to blood meal processing. To achieve this goal four objectives were set.

The results of studies from Objectives 1 to 3 are presented in chapter two of this thesis in manuscript format.

1. Isolation and characterization of an NPF in the mosquito *A. aegypti*.
   To determine if NPF was conserved in Nematocera, the suborder of Diptera in which *A. aegypti* belongs, an NPF was extracted from mosquito abdomens and purified by high-performance liquid chromatography. For all purification steps *Dm*NPF antiserum was used to track the immunoreactive peptide in a radioimmunoassay.
2. Identification of a cDNA sequence that encodes the *Aea*NPF. Primers were designed against the sequence of the isolated peptide and used in the polymerase chain reaction to amplify products that contained the nucleotide sequence of the gene. Ultimately the cDNA that encodes the prepropeptide was obtained.
3. Determination of the cellular sources of *Aea*NPF and presence in hemolymph.
   Antiserum to *Dm*NPF was employed in immunocytochemistry to localize NPF-like immunostaining in cells of the brain and midgut of larval and adult *A. aegypti*. Northern analyses demonstrated the presence of *Aea*NPF transcript in the brain and midgut of females thus supporting the immunocytochemical

observations. Presence of *Aea*NPF in the hemolymph was detected with the *Dm*NPF radioimmunoassay, and the hemolymph titer of *Aea*NPF was determined at various times throughout the course of a reproductive cycle. Mass spectroscopy of NPF in the hemolymph demonstrated that its mass was identical to synthetic *Aea*NPF, an amidated 36 amino acid peptide.

The results of the study for Objective 4 are presented in chapter three of this thesis.

4. Investigation of a possible function of *Aea*NPF. The presence of PP inhibits pancreatic secretion in mammals, and PP-like peptides have been localized in the midgut of two mosquitoes. Considering the major role that trypsin plays in protein digestion in mosquitoes, it was hypothesized that *Aea*NPF may regulate blood meal digestion. Therefore, synthetic *Aea*NPF was tested in a bioassay to determine whether it increased or decreased trypsin levels in blood fed female mosquitoes.

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CHAPTER 2

IDENTIFICATION OF A NEUROPEPTIDE F IN THE YELLOW FEVER MOSQUITO, *AEDES AEGYPTI*[1]

[1]Stanek, Dawn M., Pohl, Jan, Crim, Joe W., Brown, Mark R. 2001. To be submitted to Peptides.

ABSTRACT

STANEK, D.M., POHL, J., CRIM, J.W., BROWN, M. R.

Identification of a neuropeptide F in the yellow fever mosquito, Aedes aegypti (L). **To be submitted to Peptides 2001.** A neuropeptide F (NPF) was isolated from an extract of adult *Aedes aegypti* mosquitoes with high performance liquid chromatography, based on its immunoreactivity to *Drosophila melanogaster* NPF antiserum in a radioimmunoassay. A partial sequence was obtained for the mosquito peptide, and a cDNA coding for NPF was deduced from products obtained by the polymerase chain reaction. Similar to invertebrate NPF's and the neuropeptide Y family of vertebrates, the encoded, putative prepropeptide was processed into an NPF with 36 amino acids that is amidated at the carboxy terminus. Immunocytochemistry demonstrated expression of *Aedes aegypti* NPF (*Aea*NPF) in 6 to 8 brain cells, approximately 40 cells in adult male and larval midgut, and approximately 350 cells in the adult female midgut. The highest titers of NPF in the hemolymph of females were detected pre-blood meal and 24 hours post-blood meal. Purification of this material allowed for the determination of the native form of *Aea*NPF. Northern analyses detected *Aea*NPF transcript in the brain and midgut of female *A. aegypti*, substantiating immunocytochemical findings.

Index terms: Insect, Diptera, Midgut, Neuropeptide F

INTRODUCTION

Neuropeptide Y (NPY) has pivotal roles in the regulation of food intake, circadian rhythms, and other physiological processes in mammals [1-3]. Related peptides, peptide YY (PYY) and pancreatic polypeptide (PP), found mainly in the gut, regulate enzyme secretion and motility [4]. To date, the only other peptides from invertebrates markedly resembling NPY and its relatives are limited to the neuropeptide F's (NPF) of platyhelminths [5], mollusks [6], and the fruit fly *Drosophila melanogaster* [7]. De Bono and Bargmann [8] describe a loss-of-function mutation in the *npr*-1 gene of a nematode, *Caenorhabditis elegans,* which implicated a homologous function of the neuropeptide Y receptor in feeding behavior. To date, no ligand for the receptor has been identified. These findings suggest that regulatory functions of NPF and the NPY family might be widely conserved among invertebrates and vertebrates.

NPY, PYY and PP exert their effects through G protein-coupled receptors (Y-receptors). A putative *Drosophila melanogaster* NPF (*Dm*NPF) receptor (*Dm*NPFR1) was identified in the *Drosophila* genome database based on amino acid sequence similarity with the mammalian Y-receptors [9, personal correspondence J.W. Crim]. Subsequently, the cDNA encoding *Dm*NPFR1 has been cloned, sequenced and expressed in Chinese hamster ovary (CHO) cells [personal correspondence J.W. Crim]. In functional assays, *Dm*NPF was shown to be an endogenous ligand for *Dm*NPFR1 based on peptide binding and inhibition of adenylyl cyclase activity in CHO cells stably expressing the cloned cDNA for the *Drosophila* receptor [personal correspondence J.W. Crim]. Clustalw analysis of the deduced amino acid sequence for *Dm*NPFR1 groups the *Drosophila* receptor with the mammalian Y-receptor family [personal correspondence J.W. Crim]. As for *Dm*NPF, *Dm*NPFR1 transcripts have been localized to the brain and midgut [7, personal correspondence J.W. Crim]. The localization of the *Dm*NPF and *Dm*NPFR1 in the brain and midgut suggests that the peptide may regulate feeding behavior and digestion. In a recent study, sugar feeding led to increased *Dm*NPF

expression in the central nervous system of *Drosophila* larvae, suggesting that this peptide may regulate sensory stimulation in response to food [10], however biological functions for NPF have yet to be established.

Conservation of NPY-like peptides among vertebrates and invertebrates is investigated by observing similarities in amino acid sequence, gene organization, and function. These similarities can be scrutinized by isolating NPF in insects to determine if conservation in sequence and function is present within the insect order Diptera, commonly known as flies. Investigation of NPF in Dipterans will allow amino acid, gene organization, and functional comparisons between two suborders of Diptera, *Aedes aegypti* from the suborder Nematocera, and *D. melanogaster* from the suborder Brachycera. Including invertebrate NPF members as well as the members of the vertebrate NPY family will extend the evolutionary comparison of this peptide superfamily.

In insects, the presence of NPY-like peptides has been demonstrated through imunocytochemistry [11], and the presence of PP-like peptides have been identified in the mosquitoes *A. aegypti* [12] and *Anopheles stephensi* [13]. The presence of NPY-like peptides in the order Diptera is supported by the purification of the first arthropod NPF from the fruit fly *D. melanogaster* [7]. Due to similar localization patterns and the organization of the prepropeptide, it was determined that NPF from *Drosophila* was an ortholog of vertebrate NPY, and a member of the NPY superfamily. Described here is the purification of an NPF from an extract of adult mosquitoes and its structural characterization by biochemical and molecular techniques. Identification of NPF in the mosquito will allow for studies on the peptide, gene organization, and function of a second NPF in the order Diptera. This data may then be compared between two different suborders of Diptera and all members of the invertebrate NPF family and the NPY family of vertebrates.

MATERIALS AND METHODS

Mosquitoes: Aedes aegypti larvae were reared on a mixture of brewer's yeast, lactalbumin, and finely ground rat chow (1:1:1) and adults had access to 10% sucrose solution for two days post eclosion, and then were given water. All stages were maintained at 27°C and 16 h light: 8 h dark photoperiod. Experimental procedures utilized fourth instar larvae or sugar fed, three to five day old males and females. When necessary female *A. aegypti* were allowed to blood feed on an anesthetized rat for approximately 30 min, and replete females were placed into a separate cage for staged analyses.

Antiserum production: For an antigen, crude synthetic *Dm*NPF-1 (Molecular Genetics Instrumentation Facility, MGIF, University of Georgia) was coupled to thyroglobulin (TG; 7.7 mg TG/6 mg *Dm*NPF-1 with glutaraldehyde in phosphate buffered saline PBS; 0.01 M, pH7.2). After dialysis and lyophilization, the antigen was rehydrated (2 mg/ml of 0.01 M PBS) and emulsified in 1.0 ml complete Freund's adjuvant. After collection of preimmune serum, two rabbits were injected subcutaneously, each with 1.0 mg antigen/ml, in multiple sites along the back. Subsequent booster injections were carried out at four-week intervals with 1.0 mg antigen prepared similarly but with incomplete Freund's adjuvant. Both rabbits were bled two weeks after the first booster injection and at four-week intervals thereafter, and the serum was prepared [14] and stored at - 80° C. Only antiserum collected from one rabbit after the second boost (403C) was used for the immunoassays described below.

Radioimmunoassay (RIA) to monitor NPF isolation: Immunoreactive material in chromatography fractions was detected with the *Dm*NPF-1 RIA. Unpurified synthetic *Dm*NPF-1 was labeled with Na ^{125}I (Amersham) by the chloromine T method [15], and purified by high-performance liquid chromatography (HPLC). Unlabeled crude *Dm*NPF-1 was used in a range of 1.0 to 500 fmol/300 µl assay volume in triplicate tubes. Rehydrated fraction aliquots (100 µl/duplicate), diluted antiserum (403C in 100 µ1, final

dilution 1:7,500,000 to 1,050,000), and radiolabeled peptide (100 µl, ca. 8000 cpm) in Tris buffer (0.05 M, pH 7.2 with 1% bovine serum albumin (BSA) and 0.02% sodium azide) were incubated overnight at 4°C. Free and antibody-bound labeled peptides were separated using the charcoal method [15], and pellets with free peptide were counted on Packard Cobra II counter. A standard curve was plotted from the bound/free ratios and logarithmic values of synthetic peptide, and the amount of immunoreactive material was calculated from a regression equation for the linear portion (1-100 fmol) of the standard curve.

