Pyrokinin/PBAN peptides in the central nervous system of mosquitoes (Diptera: Culicidae)

Erica Hellmich

Iowa State University

Follow this and additional works at: http://lib.dr.iastate.edu/etd

Part of the Entomology Commons

Recommended Citation
http://lib.dr.iastate.edu/etd/11873

This Thesis is brought to you for free and open access by the Graduate College at Iowa State University Digital Repository. It has been accepted for inclusion in Graduate Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
Pyrokinin/PBAN peptides in the central nervous system of mosquitoes (Diptera: Culicidae)

by

Erica Hellmich

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Entomology

Program of Study Committee:
Russell Jurenka, Major Professor
Lyric Bartholomay
Michael Kimber

Iowa State University
Ames, Iowa

2010

Copyright © Erica Hellmich, 2010. All rights reserved.
I dedicate this to a great man who has guided me throughout my life, giving me my first experiences with nature and consistently motivating me by his words and actions.
TABLE OF CONTENTS

CHAPTER 1. General Introduction

Thesis Organization 1
Literature Review
  Blattodea 1
  Orthoptera 3
  Lepidoptera 6
  Diptera 8
  Hymenoptera 10
  Pyrokinin/PBAN Genes 11
Rationale 13
Objectives 14
Tables 15
Literature Cited 17

CHAPTER 2. Materials and Methods

Mosquitoes 24
Dissections
  Tools used for dissections 25
  Adult dissections
    Immobilization 25
    Ventral nerve cord 26
    Brain 26
    Thoracic nerve cords 27
Larvae
  Immobilization 27
  Dissection 27
Immunocytochemistry 28
  Goat anti-rabbit horseradish peroxidase 2° antibody 29
CHAPTER 3. Results

Results using GaR HRP as secondary antibody

- *Ae. aegypti* larvae 41
- *Ae. triseriatus* larva 41
- *An. stephensi* larvae 42
- *Oc. caspius* larvae 42
- *Ar. subalbatus* larvae 42
- *Ae. triseriatus* adult 43

Results using fluorescent secondary antibody

- *Ae. aegypti* larvae 43
- *An. stephensi* larvae 44
- *Cx. pipiens* larvae 44
- *Ar. subalbatus* larvae 45
- *Tox. amboinensis* larvae 45
- *Ae. aegypti* adults 46
- *Ae. triseriatus* adults 46
- *Tox. amboinensis* adult 47

Figures 48

CHAPTER 4. General Conclusions

Future Research 69
Tables and Figure 73
Literature Cited 78

ACKNOWLEDGEMENTS 84
CHAPTER 1. General Introduction

Thesis Organization

This thesis is divided into four chapters. Chapter 1 includes a literature review of the pyrokinin/PBAN family of peptides in various insect orders and the genes that encode for this family of peptides. The first chapter also includes the rationale behind the research and the research objectives. Chapter 2 is a description of the materials and methods devised to accomplish this project. Chapter 3 details the results of this research project. The general conclusions of the thesis are in Chapter 4.

Literature Review

Blattodea

In 1984, five substances containing hindgut-stimulating compounds in head extracts of the cockroach, *Leucophaea maderae*, were fractionated using HPLC. After further purification, eleven peptides were separated: eight closely related octapeptides, the leucokinins I-VIII (Holman et al., 1986b, 1987a, b); two sulfated peptides, leucosulfakinins (Nachman et al., 1986b; Nachman et al., 1986c); and the octapeptide, leucopyrokinin (Holman et al., 1986a). All eleven peptides were shown to have myotropic activity in the hindgut of the cockroach (Cook et al., 1989, 1990; Holman et al., 1986a; Nachman et al., 1986b; Nachman et al., 1986c). The octapeptide, leucopyrokinin, was the first peptide isolated in a family of peptides that would later be distinguished as the pyrokinin/PBAN family of peptides.

The primary sequence for leucopyrokinin was identified as: pGlu-Thr-Ser-Phe-Thr-Pro-Arg-Leu-NH₂ (Holman et al., 1986a). This new peptide was named Lem-
PK. Investigations by Nachman et al. (1986a) revealed the pentapeptide fragment Phe-Thr-Pro-Arg-Leu-NH₂ as the active core of Lem-PK. Subsequently, two peptides in the pyrokinin/PBAN family, Pea-PK-1 and Pea-PK-2, were isolated from the American cockroach, *Periplaneta americana* (Predel et al., 1997a). This study also marked the first time the myotropic peptides had been isolated from the retrocerebral complex, the major storage and release center of neurosecretions produced in the insect brain. The finding of a pyrokinin/PBAN family peptide in a neurohemal organ suggested its role as a hormone. The pyrokinin/PBAN family was increased to seven known peptides from the American cockroach with the isolation of Pea-PK-3, Pea-PK-4, Pea-PK-5 (Predel et al., 1999; Predel et al., 1997b) and Pea-PK-6 (Predel and Eckert, 2000).

Investigations by Predel et al. (1999) showed the pyrokinin-isoforms were differentially distributed in the American cockroach. Pea-PK-1-4 were isolated from the retrocerebral complex and Pea-PK-5 was isolated from the abdominal perisymathetic organs (aPSO), or neurohemal organs. The most recently identified isoform, Pea-PK-6, was isolated from the aPSO as well as the corpora allata (CA) (Predel and Eckert, 2000). Pea-PK-6 was the first neuropeptide to be confirmed to be stored in both organs. Pea-PK-1-4 were also found in the CA and Pea-PK-5 was again identified in the aPSO. Pea-PK-1-4 and Pea-PK-6 were found to be produced in neurosecretory cells of the subesophageal ganglion and tritocerebrum and transported via the nervi corporis cardiac-1 (NCC-1), NCC-3 and nervi corporis allati-2 (NCA-2) into the retrocerebral complex. Pea-PK-1, Pea-PK-4 and Pea-PK-6 were detected around the CA and innervated target organs such as the pharyngeal dilator muscles and aorta wall. Pea-PK-5-6 immunostained nerve fibers
showed innervations of target organs in the abdomen, such as hyperneural muscles, heart and segmental vessels.

Several studies were done to determine the efficacy of each pyrokinin on various muscles within the two species of cockroaches in which the peptides had been isolated, *Leucophaea maderae* and *Periplaneta americana*. Lem-PK was first studied in 1993 for its ability to induce contraction of various muscles in both species of cockroaches (Wagner and Cook, 1993). In *Leucophaea maderae*, Lem-PK had similar action on the hindgut, foregut and heart as did proctolin. Response to Lem-PK in the oviduct required 100 times as much peptide as the neurohormone proctolin for half as great of a response. In *Periplaneta americana*, 100 times more Lem-PK than proctolin was necessary for a response in the hindgut, and 1000 time more Lem-PK for the foregut. Studies of Pea-PK-1-5 (Predel et al., 1999), showed isoforms 1-4 had some degree of myotropic effect on the hyperneural muscles whereas Pea-PK-5 had extremely low myotropic effect. Further studies showed the most potent isoform on hyperneural muscles was PK-1 followed by PK-4>PK-3>PK-2>PK-5 (Predel and Nachman, 2001). These same results were found for the myotropic activity in the hindgut, foregut and oviduct. These studies demonstrated no muscle-specific actions of the various isoforms.

**Orthoptera**

Research in *Locusta migratoria* (Sreng et al., 1990) found PBAN-like peptides associated with the brain, corpora cardiaca, corpora allata, subesophageal ganglion and thoracic ganglia in both male and female locusts. Heat stability tests on the extracts from the corpora allata showed no change in the activity of the peptides when heated for 5
4

minutes at 100°C.