Body part extraction and solid phase extraction: Abdomens were separated from mass-reared adult mosquitoes [16] and frozen (-80° C). Whole frozen abdomens (40 g wet weight) were extracted in boiling 3% acetic acid solution (400 ml) for ca. 30 min. After cooling in an ice bath, the extract solution was centrifuged (1000 x g, 10 min; 15,300 x g, 20 min, both at 4 °C), and the supernatant solution frozen.

The thawed solution was passed through a C_{18} cartridge (10 g sorbent mass, Mega Bond Elut, Varian, Harbor City, CA), which had been conditioned with acetonitrile (CH_3CN) and then 0.1% trifluoroacetic acid (TFA) in water (60 ml each). Afterwards, the cartridge was washed with TFA solution (60 ml), and adsorbed material was step eluted with 10% and 80% CH_3CN in TFA solution (60 ml each). The same cartridge was washed with CH_3CN, and the procedure repeated. The 10% and 80% CH_3CN step eluates were lyophilized.

Because only the two 80% CH_3CN eluates contained NPF-immunoreactive material, they were combined and subjected to two steps of semi-preparative HPLC, as described below. For the second step, fractions with NPF-immunoreactive material originating from three other extracts of adults (abdomens, 30 g; mixed thoraces and abdomens, 30 g; and whole adults, 47 g) were added to the immunoreactive fractions obtained from the first HPLC step. These extracts were similarly processed and subjected to HPLC as described in this paper for the purification of other peptides.

HPLC: A Beckman chromatography 421A/114M system was used for semi-preparative HPLC and a Beckman 126/166 system for analytical HPLC. Fractions were collected in polypropylene tubes, and aliquots for the *Dm*NPF-1 RIA were taken prior to freezing. In preparation for the subsequent chromatography step, fractions containing immunoreactive material, as determined by the RIA, were partially lyophilized and pooled with the initial mobile phase solution for loading on the column.

The 80% CH_3CN eluates of the abdomen extract were rehydrated and fractionated by semi-preparative HPLC (Waters DeltaPak C_{18} column, 25 x 100 mm cartridge and guard cartridge; solvent A, with 0.1% heptafluorobutyric acid (HFBA) in water, and solvent B, 90% CH_3CN in A; gradient program: 10-100% B, 50 min; 6 ml/min; 280 nm). Immunoreactive fractions from this step were pooled with those obtained from the chromatography of other extracts (see above) and lyophilized. This material (ca. 0.5 g) was fractionated on the same column using a different ion-pairing agent in the mobile phase (solvent A, 0.1% TFA in water, and solvent B, 80% CH_3CN in A; gradient program: 10-60% B, 50 min; flow rate, 6 ml/min; monitored at 280 nm). The immunoreactive material eluted within 20-50% B range of the gradient, and four major groups of fractions were identified with the RIA, after different dilutions of the fraction aliquots were quantified. The first and second groups yielded peptides that were structurally characterized and found to have an RFa C-terminus, thus substantiating their immunoreactivity in the RIA. This work will be described in another manuscript. From the third group, a different peptide was isolated in insufficient quantity to be sequenced. The fourth group of fractions (six fractions eluting at 40-45% B) yielded NPF-related peptides, which were purified as described below.

Immunoreactive peptides were purified to homogeneity after five steps of analytical HPLC. For the first two steps, the material from the fourth group was subjected to reversed-phase HPLC on a C_8 column (Alltech, Macrosphere 300, 7 μm matrix, 250 x 4.6 mm). First, the material was eluted from the column with a gradient of

solvent B, 80% CH_3CN in A with 0.1% HFBA (gradient program: 10-60% B, 50 min; 1 ml/min; 280 nm). Second, immunoreactive material in five fractions (eluting ca. 50% B) from the previous step was eluted from the column with a gradient of solvent B as above with TFA (gradient program: 20-60% B, 50 min; 1 ml/min; 206 nm). For the third step, material eluting around 48% B in six fractions from the previous step were subjected to cation exchange HPLC on a Vydac 400 VHP column (7.5 x 75 mm) using a gradient program (0-50% solvent B, 0.5 M ammonium acetate, 50 min; 1 ml/min; 280 nm; solvent A, 0.02 M ammonium acetate, pH 4.5; both solvents with 10% CH_3CN). This chromatography step resolved three groups of fractions with immunoreactive material (Group 1 eluting 8-10% B; Group 2, 12-14% B; Group 3, 24-25% B) that were fractionated separately with two analytical HPLC steps on a C_{18} column (Phenomenex, Jupiter; 5 µm, 300 Å matrix; 250 x 4.6 mm). From Group 1, two immunoreactive peptides were isolated in quantities insufficient for structural characterization. Peptides in fractions from the other two groups were resolved on the above column with mobile phases containing HFBA, as above (solvent B with 90% CH_3CN; 20-70% B, 50 min; 1 ml/min; 280 nm). From Group 2, a single fraction (eluting at ca. 44% B) contained the majority of the immunoreactivity that eluted in a single homogenous peak in the next HPLC step using TFA in the mobile phases (20-40% B, 10 min, 40-60% B, 40 min; 206 nm; peak fractions were manually collected). This peptide was later determined to be an NPF after structural analyses led to the identification and characterization of its encoding cDNA, as reported below. A truncated form of the NPF was isolated similarly from Group 3, as determined from the structural analyses described below.

Mass spectroscopy: Portions of the isolated peptides were analyzed by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), and electrospray ionization triple quadrupole mass spectrometry (ESI-MS) at Emory University Microchemical Facility, Winship Cancer Institute. A model ReflexIII delayed-extraction MALDI-TOF mass spectrometer (Bruker Daltonics, Billarica, MA) equipped with a 337 nm nitrogen laser was run in the reflectron mode of operation. A

matrix of a-cyano-4-hydroxycinnamic acid (MALDI-Quality, Hewlett-Packard, Palo Alto, CA) was used. A model API3000 triple quadrupole mass spectrometer (PE-Sciex, Foster City, CA) equipped with a MicroIonSpray electrospray source and operated in the positive mode was used for ESI-MS analysis. The samples were introduced at 5 ul/min in 50% CH_3CN in 0.1% acetic acid.

HPLC fractions from hemolymph samples were analyzed by MALDI-MS with a Bruker Reflex time-of-flight mass spectrometer (Billerica, MA) retrofitted with delayed extraction at the Chemical and Biological Sciences Mass Spectrometry Facility, University of Georgia. The matrix was a saturated solution of 3,5 dimethoxy-4-hydroxycinnamic acid (Aldrich, Milwaukee, WI) in a 1:1 mixture of water: CH_3CN with 0.1% TFA. The spectrum was acquired in linear mode by averaging 151 laser shots and was externally calibrated using synthetic human angiotensin II and bovine insulin standards.

Peptide sequencing: Portions of the peptides isolated as described above were sequenced from the amino (N) terminus. Automated Edman degradation of the peptides was performed on an Applied Biosystems (Foster City, CA) model cLC Procise sequencing system at Emory University Microchemical Facility, Winship Cancer Institute. The samples were loaded onto a polybrene-coated glass fiber filter. The manufacturer's pulsed liquid chemistry program was used to degrade the peptides.

Polymerase chain reaction (PCR): Three types of PCR were utilized to amplify products encoding parts of the NPF amino acid sequence. These products were compiled into a sequence that contained the open reading frame (ORF) of *Aedes aegypti* NPF (*Aea*NPF). All sequencing reactions were conducted at MGIF (UGA), and primers were synthesized by MWG-Biotech (HighPoint, NC).

1. *Two-step PCR:* For the first step, amplification was conducted with a biotinylated degenerate primer 5'-GCRTGYTGNGCRTGYTTNGTYTC-3' (antisense for bases 244-266, Fig. 1), and one of four random sense primers, in different reaction mixtures:

5’-CAGTTCAAGCTTGTCCAGGAATTCNNNNNNGGCCT-3’;
5’-CAGTTCAAGCTTGTCCAGGAATTCNNNNNNGCGCT-3’;
5’-CAGTTCAAGCTTGTCCAGGAATTCNNNNNNCCGGT-3’;
5’-CAGTTCAAGCTTGTCCAGGAATTCNNNNNNCGCGT-3’. The primers were designed to anneal to DNA approximately every 1000 bases. Amplification was conducted by PCR with 0.8 μg *A. aegypti* genomic DNA [17]. Reaction mixtures contained 0.1 ug of each primer, 1.25 U Taq polymerase (Promega), and buffer solution containing 2 mM $MgCl_2$ and 0.1 mM each nucleotide in a 50 μl total volume. Amplification occurred with initial denaturation at 94°C for 2 min then 35 cycles (94°C, 45 sec; 62°C, 45 sec; 72°C, 3 min), and a final extension at 72°C for 10 min in a gradient cycler (Eppendorf).

The biotinylated product amplified in the above reactions was purified by Dynabead-Streptavidin purification using magnetized streptavidin beads [18], and subsequently used as a template for nested PCR reactions. The second amplification was conducted by PCR using 1 μl of purified biotinylated DNA PCR product, and primers 5’-CAGTTCAAGCTTGTCCAGGAATT-3’ (made to 5’ end of the random sense primers), and the degenerate NPF primer used in the first step. Reaction mixtures contained, 1.25 U of Taq polymerase (Promega), 0.1 ug of each primer, and buffer solution containing 2 mM $MgCl_2$ and 0.1 mM each nucleotide in a final volume of 25 μl. Initial denaturation was at 94°C for 2 min then 35 cycles (94°C, 15 sec; 62°C, 15 sec; 72°C, 30 sec). A one hundred forty one base pair (bp) product was amplified, purified (GeneElute, Sigma), cloned (TOPO TA, Invitrogen), and sequenced.