Schoofs et al. isolated six peptides within the pyrokinin/PBAN family in the early 1990’s. The first peptide identified in the pyrokinin/PBAN family of peptides isolated from *L. migratoria* was termed locustamyotropin I (Lom-MT I) (Schoofs et al., 1990a). The C-terminal amino acid sequence was found to be Phe-Ser-Pro-Arg-Leu-NH2. Lom-MT I is not blocked at the N-terminus like Lem-PK but is similar in activity as it also stimulates visceral muscle contraction of the hindgut of *L. maderae* and the visceral muscles of the oviduct and foregut in *L. migratoria*. Lom-MT II, isolated from *L. migratoria* (Schoofs et al., 1990b), has a C-terminal sequence of Phe-Thr-Pro-Arg-Leu-NH2, which is identical to Lem-PK isolated from *L. maderae*. These peptides differ in the first three aminoterminal residues, Lem-PK being blocked, pGlu-Thr-Ser, and Lom-MT II, Glu-Gly-Asp. Lom-MT III and IV (Schoofs et al., 1992a) were isolated from locusts and differ in a single residue in the C-terminal sequence, Lom-MT III: Phe-Val-Pro-Arg-Leu-NH2 and Lom-MT IV: Phe-Ser-Pro-Arg-Leu-NH2. All locustamyotropins have been shown to stimulate the visceral muscles of the hindgut of *L. maderae* and the oviduct of *L. migratoria*. Lom-MT IV shows greater effect on the visceral muscles than Lom-MT I-III.

A peptide similar to Lem-PK was isolated from *L. migratoria* and termed Lom-PK, locustapyrokinin. The two peptides differ by a single residue in the C-terminal sequence of Phe-X-Pro-Arg-Leu-NH2, where X is Thr in Lem-PK and is replaced by Val in Lom-PK. Lom-PK and Lem-PK have similar activity in the hindgut of *L. maderae*. Lom-PK II, also isolated from *L. migratoria*, has the C-terminal sequence Phe-Thr-Pro-Arg-Leu-NH2. Both Lom-PK I and Lom-PK II are blocked at the N-terminus like Lem-
PK. Lom-PK II stimulates the visceral muscles of the hindgut in *L. maderae* and the oviduct in *L. migratoria*.

Immunocytochemical studies were conducted to isolate the location of locustamyotropin peptides in *L. migratoria* (Schoofs et al., 1992b; Tips et al., 1993). Immunoreactivity was present in cell bodies of the inner and outer edge of the tritocerebrum and the branches of the nervi corporis allati I and II innervating the corpora allata. Axon terminals in the corpora cardiaca were stained indicating that locustamyotropins might be released into the hemolymph. Two immunoreactive cell clusters were detected within the subesophageal ganglion with their axons traveling to the nervi corporis allati II and continuing on to the corpora allata. Immunoreactive cells were present in the third thoracic ganglion, which is fused with the first three abdominal ganglia, with reactivity shown in all three abdominal ganglia. Immunoreactive fibers were detected in all three thoracic ganglia and throughout the abdominal ganglia and connectives of the ventral nerve cord. Abdominal ganglia 4-6 showed immunoreactive cells in three regions, ganglion 7 showed two regions and ganglion 8 showed three regions. Ganglia 4-8 had an immunoreactive process on the anterior of the ganglion that led to a neurohemal organ containing many immunoreactive terminals.

Another pyrokinin/PBAN peptide and a closely related peptide were isolated from *Schistocerca gregaria* (Veelaert et al., 1997). Scg-MT I, *Schistocerca gregaria* myotropin-I, was isolated with the C-terminal sequence of Phe-Ser-Pro-Arg-Leu-NH2. The closely related peptide, Scg-MT-II, has a very similar C-terminal sequence, Phe-Pro-His-Pro-Arg-Leu-NH2.
**Lepidoptera**

A 33-amino acid peptide, Hez-PBAN, was isolated from the subesophageal ganglion in *Helicoverpa zea* (Raina et al., 1987). Hez-PBAN was the first pyrokinin/PBAN peptide isolated in Lepidoptera and displayed pheromonotropic activity from larval, pupal, and adult SEG when tested on adult female moths. Hez-PBAN induced pheromone production in *H. zea* as well as six other moth species (Raina et al., 1989). Two PBAN peptides were isolated from *Bombyx mori*, Bom-PBAN-I (Kitamura et al., 1989) and Bom-PBAN-II (Kitamura et al., 1990) and one in *Lymantria dispar*, Lyd-PBAN, (Masler et al., 1994). As of 2009, PBAN had been characterized in 19 species of moths based on either peptide purification or sequence of the gene encoding PBAN (Rafaeli, 2009).

Immunocytochemistry has been done in several species, beginning with *H. zea* in 1992 (Kingan et al., 1992). In *H. zea*, three clusters of immunoreactive cells were detected in the SEG, in the mandibular, maxillary and labial neuromeres. Cells from the labial neuromere showed immunoreactive axons reaching the CC and aorta, both of which showed immunoreactivity, indicating the CC as a neurohemal release site. Axons from the mandibular and maxillary clusters extend toward the anterior of the SEG. Two pairs of axons originate in the maxillary neuromere and extend the entire length of the VNC to the terminal abdominal ganglion. Axons also lead from the maxillary cluster to nerve endings in the ventral and superior protocerebrum. Additional investigations revealed three clusters of immunoreactive cells in the SEG of day 0 pupae, larvae, and adults of *B. mori* (Sato et al., 1994) and three clusters of immunoreactive in the SEG of larvae, adult males and females of *Agrotis ipsilon* (Duportets et al., 1998).
Immunoreactivity similar to that found in *H. zea* was observed in female *Ostrinia nubilalis* (Ma and Roelofs, 1995). Two pairs of axons from the mandibular and maxillary neuromeres project into the paired ventral connectives and continue the entire length of the VNC terminating in the TAG. The labial neuromere axons exit the SEG via the nervi corporis cardiaci III to the CC, also similar to *H. zea*. Two pairs of immunoreactive cell bodies were identified in the prothoracic ganglion and two pairs in the mesothoracic ganglion. A pair of cell bodies was also found in the fused metathoracic-abdominal 1-2 ganglion. Axons originating in these cells project vertically, then posteriorly into the VNC with the destination not yet determined. A pair of immunoreactive cells was also found in the third and fourth abdominal ganglion with axons projecting vertically and anteriorly into the VNC, again with destination not determined. In the fifth abdominal ganglion another pair of immunoreactive cells was detected, and in the TAG three pairs of cell bodies were identified.

Similar immunocytochemical results from *Samia cynthia ricini* (Wei et al., 2004) and *Anterea pernyi* (Wei et al., 2008) were shown. In both species, three groups of immunoreactive cell bodies were detected, each group associated with the mandibular, maxillary and labial neuromeres. A pair of immunoreactive cell bodies was also found in the thoracic ganglion and the TAG. Choi et al. (2004) found similar staining results in the SEG of *Adoxophyes*, with three groups of immunoreactive cell bodies being found. Three pairs of reactive cell bodies were identified in each protocerebral hemisphere of the brain, results not previously seen in lepidoptera. A network of neurite varicosities from these cell bodies descended into the protocerebrum. Two pairs of immunoreactive cell bodies were detected in the first thoracic ganglion and one pair in the third thoracic
ganglion. A pair of immunoreactive cell bodies was also identified in the third and fourth abdominal ganglion with both pairs being associated with a neurohemal organ.