2. *Anchored PCR*: Fifty heads or midguts of three to five day old *A. aegypti* were dissected and transferred into 50 μl RNA*later*™ (Ambion). Once RNA*later*™ was removed, the tissues were homogenized in 250 μl of lysis/binding buffer (100 mM Tris-HCL, pH7.5; 500 mM LiCl; 10 mM EDTA, pH 8.0; 1% LiDS; 5 mM DTT),

and centrifuged at room temperature (14,000 x *g,* 10 min). To bind the mRNA, 10 µl of prewashed magnetic beads (Dynabeads mRNA DIRECT Micro Kit) were mixed with the supernatant from above, and a magnetic stand was used to separate the mRNA. First strand cDNA was synthesized from isolated mRNA (First-strand cDNA synthesis kit, Amersham Pharmacia Biotech) using a Not I-d $(T)_{18}$ primer 5'-d[AACTGGAAGAATTCGCGGCCGCAGGAAT$_{18}$]-3' in a reverse transcription reaction.

Amplification with PCR using a touchdown program was conducted with 1 µl synthesized first strand cDNA from above, and primers 5'-CCGAAGCAATCCGG CTCCTACAAGAACT-3' (sense for bases 215 to 242, Fig. 1), and 5'-AACTGG AAGAATTCGCGGCCGCAGGAAT$_{18}$-3' (Not I-d $(T)_{18}$ primer). Reaction mixtures contained 1.25 U of Taq polymerase (Promega), 0.1 ug of each primer, and buffer solution containing 2 mM $MgCl_2$ and 0.1 mM each nucleotide in a final volume of 25 µl. Initial denaturation was at 94°C for 2 min then 15 cycles (94°C, 10 sec; 64-45°C, 30 sec; 72°C, 1 min), and then 35 cycles with annealing at 45°C was completed. A six hundred seventy one bp product was amplified, purified (GeneElute, Sigma), cloned (TOPO TA, Invitrogen), and sequenced.

3. *Inverse PCR:* Genomic DNA (2.08 µg) was digested at 65°C for 2 hours with 10 U of Taq I (New England BioLabs). The genomic DNA was then extracted with phenol/chloroform, and ethanol precipitated. Digested DNA was self-ligated with T4 DNA ligase (Life Technologies, 3U in 50 µl ligation buffer, 22°C, 4 h), ethanol precipitated, and resuspended in pure water. Amplification was conducted by PCR with 100 ng of digested genomic DNA, and specific primers, 5'-CCGGGCATCGG TGAAAGAACTTAGATC-3' (antisense for bases 163 to 189, Fig. 1), and 5'-CGAA GCAATCCGGCTCCTACAAGAAC-3' (sense for bases 216 to 241, Fig. 1). Reaction mixture contained 0.1 ug of each primer, 1.25 U Taq polymerase (Perkin Elmer), and buffer containing 2 mM $MgCl_2$ and 0.1 mM of each nucleotide in a final

volume of 25 µl. Denaturation was at 94°C for 10 min then 40 cycles (94°C, 30 sec; 63.4°C, 30 sec; 72°C, 1min), and a final extension at 72°C for 10 min. A two hundred fifty bp product was amplified, purified (GeneElute, Sigma), cloned (TOPO TA, Invitrogen), and sequenced.

AeaNPF open reading frame: To confirm the compiled nucleotide sequences, specific primers were used to amplify by PCR the ORF from cDNA prepared from female heads or midguts. Amplification was conducted using 1 µl of cDNA from heads or midguts (as above), and specific primers 5'-GATTTTCTCCCTGGAAAGCC-3' (sense for bases 36 to 55, Fig.1), and 5'-TGCTGTAACTGTGCTTGC-3' (antisense for bases 763 to 780 Fig. 1). Reaction mixtures contained, 1.25 U of Taq polymerase (Perkin Elmer), 0.1 ug of each primer, and buffer solution containing 2 mM $MgCl_2$ and 0.1 mM each nucleotide in a final volume of 25 µl. Initial denaturation was at 94°C for 10 min then 35 cycles (94°C, 15 sec; 54°C, 15 sec; 72°C, 30 sec), and a final extension at 72°C for 5 min. A seven hundred and forty one bp product was amplified from both templates, purified (GeneElute, Sigma), cloned (TOPO TA, Invitrogen), and sequenced.

Immunocytochemistry: Brains and midguts from three to five day old adults and fourth instar larvae were dissected and fixed for one hour in 4% paraformaldehyde (16% stock, Electron Microscopy Sciences (Fort Washington, PA, USA), diluted in PBS). After removal of fixative solution, the tissues were processed through an ethanol/water series, washed three times with PBS containing 0.5% Triton X-100 (T), and blocked with 5% goat serum for 2 hours. Primary antiserum (403C) alone (1:1000) or preabsorbed was incubated with tissues overnight at 4°C. Tissues were then washed three times for one hour each with PBST with 1% goat serum, and incubated overnight at 4°C with Alexa Fluor® 488 goat anti-rabbit IgG (H+L) conjugate (1:1000 dilution, Molecular Probes, Eugene, OR). After rinsing three times with PBST for one hour each, tissues were mounted on slides in a 1:1 mixture of glycerol: PBS.

Negative controls for tissue staining included treatment with preimmune serum, second antibody alone, and antiserum preabsorbed with synthetic *Dm*NPF-2 (Quality Control Biochemicals, Hopkinton, MA), or Phe-Met-Arg-Phe-NH_2 (FMRFa) (Peninsula Laboratories, Torrance, CA, USA) (20-30 μg/ml of diluted antiserum, overnight, 4°C). Tissues from five or more individuals treated or staged in the same manner were examined or photographed with an Olympus BX60 microscope equipped with an epi-fluorescent light source. Cell counts are reported as and average of five or more tissues.

Identification and quantification of AeaNPF in hemolymph: A second *Dm*NPF RIA (*Dm*NPF-2 RIA) was developed using pure synthetic *Dm*NPF (*Dm*NPF-2, Quality Control Biochemicals), only the antiserum dilution (1:21,000 to 24,000 final dilution) and the standard peptide range (1 to 10,000 fmol) were changed. The putative *Aea*NPF sequence (as characterized below) was synthesized in the laboratory of Dr. Stephan Klauser (University of Zurich Hospital, Zurich, Switzerland), and its structure was confirmed by HPLC elution and mass spectroscopy. Parallel binding was demonstrated for *Aea*NPF in this *Dm*NPF-2 RIA in the linear range of 500 to 5,000 fmol.

The *Dm*NPF-2 RIA was employed to determine the hemolymph titer of NPF in females before and during the course of a blood meal due to the antibodies ability to recognize NPF in the mosquito *A. aegypti*, as evidenced by its use for the purification of the peptide from *A. aegypti*. Hemolymph was obtained by slashing thoraces of staged females and incubating them in a 1:1 mix of RIA buffer and saline solution (128 mM NaCl, 4.7 mM KCl, 1.9 mM $CaCl_2$) with a protease inhibitor cocktail (Roche Molecular Biochemicals, 2X, in 139 mM NaCl, 4.05 mM KCL, 1.85 mM $CaCl_2$, 12.5 mM HEPES, 2.5 mM trehalose, 0.3 mM $MgCl_2$, and 0.9 mM $NaHCO_3$; pH 6.5) for 10 min (8 females/200 ul/well of a ceramic plate held on ice; triplicate samples for each time point). These samples were stored at -80°C until all time points for a set were obtained. After thawing, the samples were centrifuged (14,000 x *g,* 10 min; 4°C), and each of the supernatant solutions was assayed in the *Dm*NPF-2 RIA at two dilutions. This

procedure was repeated three times to obtain NPF quantities for a total of nine samples; only those samples showing a dilution effect in the RIA were used for analysis.

To confirm the presence of NPF in hemolymph and determine its native form, hemolymph was collected from females, as above, at the times when the NPF titer was greatest and subjected to HPLC to isolate the immunoreactive form for mass spectroscopy. Hemolymph from sugar fed and 24 h post-blood meal females (100 for each time) was pooled as above, lyophilized, and resuspended in 200µl of an aqueous 20% CH_3CN solution containing 0.1% TFA. After centrifugation, the supernatant solution of each hemolymph sample was injected onto C_{18} column (Phenomenex, Jupiter; 5 µm, 300 Å matrix; 250 x 4.6 mm) and eluted by reversed-phase HPLC (solvent B with 80% CH_3CN; 20-100% B, 50 min; 1 ml/min; 206 nm). Lastly, synthetic *Aea*NPF (8.8 µg) was injected and eluted similarly. Aliquots from the fractions collected for each sample and the synthetic *Aea*NPF were lyophilized, rehydrated with RIA buffer, and briefly sonicated to solubilize the material for assay in the *Dm*NPF-2 RIA. Fractions found to contain the greatest quantity of immunoreactive peptide were vacuum centrifuged to concentrate the peptide for analysis at the Chemical and Biological Sciences Mass Spectrometry Facility (UGA).

Northern Analyses: To substantiate immunocytochemical localization, Northern analyses of brain, midgut, and ovary were conducted. A 744 bp PCR fragment containing the mosquito NPF ORF was used in a runoff transcription reaction (MAXIscript) that contained digoxigenin labeled dUTP thus producing a digoxigenin labeled RNA probe. The probe was aliquoted and stored at -80°C. Heads, midguts, and ovaries (control tissue) were dissected in saline solution (128 mM NaCl, 4.7 mM KCl, 1.9 mM $CaCl_2$) and immediately placed into RNAlater™ (Ambion) and stored at –80°C until processed. TRIZOL reagent (GibcoBRL) was used to isolate total RNA from each of the tissues. Loading buffer (5X: 32% formamide. 2.4% formaldehyde, 4X MOPS, 20% glycerol, 4 mM EDTA, and bromophenol blue) was added to forty tissue equivalents of total RNA, and RNAse free water was added to a final volume to 20 µl.

The RNA was heated to 80°C for 10 min and loaded onto a precast 1.25% Reliant MOPS gel (BMA-Rockland, ME). Electrophoresis occurred at 3 volts/cm for approximately two hours in 1X MOPS running buffer, and the material from the gel was vacuum transferred for one hour to a nylon Magnacharge membrane (Osmonics). The material on the blot was autcrosslinked to the membrane (Stratagene autocrosslinker), and prehybridization followed for one hour with Ultrahybe (Ambion). Probes were denatured for 10 min at 80°C and added to the prehybridization solution (1:20K final concentration for *Aea*NPF and actin control), which incubated with the blot overnight at 65°C. Three stringency washes, 20 min each in 0.5 X SSC (75 mM NaCl, 7.5 mM Sodium citrate, pH 7.0)/0.1% sodium dodecyl sulfate followed at 65°C. The blot was blocked for one hour at room temperature with maleic acid buffer (0.15 M NaCl, 0.1 M maleic acid, pH 7.5) containing 0.3% polyoxyethylenesorbitan monolaurate (Tween-20) and 10% blocking reagent (Roche Molecular Biochemicals). Anti-digoxigenin-alkaline phosphatase (Roche Molecular Biochemicals) was diluted in maleic acid buffer with 0.3% Tween-20 at a 1:20K dilution and incubated for 20 min at room temperature. Final washes, three times, 20 min each, were conducted at room temperature with maleic acid buffer containing 0.3% Tween-20. Chemiluminescent detection followed using the substrate CDP-Star (Roche Molecular Biochemicals).

RESULTS

Isolation and primary structure of Aedes aegypti NPF: Two NPF-like peptides were isolated from pooled abdomen/body extracts after seven HPLC steps as monitored with the *Dm*NPF-1 RIA. As shown by N-terminal sequencing, the larger peptide (3607 Da, as determined by mass spectroscopy) had a sequence of RPQDDPTSVAEAIRLLQE LETKHAQHArpr (letters in lower case represent tentative residues). The other peptide (3100 Da) had a sequence of PTSVAEAIRLLQELETKHAQHARPr. Comparison of the two sequences showed that the smaller one was a truncated form of the larger peptide.

Nucleotide sequence of the Aedes aegypti NPF gene: Based on the amino acid sequence obtained from N-terminal sequencing, degenerate primers were synthesized to amplify, by PCR, the nucleotide sequence of the purified peptide. Employing a two-step process, a 141 bp product (Fig. 1, bases 123 to 264) was amplified, purified, cloned, and sequenced to obtain the sequence encoding a portion of the purified peptide and the signal peptide. Anchored PCR followed, and a 671 bp product (Fig. 1, base 214 to poly A tail) was amplified, purified, cloned, and sequenced to obtain the sequence for the C-terminus of the isolated peptide, the C-terminal peptide, and the 3' UTR. Finally, with inverse PCR, a 248 bp product (Fig. 1, bases 1 to 187) containing the 5' UTR and part of the signal peptide, and (Fig. 1, bases 215 to 276) containing part of the NPF was amplified, purified, cloned, and sequenced.

Once the nucleotide sequences were compiled, a full length 744 bp product containing the ORF was amplified by PCR with head and midgut cDNA as template (Fig. 1, bases 36 to 780). Nucleotide sequences of the ORF amplified by PCR with cDNA of heads or midguts were compared and found to be identical.

The prepropeptide encoded by the ORF has 90 amino acids, of which the first 27 amino acids constitute a putative signal peptide. After removal of the signal peptide, proteolytic processing at a dibasic residue site, Lys_{65} and Arg_{66} (Fig. 1), would give a peptide with 37 amino acids, and amidation of the C-terminal Gly_{64} would result in a mature form with 36 amino acids (Fig. 1, underlined). The proteolytic cleavage produces a 24 amino acid C-terminal peptide. The processed 36 residue peptide is designated *Aea*NPF and shares sequence similarity with invertebrate NPF's (Fig. 2A), and members of the vertebrate NPY superfamily (Fig. 2B).

Immunocytochemistry: To investigate cellular localization of NPF-like material, immunocytochemistry was conducted on brains and midguts of larvae and adults using antiserum to *Dm*NPF. This antiserum was preabsorbed with FMRFa peptide to limit staining to cells that contained only NPF-like material.

After observing brains of six larvae, an average of eight pairs of cells per brain displayed immunostaining with the *Dm*NPF antiserum preabsorbed with FMRFa (Fig 3A) with staining patterns similar to those of adult brains. In adult female (n=6) and male brains (n=6), there was one pair of medial neurosecretory cells, two pairs of cells adjacent to the medial cells, and possibly two more pairs of cells even more laterally situated that displayed immunoreactivity to *D*mNPF antiserum (Fig.3B). The larval foregut contained a ring of cells in the cardia region that was immunoreactive to preabsorbed *Dm*NPF antiserum (Fig. 3C). Endocrine cells in the midgut of larvae were conical in shape with long apical extensions to the lumen (Fig. 3D). In larval (n=6) and male (n=6) midguts, the number of immunostained cells was less than that of the female midgut with an average of 33 and 45 immunostained cells respectively. On average three hundred and fifty endocrine cells (n=11) in the posterior midgut of females were immunoreactive to the *Dm*NPF antiserum preabsorbed with FMRFa (Fig. 3E). The apical extensions of such cells in adult midguts were much shorter than those observed in the larval midgut. In the cardia of the anterior midgut of adults, cells of the ventral ganglia were immunostained, as were axons from these cells that extended along the anterior midgut (Fig. 3F).

Parallel binding and linear displacement: A RIA was used to demonstrate that *Dm*NPF antiserum would recognize *Aea*NPF. Linear displacement of *Aea*NPF (linear range 500 to 5000 fmol) and *Dm*NPF (linear range 50 to 5000 fmol) was demonstrated in the *Dm*NPF-2 RIA, and the two lines appeared to be parallel (Fig. 4A). Over this range only 5% of the actual *Aea*NPF present in the mosquito was recognized by the *Dm*NPF antiserum.

Hemolymph titer for Aedes aegypti NPF*:* To investigate if *Aea*NPF was present in the hemolymph, its titer was quantified with the *Dm*NPF-2 RIA in female mosquitoes during the course of a blood meal. The highest titers of NPF in the hemolymph were 0.78 ± 0.31 pmol/female (average± standard error) observed in non-blood fed females, and 0.49 ± 0.21 pmol/female at 24 hours post-blood meal with a range of 0.10 to 0.31

pmol/female at the remaining time points (Fig. 4B). Hemolymph from non-blood fed females and 24 hours post-blood meal was again collected and separately fractionated by HPLC (Fig. 5). Synthetic *Aea*NPF (4130.4 Da) was similarly fractionated for comparison (Fig. 5). The eluted material was tested with the *Dm*NPF-2 RIA, and mass spectroscopy was conducted on material from the fractions containing the largest immunoreactivity. The immunoreactive material eluted 26 to 29 minutes into the purification for each hemolymph sample and 26 to 32 minutes for the synthetic *Aea*NPF (Fig. 5). Mass spectroscopy determined that the fraction collected from 26 to 27 minutes from hemolymph of non-blood fed females had a mass of 4133 Da, and fractions collected from 26 to 27 minutes from hemolymph of females 24 hours post-blood meal had a mass of 4132 Da. These masses were consistent with that of synthetic *Aea*NPF. A fraction eluting from time 30 to 31 minutes in the synthetic *Aea*NPF sample also showed immunoreactivity in the *Dm*NPF-2 RIA. Mass spectroscopy determined that the fraction collected at 30 to 31 minutes contained shorter peptides between 1000 to 1700 Da.

Northern Analyses: To substantiate the cellular localization of *Aea*NPF, Northern blot analyses were conducted on adult female head, midgut and ovary. Northern blot analysis demonstrated that *Aea*NPF transcript was present in the brain and midgut of adult female mosquitoes with a transcript size of approximately 1650 bp. No transcript of this size was observed in the ovary controls. Actin and total RNA are shown for comparison (Fig. 6).

DISCUSSION

A neuropeptide F (NPF) was isolated from an extract of adult *A. aegypti* mosquitoes with HPLC based on its immunoreactivity in the *Dm*NPF-1 RIA. The amino acid sequence of the mosquito NPF allowed for the cloning of the encoding cDNA, and deduction of the amino acid sequence of the preprohormone. As revealed with immunocytochemistry using *Dm*NPF antiserum, NPF is found in a few brain cells in larvae and adults, and on average 350 midgut endocrine cells in adult females. Related

to invertebrate NPFs and the vertebrate neuropeptide Y family, the encoded, prepropeptide was processed into an amidated peptide with 36 residues. Its presence in hemolymph was demonstrated with the highest titers of NPF-like material appearing pre-blood meal and 24 hours post-blood meal. Fractionation of hemolymph collected at time points with the highest titer allowed for the circulating form of *Aea*NPF to be confirmed as an amidated native peptide of 36 amino acids. Finally, a transcript of 1650 bp was observed in the brain and midgut of female *A. aegypti* using Northern analyses.

The gene for *Aea*NPF includes an open reading frame encoding a prepropeptide. The mature peptide begins after the signal peptide with a Ser_{28} (Fig. 1) at the N-terminus. Proteolytic processing occurs at the endoproteinase site, Lys_{65} and Arg_{66} (Fig. 1), and cleaves the C-terminal peptide from the mature peptide. Subsequently Gly_{64} is converted to a C-terminal amide on the NPF. The prepropeptide organization is common to all NPYs and true NPF members, and this organization supports *Aea*NPF as a true NPF and a member of the NPY superfamily.

Once the peptide sequence was determined it enabled the comparison of *Aea*NPF to other members of the NPF family. *Aedes aegypti* NPF appears most similar to *Dm*NPF with seven conserved and fourteen identical residues (Fig. 2A). This is not surprising, but significant as these peptides are the only known NPFs from insects. Further, in the order Diptera or flies, the genus *Drosophila* is placed in the suborder Brachycera, while the mosquito *A. aegypti* is a member of the suborder Nematocera. Although these Diptera are from different suborders, both peptides contain an amidated mature peptide of 36 amino acids, and a C-terminal peptide, which is common to the NPY family.