Various physiological functions have been attributed to PBAN-like peptides in lepidoptera. Stimulation of pheromone biosynthesis was first seen in *H. zea* (Raina et al., 1989). Melanization and reddish coloration in moth larvae were also attributed to PBAN-like peptides (Altstein et al., 1996; Matsumoto et al., 1990; Matsumoto et al., 1992; Raina et al., 2003). PBAN-like peptides were also found to have an affect on the induction of embryonic diapause in *B. mori* (Imai et al., 1991; Suwan et al., 1994) and the termination of diapause in *Heliothis virescens* and *Helicoverpa armigera* (Xu and Denlinger, 2003; Zhang et al., 2004). In addition, ecdysone biosynthesis in the prothoracic glands of *B. mori* has recently been shown to involve PBAN-like peptides (Watanabe et al., 2007).

**Diptera**

Research on fleshfly larvae, *Sarcophaga bullata*, indicated that a peptide was involved in pupariation behaviors and puparial tanning (Zdarek et al., 1997). Injection of Lem-PK resulted in accelerated pupariation behavior and subsequent puparial tanning. When a posterior segment was ligated on the larvae, isolating it from the CNS, injection of Lem-PK anterior to the ligation showed no muscular contractions or pupariation behavior. However, it did accelerate tanning of the ligated segment. These results suggested that Lem-PK elicits cuticular changes via a peripheral mechanism.

Investigations continued to explore the relationship of the chemical structure and conformation of analogues of pyrokinin/PBAN and pupariation acceleration activity (Nachman et al., 1997). The C-terminal pentapeptide, FTPRLamide, demonstrated
equipotency in the pupariation behavior, but only 10% of the potency on the tanning aspect compared to Lem-PK. The tetrapeptide TPRLamide showed equipotency to Lem-PK in both aspects and the tripeptide PRLamide showed 10% of the response. These results showed the tripeptide as the active core for pupariation acceleration and that a complete response is achieved with the tetrapeptide. Further research (Zdarek et al., 1998) indicated the two C-terminal amino acid residues are the most critical for pupariation acceleration. Lom-MT II-IV showed a decrease in potency in pupariation behavior and an even greater decrease in tanning as compared to Lom-MT-I and Lem-PK. This shows the potential for N-terminally modified pyrokinin in distinguishing between the behavioral and tanning aspects of pupariation (Zdarek et al., 1998).

The naturally occurring pupariation factor was isolated from the grey flesh fly, Neobellieria bullata (Verleyen et al., 2004). It is an octapeptide with the sequence Ser-Val-Gln-Phe-Lys-Pro-Arg-Leu-amide and designated as Neb-PK-2. The immunohistochemical distribution pattern of Neb-PK-2 is very similar to that of Drosophila pyrokinin-2 (see below), from which it differs by only one amino acid residue. Therefore this pyrokinin is most likely involved in the higher Diptera that utilize a puparium for pupation. This was confirmed in another study testing all five pyrokinins identified from N. bullata (Nachman et al., 2006).

Pyrokinin/PBAN peptides from Drosophila melanogaster have also been studied (Choi et al., 2001). Extracts from D. melanogaster larval CNS were injected into H. zea and H. armigera and stimulated pheromone biosynthesis. Immunoreactivity was observed in 3rd instar feeding, wandering and pre-pupal stage larvae, pupae, adult males and females. Similar immunoreactive staining was detected throughout the life stages.
Three groups of immunoreactive neurons were found in the SEG of larvae whereas adults had four. In larvae the most anterior group of neurons projected a set of neurites to the brain, forming an arch-like process in the anterior protocerebrum while this process originated in the second group of neurons in adults. In adults, a pair of neurons was found in the dorsolateral part of each protocerebrum. All developmental stages in *D. melanogaster* had three pairs of immunoreactive neurons present in the ventral nervous system, which appear to send axons to a neurohemal organ. Adults contained a network of varicosities in the abdomen that was not seen in immature flies. Similar immunoreactivity was seen in brain/SEG of the adult blowfly, *Protophormia terraenovae* (Shiga et al., 2000).

**Hymenoptera**

Recently, work has begun on the characterization of pyrokinin/PBAN peptides in fire ants, *Solenopsis invicta*. Immunocytochemistry has shown similar patterns to those previously seen in moths (Choi et al., 2009). Three clusters of neurons with PBAN-like immunoreactivity and corresponding to the mandibular, maxillary and labial neurons have been identified in the SEG. The corpora cardiaca was shown to contain abundant immunoreactive material. Five pairs of immunoreactive neurons were detected in the ventral nerve cord, one in the first thoracic ganglion, one in the second thoracic ganglion, and the remaining three pairs in the third, fourth and fifth abdominal ganglia. The neurons in the abdominal ganglia were associated with neurohemal organs. Neurites were found running throughout the ventral nerve cord, which is similar to previous findings from other species studied to date.
Soi-PBAN, the first *S. invicta* peptide from the pyrokinin/PBAN family, is composed of a 26 amino acid chain, GSGEDLSGYDAYEVDEDDHPLFVPRLamide (Choi and Vander Meer, 2009). The gene sequence for Soi-PBAN shows 56% similarity to PBAN found in *Apis mellifera* (Hummon et al., 2006) and 30% similarity to lepidopteran moth PBAN. When injected into decapitated *H. zea* females, Soi-PBAN induced pheromone production, which indicates cross-reactivity.

Five additional *Solenopsis* PBAN genes have been isolated from 4 species, *S. geminata*, *S. richteri*, *S. pergandii*, *S. carolinensis*, and a hybrid of *S. invicta* and *S. richteri* (Choi et al., 2010). The cDNA coding region for PBAN in *S. germinata* (Sog-PBAN) showed 98% similarity with that of *S. invicta* but has the 27 amino-acid sequence GSGEDLFSYGDAYEVDEDDHPLFVRLamide. The cDNA sequences for PBAN from *S. invicta*, *S. richteri*, and the hybrid are nearly identical, having only a single DNA codon difference, which does not alter the amino acid sequence. The PBAN coding region in *S. pergandii* is identical to that of *S. germinata*. The cDNA sequence for *S. carolinensis* PBAN (Soc-PBAN) showed a 92% similarity to the other PBANs isolated from fire ants. Soc-PBAN, GSGEDLFSFGDAYEADEDDHTLFVPRlamide, differs from Sog-PBAN by three amino acids.

**Pyrokinin/PBAN genes**

In *D. melanogaster*, two genes, CAPA (Kean et al., 2002) and hugin (Meng et al., 2002) have been shown to produce pyrokinin/PBAN family peptide precursors. The CAPA gene encodes two perviscerokinins (PVKs), CAPA-PVK-1 and CAPA-PVK-2, and a single pyrokinin, CAPA-PK. Sequencing of other insect genomes has resulted in
identification of similar CAPA genes (Table 1). The hugin gene encodes for a peptide similar to the pyrokinins, Drm-PK-2, and to an ecdysis-triggering hormone-like peptide (Meng et al., 2002). The genes encoding the precursors similar to the hugin gene have been identified in other insects but are encoding PBAN-like peptides.