For insects, evidence for the presence of NPF-like peptides has come from immunocytochemical studies often using antiserum against bovine PP (reviewed in [11]). Cells immunoreactive to the *Dm*NPF antiserum were observed in both the nervous system and in the midgut endocrine system of *A. aegypti*, respective locations shown for *Dm*NPF [7]. In the present study *Dm*NPF antiserum was utilized due to its

cross reactivity with the mosquito NPF as evidenced by its use for purification, and linear binding of *Aea*NPF and *Dm*NPF peptide in the *Dm*NPF-2 RIA (Fig. 4A). However, insects also contain peptides related to FMRFa, a molluscan cardioexcitatory peptide, and in *A. aegypti,* decapeptides with terminal RFa residues, the *Aedes* "head peptides" [19], have been characterized. Cross-reactivity between many peptides and the terminal RFa in *Dm*NPF antiserum hinders definitive interpretations of studies based solely on immunocytochemical approaches [11]. Therefore *Dm*NPF antiserum was preabsorbed with FMRFa to limit staining to cells containing NPF-like material only. The results from immunocytochemical localization were substantiated by the amplification of *Aea*NPF from cDNA prepared from heads and midguts of adult females, demonstrating the presence of the nucleotide sequence encoding the peptide in similar locations as the mature peptide.

To determine the location of the transcript that encoded the protein, *Aea*NPF mRNA localization was conducted. Northern analyses demonstrated the presence of *Aea*NPF transcript in the brain and midgut of sugar fed females. The *Aea*NPF cDNA identified was approximately 800 bp long while the transcript visualized with Northern blot analyses was 1650 bp. The discrepancy in size between the mRNA and cDNA is addressed by the RNA transcript containing a longer poly A tail, and a longer 5'UTR.

Demonstration of the presence of *Aea*NPF in the hemolymph was an important breakthrough as characterization studies of neuropeptides rarely contain data demonstrating the presence of peptides in circulation [20]. If peptides act as hormones, then by definition, their presence in the hemolymph should be detectable. Observation of the hemolymph titer of *Aea*NPF and the changes that occurred during a reproductive cycle demonstrated *Aea*NPF presence within the circulatory system, thus showing *Aea*NPF acts in a hormonal fashion. The titer of *Aea*NPF was highest before a blood meal with a concentration of 790 nM and then dropped immediately once a blood meal was imbibed. A second peak with a concentration of 490 nM of *Aea*NPF was observed 24 hours post-blood meal, after which the titer dropped once again. In a similar study,

the hemolymph titer of *Aedes* "head peptide" (Aea-HP-I) was observed throughout the course of a reproductive cycle in the mosquito *A. aegypti*. [19]. The Aea-HP-I titer was low after a blood meal and remained so until approximately 36 hours post-blood meal where a peak occurred. Then the titer decreased until low levels were observed at 54 hours post-blood meal. The titer of Aea-HP-I was different from that observed for *Aea*NPF suggesting an alternative cause for release into circulation as evidenced by Aea-HP-I role in host seeking behavior [19].

To confirm that hemolymph samples from non-blood fed and 24 hours post-blood meal time points contained NPF it was necessary to purify the peptide from the hemolymph for mass spectroscopy. After purification, mass spectroscopy was conducted on the peptides from the hemolymph that were immunoreactive in the *Dm*NPF-2 RIA. Synthetic *Aea*NPF was purified in a similar fashion and analyzed. Mass spectroscopy confirmed that the peptide purified from the hemolymph had a mass identical to that of the *Aea*NPF. These peptides also eluted in HPLC at the same time as synthetic *Aea*NPF.

The isolation and characterization of *Aea*NPF, and its presence in the hemolymph indicate that this neuropeptide is a hormone. The action of mammalian NPYs are transmitted through a family of conserved G protein-coupled receptors. Activation of the Y receptors by NPY-related peptides has been shown to cause an inhibition of adenylyl cyclase, an increase in intracellular calcium, or both [21-23]. Indeed, a G protein-coupled receptor has been identified for *Dm*NPF, and data suggests that *Dm*NPF [7] appears to be the endogenous ligand for the identified receptor [personal correspondence J.W. Crim]. The specific binding of iodinated *Dm*NPF and inhibition of adenylyl cyclase in CHO-K1 cells stably transfected with the *Dm*NPF receptor supported this conclusion. We anticipate the presence of a homologous *Aea*NPF receptor in the mosquito. Once the *Aea*NPF receptor is identified and cloned, a detailed characterization of its signal transduction pathway can be determined in response to *Aea*NPF. Structural conservation is assumed to occur among peptides and their

receptors, leading to a possibly conserved function in enzyme secretion and feeding behavior. Further studies with *Aea*NPF will aid in the elucidation of the physiological roles of this peptide hormone.

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  1 GAGCGCATTGCTGAACTTGCATTTCTCTATTTATAGATTTTCTCCCTGGAAAGCCCCCAC
 61 CTCTAGAACCCAATTTATCACACAGCAAAAATGACATTCTCAACTTCGTCCAGCTTCTCC
                                   M  T  F  S  T  S  S  S  F  S
                                                                10

121 CGGCGTGCCCTTGTTGCCCTGCTGGTATGCACTCTGCTCATAGATCTAAGTTCTTTCACC
    R  R  A  L  V  A  L  L  V  C  T  L  L  I  D  L  S  S  F  T
                               20                               30

181 GATGCCCGGCCCCAGGATGACCCAACATCTGTAGCCGAAGCAATCCGGCTCCTACAAGAA
    D  A  R  P  Q  D  D  P  T  S  V  A  E  A  I  R  L  L  Q  E
                               40                               50

241 CTGGAGACAAAACACGCCCAACACGCTAGGCCAAGATTCGGCAAACGAAGTTATTTAAAT
    L  E  T  K  H  A  Q  H  A  R  P  R  F  G  K  R  S  Y  L  N
                               60                               70

301 CCAGCCGGCTATGGACAGGATGAGCAGGAGGATGATTGGCAGGATTCAACGTTTACAAGA
    P  A  G  Y  G  Q  D  E  Q  E  D  D  W  Q  D  S  T  F  T  R
                               80                               90

361 TGAACAAAATAGCATCAACTAAGCGATGATAAGCGATGACTCAGATAACAATCTGCAGCG
    *

421 AACATAGCTAACCAAGCCAACATGGAACCATTTCCAGCTCTCCATTTCAACACAATTCAT
481 TCGTAAAACTATTGATAACATGTTAGAAGACTTATTAGAGAAATATATTTGAACAGACAA
541 CGATTGTCATAATTGGATTCCGATGTTTGGAGGATAATATAACAATGTATAATCTTTCTT
601 GACATGTAATCAATATACTTTGAGTATGTAATACATTATGTGCTGCAAAATCGGACATTT
661 CAATAATATTTTAAGGACGATCTGATTACAAATGACTAAAGTTATTGCTACGATTGTTTT
721 CAACAAATGAATCACTTGTACATATATTGGAACGTATATTATGCAAGCACAGTTACAGCA
781 TTAGAATAACCTGGCAACAAATGCTGCCAAATGATGATTCTGATCATTGAACAGAAATAA
841 AGTTCTCAAATAGAACAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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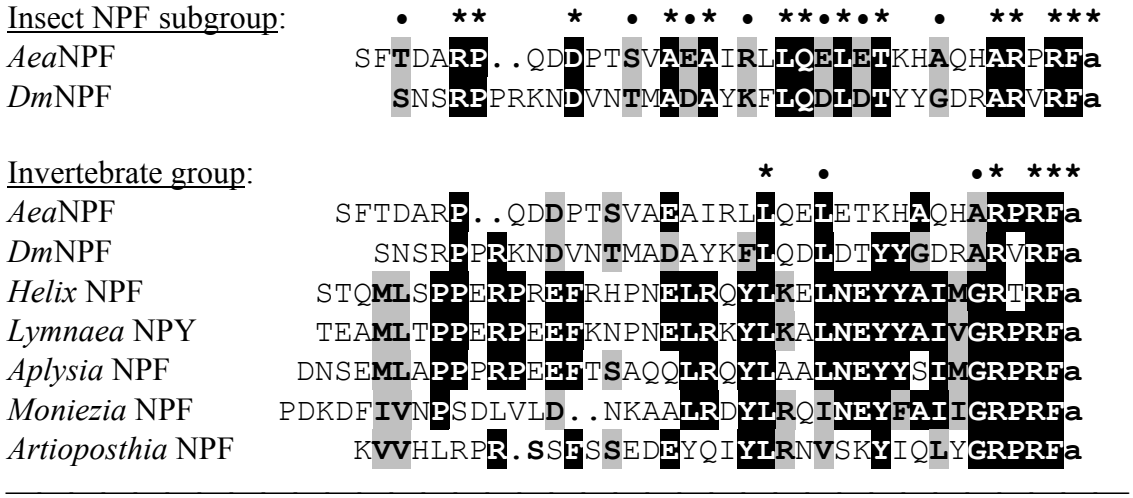
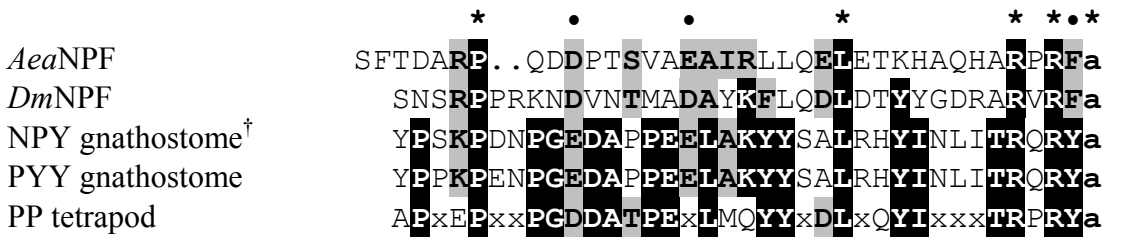
Figure 1. Nucleotide and amino acid sequences of *Aea*NPF. The amino acid sequence for the signal peptide is shown in ***bold italic***, and the native form of NPF is underlined. The box denotes the endoproteinase cleavage site where the C-terminal peptide is cleaved from the mature peptide and Gly_{65} is subsequently converted to a C-terminal amide. A polyadenylation signal sequence present in the 3'UTR is double underlined.

A. Pileup comparisons of amino acid sequences for invertebrate NPF's.



B. Pile comparisons for arthropod NPF's relative to members of the vertebrate NPY/PYY/PP family.



indicates majority identical; , majority conserved *viz*. *Aea* NPF; *****, all identical; •, all conserved. †data from Larhammar (1996).

Figure 2. Amino acid sequence comparisons for *Aea*NPF

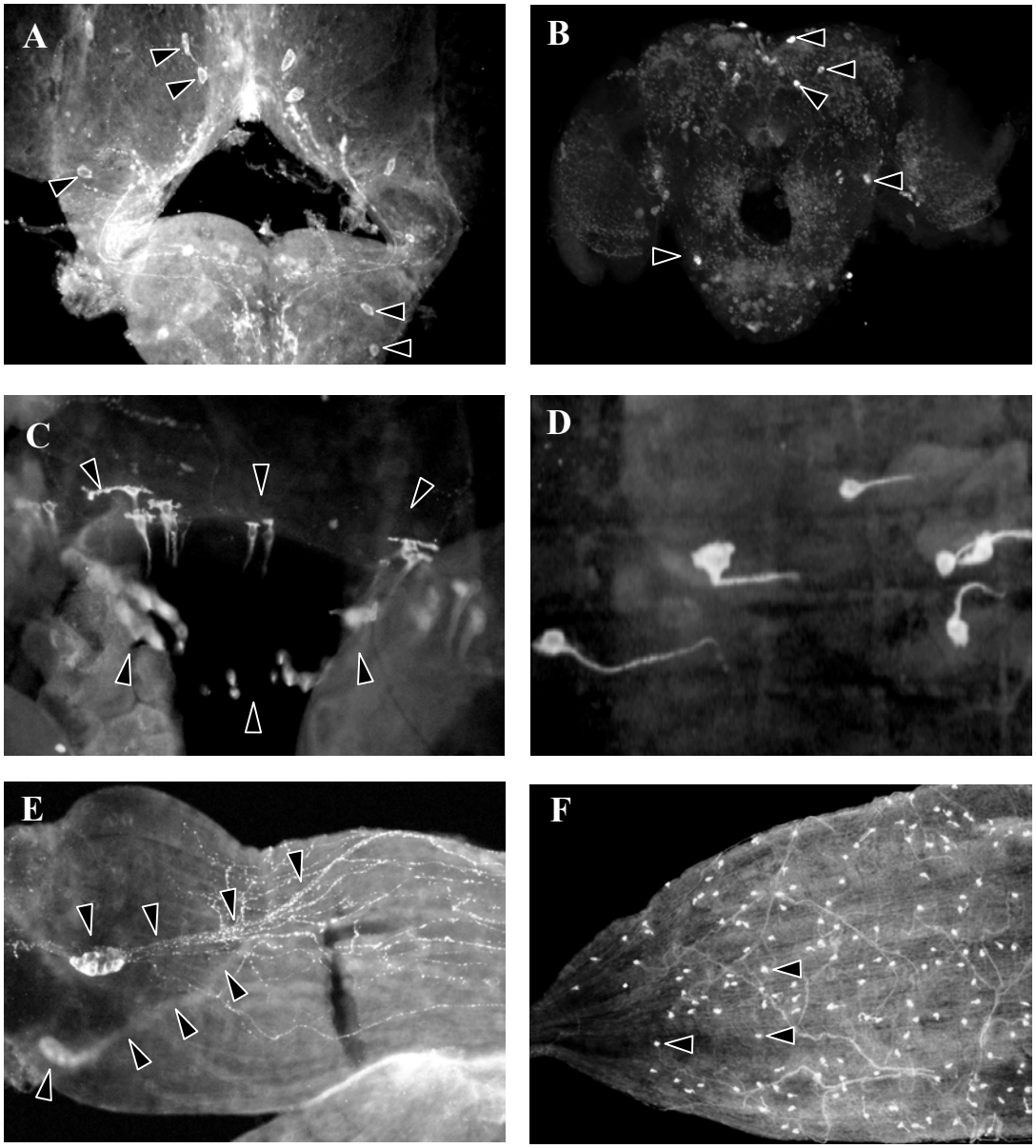


Figure 3. *Aea*NPF immunostaining in the brain and midgut of larval and adult *A. aegypti*. A. Immunolocalization of *Aea*NPF in the larval brain (arrows top), and with two cells located in the subesophegeal ganglia (arrows bottom) X110. B. In adult brains, one pair of medial neurosecretory cells, 2 pairs of cells adjacent to the medial cells, and possibly 2 more pairs of cells even more laterally situated displayed immunoreactivity to *D*mNPF antiserum (arrows) X83. C. The larval foregut contained a ring of cells in the cardia region that was immunostained (arrows) X116. D. Endocrine cells of the larval midgut were conical in shape with long apical extensions extending to the lumen X217. E. In the cardia of the anterior midgut of adults, cells of the ventral ganglia (arrows left) were immunostained as were the axons from these cells that extended along the anterior midgut (3 arrows to right of each cell) X164. F. Approximately three hundred and fifty endocrine cells in the posterior midgut of females were immunostained when *Dm*NPF antiserum preabsorbed with FMRFa was used (arrows) X60.

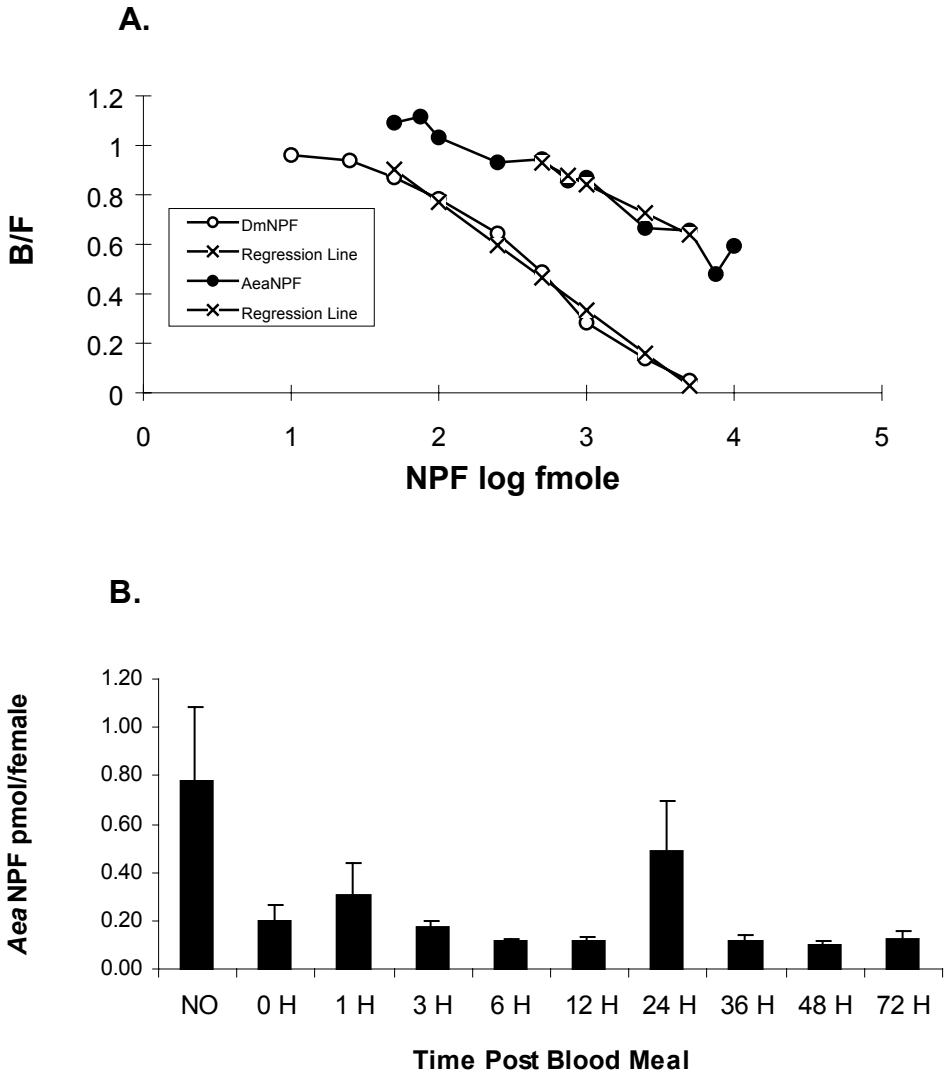


Figure 4. A. Parallel binding of *Aea*NPF (linear range 500 to 5000 fmol) and *Dm*NPF (linear range 50 to 5000 fmol) is demonstrated in the *Dm*NPF-2 RIA. B. Titer of *Aea*NPF in the hemolymph was quantified during different time points during a reproductive cycle using the heterologous *Dm*NPF-2 RIA. Data presented are per female averages from 9 replicates quantified as 2 female equivalents (n=9) for each time point with error bars reported as standard error. The highest titers of *Aea*NPF were present pre-blood meal (NO) and 24 hours post-blood meal (24 H).