The CAPA gene produces neuropeptides found in the abdominal ventral nerve cord of insects. They are synthesized in the median neurosecretory neurons of abdominal ganglia and are likely released as hormones from the abdominal perisym pathetic organs (PSO) into the hemolymph. The first CAPA peptide was identified from *Periplaneta americana* and designated periviscerokinin-1 (PVK-1) (Predel et al., 1995a). PVK-1 was the first instance of a neuropeptide having been isolated from an insect PSO and indicated that abdominal ganglia may produce different neurohormones than those being produced in other areas. PVK-1 was also shown to be an ortholog of the *Drosophila* peptide encoded on the CAPA, or “capability”, gene (Kean et al., 2002). The name is in reference to the gene’s ability to encode for neuropeptides related to cardioacceleratory peptides (CAPs) in the VNC/PSOs of *Manduca sexta*. Many CAPs have been isolated from arthropods and mollusks (Table 1).

In *Manduca sexta*, CAPA was isolated in 15-16 pairs of cells in the brain of 1st and 2nd instar larvae (Loi and Tublitz, 2004). Larval and adult stages contain two pairs of CAPA-expressing cells in the SEG and one pair in the thoracic ganglia. The abdominal ganglia of larvae have four pairs of expressing cells while in adults the number is reduced to three. A study conducted by Neupert et al. (2009) revealed the products of two different genes, CAPA and PBAN, expressed in the labial neuromeres of the SEG of *M. sexta*. The mandibular and maxillary neuromeres only expressed peptide products of the
PBAN-gene. In *D. melanogaster*, only the CAPA gene is expressed in labial neuromeres (Kean et al., 2002), and in *P. americana* the PBAN gene is expressed (Predel et al., 2007).

Isolation of the CAPA-expressing cells in *P. americana* also revealed that each of the six neuropeptides, Pea-PK 1-6, which includes three CAPA gene related products, are packaged in separate vesicles (Pollák et al., 2005). This packaging was found in the neurosecretory cells of the abdominal PSO and interneurons of the brain. These results suggested that the CAPA gene was cleaved to produce multiple peptides and packaged and distributed independently.

The receptors for these two genes have been studied in many insects with the most relevant being the work done in *Anopheles gambiae* (Olsen et al., 2007). In *A. gambiae* the two CAPA peptides, CAPA-1 and CAPA-2, bind to a specific CAPA receptor that does not react with other neuropeptides, whereas PK-1, also known as CAPA-PK, activates a specific PK-1 receptor. In *A. gambiae* the coding region for the pyrokinin peptides, similar to the pyrokinin gene produced from the hugin gene in *D. melanogaster*, gives rise to two pyrokinins, PK-2-1 and PK-2-2, and a pyrokinin-1, PK-1. Each PK-2 activates a specific PK-2 receptor. In *D. melanogaster* there are two receptors that are activated by the hugin peptides. There is also much cross talk in *A. gambiae* between the products of the two gene precursors and the receptors (Olsen et al., 2007).

**Rationale**

Pyrokinin/PBAN peptide sequences have been identified in many insect species. At this time the function of these peptides is known only from a small number of insect
species and has been shown to be important in pheromone biosynthesis, melanization of larvae and embryonic diapause, among other functions. Mosquitoes are very important vectors of arboviruses and other parasites. Identification and characterization of PBAN-like peptides in mosquitoes offers a further understanding of their physiology. Furthermore, study of these peptides may provide valuable insight toward control of the insect vector or the pathogen within the vector.

**Objectives**

**General objective:** Using immunocytochemistry, identify and locate pyrokinin/PBAN-like peptides in the central nervous system of adult and larval mosquitoes.

**Specific objectives:**

1. **Devise techniques to:**
   a. dissect central nervous system from mosquito adults.
   b. dissect central nervous system from mosquito larvae.
   c. successfully complete immunocytochemistry.
   d. mount central nervous system for microscopy visualization.

2. Compare immunocytochemical results to those from previously studied insects.
Table 1. Selected sequences of CAPA peptides from insects.

<table>
<thead>
<tr>
<th>Species</th>
<th>CAPA-PVK-1</th>
<th>CAPA-PVK-2</th>
<th>CAPA-PK</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drosophila melanogaster</td>
<td>GANMGLYAFPRV</td>
<td>ASGLVAFPRVA</td>
<td>TGPSASSGLWFGPRL</td>
<td>Keen et al., 2002; Predel et al., 2004; Baggerman et al., 2005; Wegener et al., 2006</td>
</tr>
<tr>
<td>Aedes aegypti</td>
<td>GPTVGLFAFPRV</td>
<td>pQGLVFPRPV</td>
<td>AGNSGANSGMWFGPRL</td>
<td>Predel and Wegener 2006</td>
</tr>
<tr>
<td>Anopheles gambiae</td>
<td>GPTVGLFAFPRV</td>
<td>pQGLVFPFRV</td>
<td>AGGTGANSAMWFGPRL</td>
<td>Pollock et al., 2004; Riehle et al., 2002</td>
</tr>
<tr>
<td>Bombyx mori</td>
<td>PDGVNLYPFPFRV</td>
<td>QLYAFPRV</td>
<td>XXPGMWFPRV</td>
<td>Predel and Wegener 2006</td>
</tr>
<tr>
<td>Locusta migratoria</td>
<td>AAGLQFPFRV</td>
<td>GLLAFPRV</td>
<td>DGGEPAAPLWFGPRL</td>
<td>Clynen et al., 2003; Predel and Gade 2002</td>
</tr>
<tr>
<td>Schistocerca gregaria</td>
<td>AAGLQFPFRV</td>
<td>GLLAFPRV</td>
<td>DGAETPGAAALWFGPRL</td>
<td>Clynen et al., 2003; Predel and Gade 2002</td>
</tr>
<tr>
<td>Periplaneta americana</td>
<td>GASGLIPVMRN</td>
<td>GSSGLISMPRV</td>
<td>GGGSGGETSGMWFPRP</td>
<td>Predel et al., 1995; Predel et al., 1998; Predel et al., 1999; Predel et al., 1999</td>
</tr>
<tr>
<td>Leucophaea maderae</td>
<td>GSSGLIPFGRT</td>
<td>GSSGLISMPRV</td>
<td>FGETSGETKGMWFPRP</td>
<td>Predel et al., 2000; Predel unpublished data</td>
</tr>
</tbody>
</table>
Table 2. Amino acid sequence and accession number of PBAN-like sequences.

<table>
<thead>
<tr>
<th>Species</th>
<th>Peptide Name</th>
<th>Sequence</th>
<th>Accession No.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. americana</td>
<td>Pea-PK-1</td>
<td>HTAGFIPRL</td>
<td>P82691</td>
<td>Predel et al., 1997b</td>
</tr>
<tr>
<td></td>
<td>Pea-PK-2</td>
<td>SPPFAAPRL</td>
<td>P82692</td>
<td>Predel et al., 1997b</td>
</tr>
<tr>
<td></td>
<td>Pea-PK-3</td>
<td>LVFPFRPRL</td>
<td>P82618</td>
<td>Predel et al., 1999</td>
</tr>
<tr>
<td></td>
<td>Pea-PK-4</td>
<td>DHLPHDVSFAIPRL</td>
<td>P82619</td>
<td>Predel et al., 1999</td>
</tr>
<tr>
<td></td>
<td>Pea-PK-5</td>
<td>GGGGSGETGMWFGPRL</td>
<td>P82617</td>
<td>Predel et al., 1999</td>
</tr>
<tr>
<td></td>
<td>Pea-PK-6</td>
<td>SESEVPGMWFGPRL</td>
<td>P82693</td>
<td>Predel et al., 2000</td>
</tr>
<tr>
<td>L. maderae</td>
<td>Lem-Pk</td>
<td>pQTSFTPRL</td>
<td>P13049</td>
<td>Holman et al. 1986a</td>
</tr>
<tr>
<td>L. migratoria</td>
<td>Lom-PK-1</td>
<td>pQDSGEWIPQFPVPR</td>
<td>P20404</td>
<td>Schoofs et al., 1993</td>
</tr>
<tr>
<td></td>
<td>Lom-PK-2</td>
<td>pQSVPTFPRL</td>
<td>P41488</td>
<td>Schoofs et al., 1993</td>
</tr>
<tr>
<td></td>
<td>Lom-MT-1</td>
<td>GVPPAAQWFSRPR</td>
<td>P22395</td>
<td>Schoofs et al., 1993</td>
</tr>
<tr>
<td></td>
<td>Lom-MT-2</td>
<td>EGDFTPRL</td>
<td>P22396</td>
<td>Schoofs et al., 1993</td>
</tr>
<tr>
<td></td>
<td>Lom-MT-3</td>
<td>RQQFPVPR</td>
<td>P41489</td>
<td>Schoofs et al., 1993</td>
</tr>
<tr>
<td></td>
<td>Lom-MT-4</td>
<td>RLHNGMPFSOPRL</td>
<td>P41490</td>
<td>Schoofs et al., 1993</td>
</tr>
<tr>
<td>S. gregaria</td>
<td>Scg-MT-1</td>
<td>GAAPAQFSRPR</td>
<td></td>
<td>Veelaert et al., 1997</td>
</tr>
<tr>
<td></td>
<td>Scg-MT-2</td>
<td>TSSLFPHPR</td>
<td></td>
<td>Veelaert et al., 1997</td>
</tr>
<tr>
<td>H. zea</td>
<td>Hez-PBAN</td>
<td>LSDDMPATPADQEMYRQDPEQIDsRTKYFSRPR</td>
<td>AAA20661</td>
<td>Davis et al., 1992</td>
</tr>
<tr>
<td>B. mori</td>
<td>Bom-PBAN-I</td>
<td>LSDDMPATPADQEMYEDPEEMESRTYFSRPR</td>
<td>AAB24327</td>
<td>Kitamura et al., 1989</td>
</tr>
<tr>
<td></td>
<td>Bom-PBAN-II</td>
<td>RLSEDMPATPADQMYQPDPEEMESRTYFSRPR</td>
<td>AAB24327</td>
<td>Kitamura et al., 1990</td>
</tr>
<tr>
<td>L. dispar</td>
<td>Lyd-PBAN</td>
<td>LADDMPATMADQVYRPEEDEISRKNYFSRPR</td>
<td>AAB32665</td>
<td>Masler et al., 1994</td>
</tr>
<tr>
<td>S. invicta</td>
<td>Soi-PBAN</td>
<td>GSGEDLSYGDAYEVDEDHHPLFVPRL</td>
<td>ACL35348</td>
<td>Choi and Vander Meer, 2009</td>
</tr>
<tr>
<td>S. geminata</td>
<td>Sog-PBAN</td>
<td>GSGEDLSYGDAYEVDEDHHPLFVPRL</td>
<td>ADI88478</td>
<td>Choi et al., 2010</td>
</tr>
<tr>
<td>S. richteri</td>
<td>GSGEDLSYGDAYEVDEDHHPLFVPRL</td>
<td>ADI88481</td>
<td>Choi et al., 2010</td>
<td></td>
</tr>
<tr>
<td>S. inv. x ric.</td>
<td>Sop-PBAN</td>
<td>GSGEDLSYGDAYEVDEDHHPLFVPRL</td>
<td>ADI88482</td>
<td>Choi et al., 2010</td>
</tr>
<tr>
<td>S. pergandii</td>
<td>Sop-PBAN</td>
<td>GSGEDLSYGDAYEVDEDHHPLFVPRL</td>
<td>ADI88479</td>
<td>Choi et al., 2010</td>
</tr>
<tr>
<td>S. carolinensis</td>
<td>Soc-PBAN</td>
<td>GSGEDLSYGDAYEVDEDHHPLFVPRL</td>
<td>ADI88480</td>
<td>Choi et al., 2010</td>
</tr>
</tbody>
</table>
Literature Cited


Cook, B., Holman, G., Wagner, R., Nachman, R., 1989, Pharmacological actions of a new class of neuropeptides, the leucokinins I-IV, on the visceral muscles of Leucophaea maderae. Comp Biochem Physiol C 93, 257-262.


Duportets, L., Gadenne, C., Dufour, M., Couillaud, F., 1998, The pheromone biosynthesis activating neuropeptide (PBAN) of the black cutworm moth, Agrotis
**ipsilon:** immunohistochemistry, molecular characterization and bioassay of its peptide sequence. Insect Biochem Mol Biol 28, 591-599.


Holman, G.M., Cook, B.J., Nachman, R.J., 1986b, Primary structure and synthesis of two additional neuropeptides from *Leucophaea maderae*: Members of a new family of cephalomyotropins. Comp Biochem Physiol C 84, 271-276.

Holman, G.M., Cook, B.J., Nachman, R.J., 1987a, Isolation, primary structure and synthesis of leucokinins VII and VIII: The final members of this new family of cephalomyotropic peptides isolated from head extracts of *Leucophaea maderae*. Comp Biochem Physiol C 88, 31-34.


Nachman, R.J., Holman, G.M., Cook, B.J., Haddon, W.F., Ling, N., 1986b, Leucosulfakinin-II, a blocked sulfated insect neuropeptide with homology to cholecystokinin and gastrin. Biochem Biophys Res Commun 140, 357-364.


Schoofs, L., Mark Holman, G., Hayes, T.K., Nachman, R.J., De Loof, A., 1990b, Isolation, identification and synthesis of locustamyotropin II, an additional neuropeptide of Locusta migratoria: Member of the cephalomyotropic peptide family. Insect Biochem 20, 479-484.


CHAPTER 2: Materials and Methods

Mosquitoes

Mosquitoes were maintained at Iowa State University in controlled conditions (27°C ± 1°C and 80% ± 5% relative humidity) with a 16:8 hour photoperiod. Larvae were fed ground Tetramin™ and adults were fed 10% sucrose.

• *Aedes aegypti* (Liverpool) – started from a colony at the University of Wisconsin Madison in 2005 which was originally obtained from a colony from the University of London in 1977

• *Aedes triseriatus* – collected in Iowa and colonized at Iowa State University in 2002

• *Anopheles stephensi* line STE2 (MRA-128) – acquired from the MR4 Institute, ATCC® Manassas, Virginia, which was donated by William E. Collins, and colonized at Iowa State University in 2009

• *Armigeres subalbatus* – started from a colony at the University of Wisconsin Madison in 2005 which was originally obtain from the National Institute of Preventive Medicine, Taipei, Taiwan, in 1992

• *Culex pipiens* – collected in Iowa and colonized at Iowa State University in 2002

• *Toxorhynchites amboinensis* – acquired from the University of Illinois Natural History Survey in 1991
• *Ochlerotatus caspius* – collected in Nile Delta, Egypt, and colonized at Iowa State University in 2008