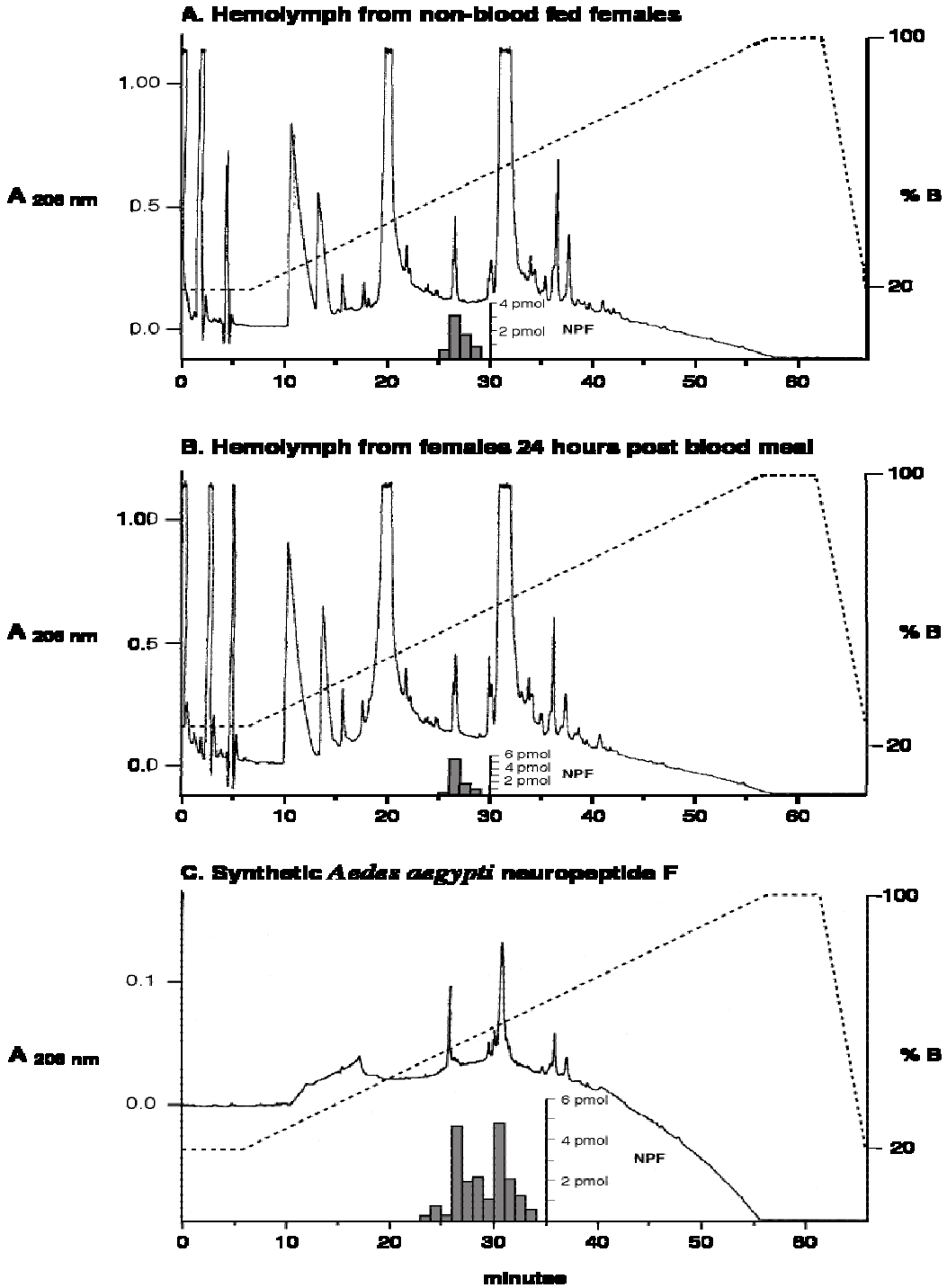


Figure 5. HPLC purification of hemolymph from female mosquitoes. A. Non-blood fed, B. 24 hours post-blood meal, and C. 8.8 µg synthetic *Aea*NPF. Material was separated with a gradient of solvent B (0.1%TFA in 80% CH_3CN; 20-100% B, 50 min; 1 ml/min; 206 nm) and assayed in the *Dm*NPF-2 RIA. Immunoreactive peptides eluting at 27 min form both hemolymph samples had a mass identical to synthetic *Aea*NPF (4130.4 Da), eluting at 27 min. Peptides eluting at 31 min were determined to be fragments of the synthetic peptide.

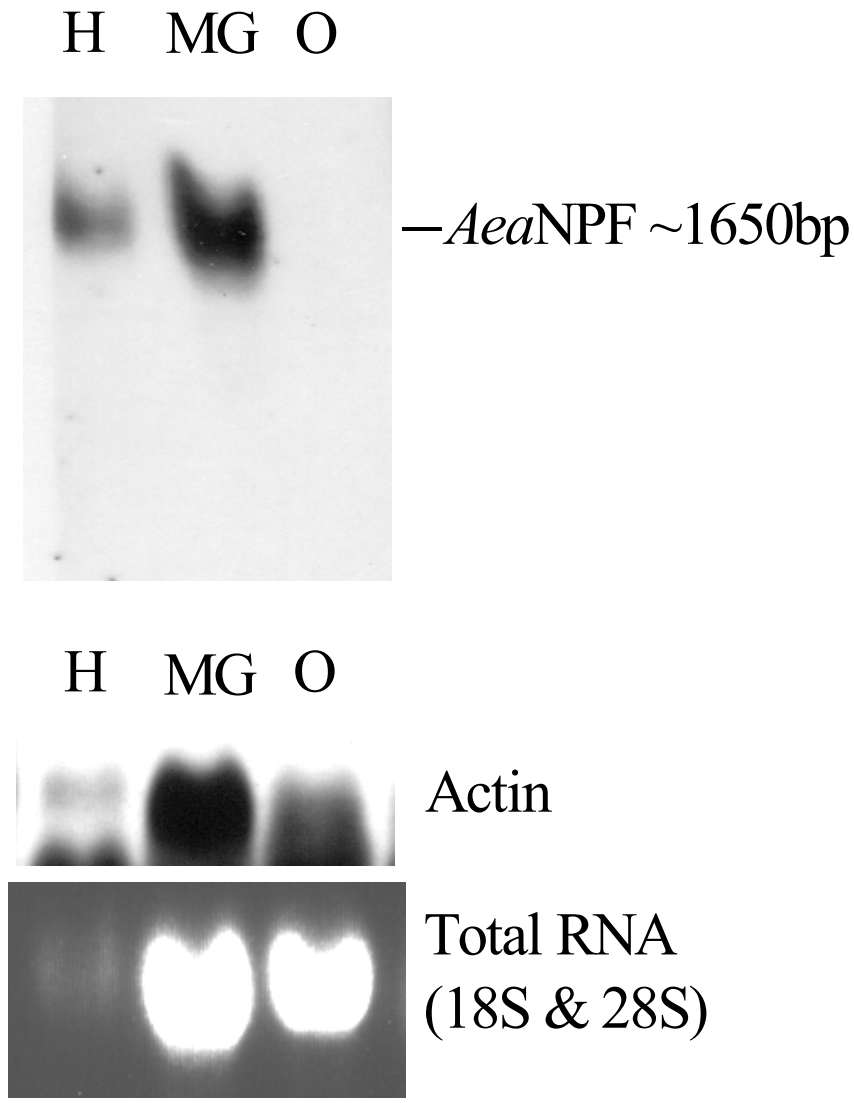


Figure 6. Northern analysis demonstrated the presence of *Aea*NPF transcript in the head (H), and midgut (MG) of sugar fed females, with ovary (O) as control. Actin and total RNA are shown for comparison.

CHAPTER 3

BIOASSAY FOR EFFECTS OF *AEDES AEGYPTI* NPF ON TRYPSIN SYNTHESIS IN THE FEMALE MOSQUITO, *AEDES AEGYPTI*

INTRODUCTION

All organisms rely on a continuous expenditure of energy. Energy and basic nutrients, such as amino acids, simple sugars, and fatty acids, are provided by the ingestion of food, which must be broken down into these molecules before becoming available for use. The diet of mosquitoes consists mainly of water and nectar, but females of some species also require a protein rich blood meal [1]. Once ingested, nectar and other plant juices are passed to the crop, but blood meals are received by the midgut. This separation of nectar and blood permits females to store a nectar meal in the crop, passing it slowly to the midgut for absorption, while the midgut is left empty to receive a blood meal at any time.

Protein is the predominant constituent of blood, and the mosquito midgut secretes a number of proteolytic enzymes to aid in its digestion. Protein digestion involves two steps and two classes of enzymes. First, protein molecules are cleaved into large peptides by endopeptidases and subsequently, large peptides are progressively shortened by exopeptidases, which remove single amino acids or dipeptides from each end. These enzymes are categorized according to their catalytic mechanisms. Trypsin and chymotrypsin are members of the subclass serine protease, which have catalytically active serine and histidine residues at their active center. Trypsin is the most prevalent endopeptidase in the midgut of the female mosquito *Aede*s *aegypti* [2,3] and accounts for 75% of protein digestion in this mosquito [4]. The activity of the trypsin increases to a maximum between 20 to 28 hours post blood meal [2].

Ingestion of a blood meal induces two phases of trypsin synthesis in the midgut of the mosquito *A. aegypti*, and each phase involves the synthesis of a different form [3]. The first phase occurs four to six hours post blood meal and is characterized by the release of small amounts of "early" trypsin, while the second phase, which occurs eight to thirty six hours post blood meal, is characterized by the release of large amounts of "late" trypsin. The early trypsin gene, under the control of juvenile hormone, is transcribed a few hours post adult emergence, and the mRNA is stored in the midgut epithelium cells until translation is triggered. Once the mosquito takes a blood meal, early trypsin is translated and released into the lumen to begin limited digestion of the meal. The mechanism initiating translation of early trypsin is unknown, but an increase in the size of the amino acid pool in the midgut is sufficient to activate translation of early trypsin mRNA [3]. Transcription and translation of late trypsin are regulated by uncharacterized proteolysis products generated by the actions of early trypsin on the blood meal protein [3]. Regulation at both the transcriptional and translational levels offers the midgut the ability to synthesize varying amounts of trypsin needed to digest different sized blood meals [3].

Effects of the endocrine system on blood digestion have been demonstrated in the mosquito by decapitation, brain neurosecretory cell ablation, and ovariectomy. In each experiment trypsin activity was reduced to less than 50% when the operations were performed prior to or immediately after the blood meal [2]. This indicates factors from the brain and ovaries affect trypsin levels. As well, endocrine cells in the midgut of the mosquito have been implicated in the regulation of trypsin secretion and protein digestion [2]. A decrease in the number of cells immunoreactive to FMRFa antiserum was observed in the midgut within six hours after a blood meal [5], suggesting release of immunoreactive material. Endocytosis of luminal material has been observed in some mosquito midgut endocrine cells [5,6], suggesting that endocrine cells survey the contents of the meal and release peptides accordingly, but this has yet to be demonstrated. Briegel and Lea observed endocrine control of tryptic activity during the

later phase of digestion [7]. Finally, it has been shown that head and ovarian factors control trypsin activity up to 16 hours after a blood meal [8,9].