**Dissections**

**Tools used for dissections**

• Dissecting tray
• Spring scissors (Fine Science Tools)
• Dumont #5 Standard tip Inox forceps
• 1 mm ID Ring Forceps (Fine Science Tools)
• Probes, 0.2 mm minuten pin in end of dowel
• 0.2 mm minuten pins
• 24 well microtiter clear plate
• PBS; 0.1 M NaH$_2$PO$_4$, 0.15 M NaCl, 0.003 M KCl, pH 7.4
• 4% formaldehyde in PBS
• Zeiss Stemi 2000 stereomicroscope

**Adult dissections**

**Immobilization**

Adult mosquitoes were immobilized by placing mosquitoes in the freezer (-20°C) for 5 minutes until dead. Note that the use of 4% formaldehyde to immobilize specimens failed to produce consistent results because the exoskeleton became too fragile.
Ventral Nerve Cord

The mosquito was placed on the dissecting tray and legs and wings were removed with scissors. The mosquito was then pinned through the abdomen with the right side down (Figure 1). A lateral cut was made in the upper abdomen, making sure not to cut deeper than necessary to open the abdomen, followed by an incision along the dorsal midline from the lateral cut to the posterior end. The abdomen was then pinned down to show internal organs and bathed in phosphate buffered saline (PBS) to keep the specimen from drying (Figure 2). Some dissections were attempted using 4% formaldehyde in place of PBS; however, this technique did not increase the success of dissections. Due to the precautions necessary when using formaldehyde, this technique was not continued and PBS was used during subsequent dissections. The digestive tract, midgut, hindgut, and ovaries were removed (Figure 3). Using probes the ventral nerve cord (VNC) was located and carefully removed. The VNC was transferred to a 24-well microtiter plate containing 4% formaldehyde using ring forceps.

Brain

Mosquitoes were placed on dissecting tray and pinned through the thorax with the right side down (Figure 1). PBS was used to cover the head of the mosquito. The proboscis and maxillary palps were slowly removed from head capsule with forceps. This technique periodically allowed removal of the brain and subesophageal ganglion (SEG). If the brain and SEG were not removed, the head was slowly smashed in a posterior to anterior direction to dislodge the tissues from the head capsule. The brain and SEG were then transferred to 24-well microtiter plate containing 4% formaldehyde using ring forceps.
Thoracic Nerve Cord

Mosquitoes were placed on dissecting tray and legs and wings were removed. They were then pinned through thorax, dorsal side down (Figure 4). Using probes, the thorax was carefully opened to reveal the thoracic nerve cord. The thoracic nerve cord was removed and transferred to 24-well plate containing 4% formaldehyde using ring forceps.

Larvae

Immobilization

Larvae were immobilized by placing directly in 4% formaldehyde for 2-3 hours. Several other immobilization techniques were tested including placing larvae on dissecting tray, pinning down, cutting open and then applying 4% formaldehyde. This technique was not continued as it was much more difficult and led to very little success.

Dissection

Larvae were placed ventral side down on dissecting tray and pinned through thorax (Figure 5). A lateral cut was made across the anterior portion of abdomen. Incisions were then made along the dorsal midline from the lateral cut to the anterior portion of the thorax and from the lateral cut to the posterior portion on the abdomen. Larvae were pinned open and tissues were kept hydrated with PBS (Figure 6). The digestive tract consisting of the pharynx, crop, proventriculus, midgut, Malpighian tubules and hindgut, was removed (Figure 7). The VNC and thoracic nerve cord were located and carefully removed using probes. The head capsule was broken apart carefully using probes. Nerves connecting the brain and SEG to the optical nerves and muscles in the
head were severed and the brain and SEG were removed. Using this technique, if all was done carefully, it was possible to remove the entire nerve cord from the brain to the terminal abdominal ganglion (TAG). The nerve cord was then transferred using ring forceps to a 24 well microtiter plate containing 4% formaldehyde.

**Immunocytochemistry**

The PBAN-antiserum was a gift to our lab from Wendell Roelofs, Cornell University.

“A truncated Hez-PBAN (T-PBAN), with the sequence Cys-Nle-Asp-Pro-Glu-Gln-Ile-Asp-Ser-Arg-Thr-Lys-Tyr-Phe-Ser-Pro-Arg-Leu-amide, was synthesized at the Cornell Biotechnology Core Facility. Two non-native amino acid residues, Cys and Nle, were introduced at the N-terminus of the peptide sequence to allow conjugation of the synthetic peptide to a carrier, and for monitoring of the conjugation reaction, respectively. T-PBAN was conjugated to bovine serum albumin (BSA) with m-maleimidobenzoyl-N-hydroxysuccinimide ester (Harlow and Lane, 1988). The amount of T-PBAN coupled to BSA was estimated by amino acid analyses to be 16.4 mol T-PBAN/mol BSA.

Antiserum was prepared at the Cornell Veterinary College Antibody Production Facility. A rabbit was immunized by an s.c. injection of 2 ml complete Freund’s adjuvant (Freund and McDermott, 1942) containing 500 pg of BSA-conjugated T-PBAN. Two 1-ml booster injections, each containing 250 pg of BSA-conjugated T-PBAN mixed in incomplete Freund’s adjuvant (Freund and McDermott, 1942) were administered 23 and 35 days after the first injection, respectively. The PBAN-antiserum, 1301-2, used in the present study was
obtained from the immunized rabbit 15 days after the second booster injection without further purification.” (Ma and Roelofs 1995)

Specificity of the PBAN-antiserum was tested by Ma and Roelofs (1995) using a dot-blot immunoassay. The results of this test showed the antiserum was specific for Hez-PBAN, Bom-PBAN I and T-PBAN and did not bind to leucopyrokinin or a fragment of leucopyrokinin. Again the specificity was tested by Ma et al. (1996) by preincubation of the PBAN-antiserum with T-PBAN resulting in no PBAN-like immunoreactivity.

**Goat anti-rabbit horseradish peroxidase 2° antibody**

Mosquito central nervous system (CNS) tissues were fixed for 3 hours in 4% formalin/PBS in a 24-well microtiter plate. All subsequent solution treatments took place in corresponding wells in the 24-well microtiter plate by transferring the tissue from one well to another. Tissues were then incubated overnight in PBS containing 2% Triton X-100 (PBS-T). Following the incubation in detergent, tissues were incubated in PBAN antiserum, diluted 1:2000, for 4-6 hours and washed in PBS-T overnight. The tissues were incubated in secondary antibody of goat anti-rabbit conjugated with horseradish peroxidase (Sigma-Aldrich), diluted 1:200, for 4-6 hours and washed in PBS-T overnight. Tissues were then washed in PBS for 30 minutes. A diaminobenzidine (DAB) stock solution was prepared by dissolving a DAB tablet and an H$_2$O$_2$ tablet in 1 ml water following the manufacturer’s protocol (Sigma-Aldrich). Following the PBS wash, tissues were transferred to a well containing a 1:4 dilution of the DAB stock solution. The tissues were transferred to PBS and rinsed for 10 minutes after satisfactory color development had occurred. The rinsed, immuno-stained tissues were then transferred to a microscope
slide. Tissues were carefully dehydrated in a graded series of ethanol (70%, 80%, 95%, 100%, roughly 5 minutes each) and cleared with methyl salicylate. A drop of xylene and a drop of permount were used to mount the tissues.

**Goat anti-rabbit Alexa Fluor® 488 2° antibody**

Mosquito CNS tissues were fixed for 3 hours in 4% formalin/PBS in a 24-well microtiter plate. The following treatments occurred in corresponding wells in the 24-well microtiter plate by transferring the tissue from one well to another. Tissues were incubated overnight in PBS containing 2% Triton X-100 (PBS-T). Tissues were then incubated in BPAN antiserum, diluted 1:2000, for 4-6 hours and washed in PBS-T overnight. After addition of the secondary antibody, goat anti-rabbit Alexa Fluor® 488 (Invitrogen), diluted 1:200, the tissues were incubated for 4-6 hours and washed in PBS-T overnight. Following the overnight wash, tissues were washed in PBS for 30 minutes and transferred to a slide in 5µl of PBS. Tissues were mounted with 15 µl Vectashield (Vector Laboratories Inc.) and covered with a coverslip. Slides were visualized under a Nikon Eclipse 50i fluorescence microscope and images were captured with a Digital Sight DS-2Mv camera and NIS Elements, version 3.0 software (Nikon, Melville, NY).

**Control**

Central nervous system tissues from larval *Ar. subalbatus* and adult *Ae. aegpit* were used for negative controls to tests for non-specific binding of the secondary antibody. After dissections, CNSs were fixed for 3 hours in 4% formalin/PBS in a 24-well microtiter plate. The following treatments occurred in corresponding wells on the 24-well microtiter
plate by transferring the tissue from one well to another. Tissues were incubated overnight in PBS containing 2% Triton X-100 (PBS-T). Tissues were then incubated in goat anti-rabbit Alexa Fluor® 488 (Invitrogen), diluted 1:200, for 4-6 hours and washed in PBS-T overnight. Following the overnight wash, tissues were washed in PBS for 30 minutes and transferred to a slide in 5µl of PBS. Tissues were mounted with 15 µl Vectashield (Vector Laboratories Inc.) and covered with a coverslip. Slides were visualized under a Nikon Eclipse 50i fluorescence microscope and images were captured with a Digital Sight DS-2Mv camera and NIS Elements, version 3.0 software (Nikon, Melville, NY). Controls showed no binding of the secondary anti-body (Figure 8). Control images were captured with a 12 second exposure, whereas results that included the PBAN antiserum were captured with a 2 second exposure or less.
Figure 1. Adult after legs and wings are removed, placed with right side down and pinned through thorax, arrowhead denotes pin.
Figure 2. Adult after being cut and pinned open, arrowheads denote pins.
Figure 3. Adult after digestive tract and reproductive organs are removed. Arrow points to ventral nerve cord.
Figure 4. Adult with legs and wings removed, placed dorsal side down with two minuten pins through thorax, arrowhead denotes pin, for thoracic nerve cord dissection.
Figure 5. Larva ventral side down with two minuten pins through the thorax, arrowheads denote pins. Red lines denote where incisions were made.
Figure 6. After cuts are made and larva is pinned open, arrowheads denote pins.
Figure 7. Larva after digestive tract is removed. Arrow points to ventral nerve cord.
Figure 8. Fluorescent micrograph of *Ar. subalbatus* control larva to show absence of non-specific binding. CNS was incubated with goat anti-rabbit Alexa Fluor® 488 only to confirm there was no nonspecific binding of secondary antibody. Green coloration is autofluorescence of CNS after 12-second exposure time. *Bar* 0.1 mm.
Literature Cited


Chapter 3. Results

The ventral nervous system of members of the family of Culicidae consists of a two lobed brain, subesophageal ganglion (SEG), and the ventral nerve cord (VNC). The VNC is comprised of three thoracic ganglia and the abdominal ganglia (Figures 1 and 2). Larval mosquitoes have eight abdominal ganglia whereas adults have six. The larval mosquitoes studied were not distinguished between 3rd or 4th instar unless otherwise stated. Results under each grouping were similar between multiple individuals unless otherwise stated.

Results using GaR HRP as secondary antibody

*Ae. aegypti* larvae

PBAN-like immunoreactivity was observed in the VNC of *Ae. aegypti* larvae (Figure 3a). A pair of stained axons was observed running the length of the VNC and terminating in the terminal abdominal ganglion (TAG) (Figure 3b). In the abdominal ganglia 1-7, a pair of immunoreactive neurons was seen along the ventral mid-line (Figure 3a). These neurons had axons projecting anteriorly, leaving the VNC and terminating in a neurohemal organ (Figure 3c). An unpaired immunoreactive cell was seen in the first abdominal ganglia of all individuals (Figure 3d) and was present in some individuals in abdominal ganglia 2-4.

*Ae. triseriatus* larva

PBAN-like immunoreactivity was observed in the VNC of a *Ae. triseriatus* larva (Figure 4). Only one individual was tested and exhibited high background levels due to
the staining process. Abdominal ganglia 2-7 each contained a pair of immunoreactive neurons located along the ventral mid-line.

**An. stephenesi larvae**

PBAN-like immunoreactivity was observed in the VNC of *An. stephenesi* larvae (Figure 5). Axons were observed running the length of the VNC (Figure 5a) and terminating in the TAG (Figure 5b). A pair of immunoreactive neurons located along the ventral mid-line was observed in abdominal ganglia 2-7.

**Oc. caspius larvae**

PBAN-like immunoreactivity was observed in the VNC of *Oc. caspius* larvae (Figure 6). No immunoreactive staining was distinguishable in the thoracic ganglia (Figure 6a) due to high background staining. A pair of immunoreactive neurons located along the ventral mid-line was observed in abdominal ganglia 2-7 (Figures 6b-e). Several pairs of these neurons had axons projecting anteriorly leaving the VNC and terminating in a neurohemal organ (Figure 6b).

**Ar. subalbatus larvae**

PBAN-like immunoreactivity was observed in the VNC of 4th instars (Figure 7). Two groups of immunoreactive neurons were observed in the SEG (Figure 7a). A lightly stained pair of immunoreactive neurons located at the ventral mid-line was observed in the first thoracic ganglion in one individual (Figure 7b), but was not present in any other individuals studied. A pair of immunoreactive neurons located along the ventral mid-line
was observed in abdominal ganglia 2-7 (Figure 7a). Axons originating in the SEG were found to run the length of the VNC and terminate in the TAG (Figure 7c).

**Ae. triseriatus adult**

PBAN-like immunoreactivity was observed in an abdominal ganglion of an adult *Ae. triseriatus* female (Figure 8). A pair of immunoreactive neurons located along the ventral-midline was observed. Axons were observed associated with this pair of neurons and projecting anteriorly, leaving the VNC and terminating in a neurohemal organ.

**Results using fluorescent secondary antibody**

**Ae. aegypti larvae**

PBAN-like immunoreactivity was observed in the CNS of larval *Ae. aegypti* (Figure 9). Three groups of immunoreactive neurons were observed in the SEG (Figure 9a). Axons originating from the two most anterior groups were observed leaving the SEG (Figure 9a) and traveling the entire length of the VNC and terminating in the TAG (Figure 9d). The ends of these axons in the TAG were associated with multiple varicosities. These axons were also associated with varicosities in each thoracic ganglia and abdominal ganglia. A pair of reactive neurons was observed in the first thoracic ganglion and a pair of reactive neurons along the ventral mid-line was observed in each of the abdominal ganglia (Figures 9b and c). Axons associated with these pairs were seen leaving the VNC and terminating in a neurohemal organ (Figure 9c). In some abdominal ganglia a third, unpaired immunoreactive neuron was observed (Figure 9b).
An. stephensi larvae

PBAN-like immunoreactivity was observed in the CNS of larval An. stephensi (Figure 10). In the SEG, three groups of immunoreactive neurons were observed (Figure 10a) with axons originating from the anterior two groups and running the length of the VNC. The axons were associated with varicosities in all thoracic ganglia (Figure 10b) and abdominal ganglia. The axons terminated in the TAG and were associated with a number of varicosities (Figure 10d). A pair of immunoreactive neurons was observed in the first thoracic ganglion (figure 10b) and in abdominal ganglia 2-7 along the ventral mid-line (Figure 10c). In the abdominal ganglia, the paired neurons were associated with axons observed leaving the VNC and terminating in a neurohemal organ (Figure 10c).