Neuropeptide F (NPF) is the invertebrate ortholog of the neuropeptide Y (NPY) family, which is composed of NPY, peptide YY (PYY), and pancreatic polypeptide (PP). Peptide YY and PP, found mainly in the gut, have been shown to regulate gut enzyme secretion in rats [10]. Studies have also shown that PP-like peptides act to inhibit pancreatic enzyme secretion [11]. Previously, a PP-like peptide was localized in the nervous system of two dipterans [12-14] and the midgut of the mosquitoes *A. aegypti* [5] and *Anopheles stephensi* [15]. With the identification of *Aedes aegypti* NPF (*Aea*NPF) (Chapter 2), and its localization in more than 300 endocrine cells in the female midgut, it was hypothesized that *Aea*NPF regulates trypsin synthesis. Therefore, synthetic *Aea*NPF was tested in a bioassay to determine its effects on trypsin levels in females after a blood meal.

MATERIALS AND METHODS

Trypsin synthesis bioassay: Three to five day old females sugar fed for two days and then sustained on water were blood fed, decapitated, and then injected with 0.5 µl of saline (128 mM NaCl, 4.7 mM KCl, 1.9 mM $CaCl_2$) or varying amounts of synthetic *Aea*NPF. Positive controls were blood fed and injected with saline for comparison. Experimental animals were incubated at 27°C for 16 h, and individual midguts were dissected and transferred to 200 µl of 20 mM Tris pH 8.0 with 20 mM $CaCl_2$. Midguts were sonicated, centrifuged (14,000 x *g*, 10 min), and the supernatant was collected. Aliquots of midgut extract (10 µl or 0.05 midgut equivalents) and standard amounts of pancreatic bovine trypsin (Sigma, T7309) (10 µl) were measured for trypsin activity using 100 µl of 4 mM N∝Benzoyl-L-Arginine-p-Nitroanilide (BAPNA) a trypsin substrate that changes intensity due to the presence of trypsin. Absorbance was measured in an endpoint assay at 405 nm using a Spectra Max 340 micro titer plate

reader to measure the colormetic reaction. The change in absorbance from time zero to 30 min post addition of substrate was calculated per 0.05 midgut equivalents. The experiment was conducted on three groups of mosquitoes with eight individuals for each treatment. The changes in absorbance due to the colormetric reaction of the substrate with trypsin were analyzed for statistical difference using ANOVA all pairwise multiple comparison Tukey test.

RESULTS AND DISCUSSION

To investigate if *Aea*NPF affected trypsin synthesis in the midgut of the mosquito *A. aegypti*, varying amount of synthetic *Aea*NPF were injected into blood fed decapitated females. For controls, saline solution alone was injected into both intact and decapitated blood fed females. A significant decrease in trypsin levels for decapitated saline injected females (DC) relative to non-decapitated saline injected females (No DC) was obtained in this study. In intact saline injected individuals, 820.3±66.0 μg of trypsin per midgut (average±standard error) was detected while in decapitated and saline injected individuals only half of this amount of trypsin per midgut was present. There was neither a significant increase nor decrease in trypsin levels between decapitated and saline injected females (DC), and those decapitated and injected with various amounts of synthetic *Aea*NPF (Fig. 1). The experimental females contained a range of trypsin levels (372.0 to 504.0 μg per midgut) when injected with a range of *Aea*NPF (0.05 to 100 pmol/midgut), but none of the levels were statistically different from those levels observed in the decapitated and saline injected females (428.0±28 μg per midgut).

A decrease in trypsin levels between the non-decapitated saline injected individuals (No DC) and decapitated and saline injected individuals (DC) was expected because many factors are released from the brain upon the ingestion of a blood meal [17,18]. It has also been demonstrated that factors from the head increase trypsin levels [8]. Decapitation limits the action of head factors on the midgut thus preventing any increase in trypsin levels in response to the presence of head factors.

It is not entirely clear why trypsin levels neither increased nor decreased between the decapitated and saline injected females (DC) and those similarly injected with different doses of synthetic *Aea*NPF. Decapitation eliminated head factors that increased trypsin levels, and the injection of synthetic *Aea*NPF was unable to restore the trypsin levels observed in intact individuals injected with saline. Thus, *Aea*NPF is not the "head factor" that stimulates trypsin synthesis. The hypothesis that *Aea*NPF acts to increase trypsin synthesis was not supported, as trypsin levels from individuals injected with *Aea*NPF did not change compared to saline injected individuals. There was no observed decrease in trypsin levels in response to injection of *Aea*NPF indicating that *Aea*NPF does not have an inhibitory effect on trypsin levels.

Although unlikely, it is possible that *Aea*NPF may regulate trypsin synthesis in the mosquito, but additional experiments should be conducted before a definitive conclusion can be drawn. A second possible reason for the lack of an observed effect of NPF on trypsin synthesis comes from a recent study [19]. De Jong-Brink demonstrates that NPF in the mollusk*, Lymnaea stagnalis* is involved in the regulation of reproduction. Thus, NPF in *A. aegypti* may have a similar function. Future studies with this newly observed role should be conducted and will benefit the search for the function of *Aea*NPF in mosquitoes.

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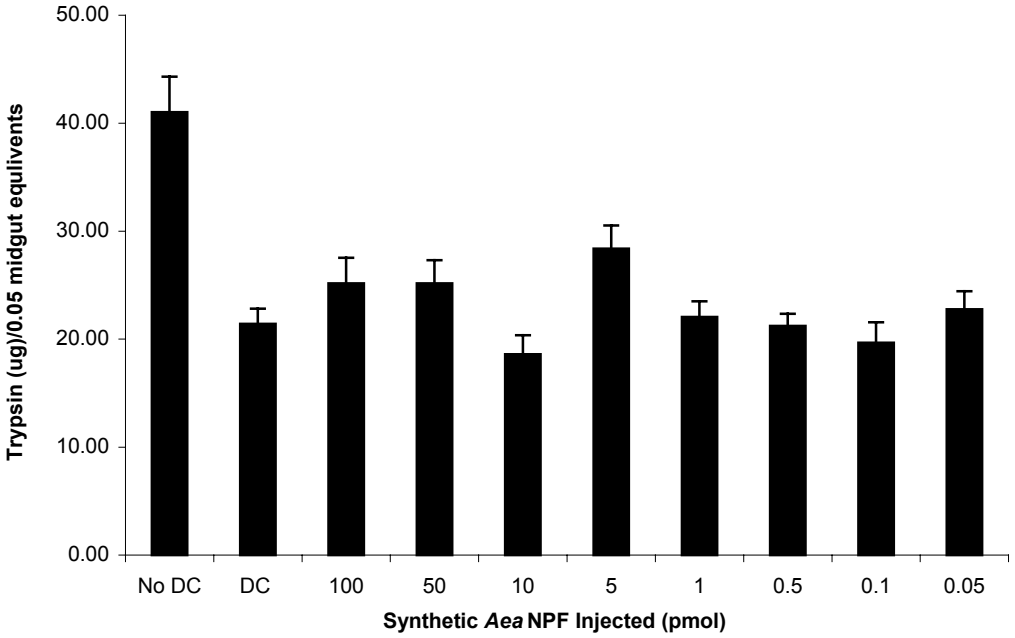

Figure 1. Trypsin (μg) per 0.05 midgut equivalents as measured in a colormetic assay at absorbance 405 nm and quantified against a trypsin standard line. Female *A. aegypti* mosquitoes (3 groups of mosquitoes with 8 females for each treatment) were given a blood meal and either left intact and injected with saline (No DC), decapitated and injected with saline (DC), or decapitated and injected with *Aea*NPF. Individual mosquitoes were incubated at 27°C for 16 h, and trypsin levels were assayed between time zero and 30 min post addition of substrate.

CHAPTER 4

CONCLUSIONS

Biochemical and molecular techniques were employed to isolate and characterize *Aedes aegypti* neuropeptide F (*Aea*NPF) and to investigate a potential role for this peptide in trypsin synthesis. First, NPF was purified from the mosquito, *A. aegypti* through semi-preparative and analytical ion exchange high performance liquid chromatography. Degenerate primers designed from the partial sequence of the purified peptide were used to amplify products that contained the cDNA encoding the prepropeptide. Immunocytochemistry and Northern blot analyses demonstrated the cellular localization of peptide and mRNA transcript in the brain and midgut of the mosquito. Quantification of the *Aea*NPF titer in the hemolymph of the mosquito *A. aegypti* determined the time and amount of peptide present in circulation during the course of a reproductive cycle. Based on the peptides presence in cells of the brain and midgut of the mosquito and changes in hemolymph titer during a reproductive cycle, it was proposed that *Aea*NPF had a function in trypsin synthesis. However, bioassay results demonstrated that *Aea*NPF was not the "head factor" that increased trypsin synthesis because injection of this peptide into blood fed decapitated females did not restore trypsin levels to that of intact, blood fed ones. As well, trypsin levels were not reduced in blood fed decapitated females when *Aea*NPF was injected relative to saline injected females. Although the exact function of *Aea*NPF in *A. aegypti* is still unclear, these studies have bought us one step closer to determining its role.

The research highlighted above has two practical applications. Mosquitoes pose a significant health risk to humans and domestic animals due to the pathogens that they transmit. Research in mosquitoes on basic mechanisms involved in feeding and the regulation of reproduction is vital. Basic research on the endocrinology of reproduction

in these pests may lead to the development of new methods for pest control. A second benefit of this research is that it has provided more evidence for the conservation of peptide families in invertebrates and vertebrates. Neuropeptide F has currently been isolated from two insects of the order Diptera [1] [Chapter 2]. First, from the fruit fly *Drosophila melanogaster* which is a member of the suborder Brachycera, and second from the mosquito *A. aegypti* a member of the suborder Nematocera. Neuropeptide Fs have also been identified in platyhelminths [2], and mollusks [3]. With the vast amount of genomic sequence data available, all aspects of peptide research including amino acid sequence, gene organization and functional studies will be necessary for the proper characterization of newly discovered peptides. Evolutionary comparisons of amino acid sequence, gene organization, and function may then be conducted to examine how peptides with similar functions have evolved, and the basic elements necessary for function. The role of NPF in the insect can be examined and compared to other insects and other invertebrates. As well, even the vertebrate orthologs of NPF such as the members of the NPY family can be included in these comparisons.

Although the precise function of NPF in *A. aegypti* is still unknown, this research has provided a strong foundation for future studies of NPF in invertebrates. It is my hope that this basic knowledge will aid future attempts in mosquito control and evolutionary comparisons of peptides.

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