Cx. pipiens larvae

PBAN-like immunoreactivity was observed in the CNS of larval Cx. pipiens (Figure 11). Three groups of immunoreactive neurons were observed in the SEG (Figure 11a). An axon was observed leaving the SEG and extending to the corpora cardiaca where much immunoreactivity was observed (Figure 11a). A pair of immunoreactive neurons was observed in the first thoracic ganglion and also varicosities associated with axons running the length of the ventral nerve cord were detected (Figure 11b). In abdominal ganglia 2-7, a pair of immunoreactive neurons was observed along the ventral mid-line and was associated with a neurohemal organ located outside the VNC (Figure 11c).
**Ar. subalbatus larvae**

PBAN-like immunoreactivity was observed in the CNS of larval *Ar. subalbatus* (Figures 12 and 13). Two pairs of immunoreactive neurons were observed in the brain along with an arch-like process in the protocerebrum (Figure 12a). Three groups of immunoreactive neurons were observed in the SEG (Figures 12 a and b). Axons leaving the SEG were observed traveling to the corpora cardiaca and terminated with enhanced immunoreactivity (Figure 12b). Axons were observed leaving the SEG, running the entire length of the VNC (Figure 13) and terminating in the TAG (Figure 13d). Varicosities were observed associated with these axons in the thoracic ganglia (Figure 13b) the abdominal ganglia (Figure 13c) and the TAG (Figure 13d). In the first thoracic ganglion (Figure 13b) and abdominal ganglia 2-7 (Figure 13a) a pair of immunoreactive neurons was observed. In the abdominal ganglia, the pair of neurons was associated with a neurohemal organ (Figure 13c) located outside the VNC.

**Tx. amboinensis larva**

PBAN-like immunoreactivity was observed in the CNS of a larval *Tx. amboinensis* (Figure 14). Axons were observed running the length of the abdominal ganglia and terminating in the TAG (Figure 14a). Varicosities associated with the axons were observed in each abdominal ganglion. In abdominal ganglia 2-7, a pair of immunoreactive neurons located along the ventral mid-line was observed (Figure 14a). Associated with each pair was a neurohemal organ located outside the VNC (Figure 14a). In abdominal ganglia 2, 4 and 5, a third unpaired immunoreactive neuron was detected posterior to the paired immunoreactive neurons (Figure 14a). In the brain, two pairs of
immunoreactive neurons were observed (Figure 14b). An arch-like process was also seen in the dorsal portion of the brain (Figure 14b). In the SEG, three groups of immunoreactive neurons were observed (Figure 14b).

*Ae. aegypti* adults

PBAN-like immunoreactivity was observed in the CNS of adult *Ae. aegypti* females (Figure 15). A pair of immunoreactive neurons was located in the dorsolateral portion of the protocerebrum (Figure 15a). Two groups of immunoreactive neurons, one on each lobe, were seen located where the two lobes of the protocerebrum meet (not pictured). Axons originating in the SEG were seen in the circumesophageal connectives and continued to an arch-like process in the protocerebrum. Three groups of reactive neurons were found in the SEG (Figure 15b). Axons extended the length of the thoracic ganglia and had many associated varicosities (Figure 15c). A pair of reactive neurons was observed in the first thoracic ganglion (Figure 15c) and in all abdominal ganglia.

*Ae. triseriatus* adults

PBAN-like immunoreactivity was observed in the CNS of adult *Ae. triseriatus* females (Figure 16). Axons were observed running the length of the VNC and were associated with varicosities in the thoracic ganglia (Figure 16a) and TAG (Figure 16c). A pair of reactive neurons along the ventral mid-line was observed in all abdominal ganglia (Figure 16a) and each pair was associated with a neurohemal organ (Figures 16b and c).
**Tx. amboinensis adults**

PBAN-like immunoreactivity was observed in the brain/SEG of an adult *Tx. amboinensis* females (Figure 17). Three groups of reactive cells were observed in the SEG. A pair of axons was observed leaving the SEG through the circumesophageal connectives and forming an arch-like process in the protocerebrum. A group of reactive neurons was observed in the dorsal portion of the protocerebrum where the two lobes meet. A pair of reactive neurons was also observed in the dorsolateral portion of the protocerebrum (Figure 17).
Figure 1. Representation of CNS in larval Culcidae.
Figure 2. Representation of CNS in adult Culicidae.
Figure 3 Photomicrographs showing the immunolocalization of PBAN-like peptides in *Ae. aegypti* larvae CNS. (a) Composite photomicrograph of ventral nerve cord showing three thoracic ganglia and a pair of reactive neurons in abdominal ganglia 2-7. (b) View of axons (arrow) in ventral nerve cord, which originated in SEG. (c) View of abdominal ganglia with neurohemal organ (arrow) associated with pair of reactive neurons. (d) First abdominal ganglia shows a pair of reactive neurons (arrow head) and a third unpaired reactive neuron (arrow). *Bar* 0.1 mm
Figure 4. Composite photomicrograph showing the immunolocalization of PBAN-like peptides in an *Ae. triseriatus* larva CNS. View of ventral nerve cord with thoracic ganglia and a pair of reactive neurons in abdominal ganglia 2-7. Arrow points to a bubble, which is an artifact of the mounting process. *Bar* 0.1 mm
Figure 5. Composite photomicrographs showing the immunolocalization of PBAN-like peptides in *An. stephensi* larvae CNS. (a) Second thoracic ganglia thru abdominal ganglia 5 showing a pair of reactive neurons in abdominal ganglia 2-5. (b) Abdominal ganglia 5-8 showing a pair of reactive neurons in abdominal ganglia 5-7. Arrow points to axon terminals in TAG which originated in SEG. *Bar* 0.1 mm
Figure 6. Photomicrographs showing the immunolocalization of PBAN-like peptides in *Oc. caspius* larvae CNS. (a) Thoracic ganglia. (b) Abdominal ganglia (b) 1-3, (c) 4-5, (d) 6, (e) 7-8 showing a pair of reactive neurons in abdominal ganglia 2-7.

*Bar* 0.1 mm
Figure 7. Photomicrographs showing the immunolocalization of PBAN-like peptides in an *Ar. subalbatus* larva CNS. (a) Composite view of central nervous system with two groups of reactive neurons in the SEG (arrow heads) and a pair of reactive neurons in abdominal ganglia 2-7. (b) A pair of reactive neurons (arrow) in first thoracic ganglia. (c) Axons running through the first abdominal ganglion which originated in SEG and terminates in the TAG (arrow). *Bar* 0.1 mm
Figure 8. Photomicrograph showing the immunolocalization of PBAN-like peptides in a female *Ae. aegypti* CNS. View of an abdominal ganglion with a pair of reactive neurons (arrow) associated with a neurohemal organ (arrow head). *Bar* 0.1 mm
Figure 9. Fluorescent micrographs showing the immunolocalization of PBAN-like peptides in *Ae. aegypti* larvae CNS. (a) SEG showing three groups of reactive neurons (arrowheads) and axons leaving the SEG (arrow). (b) 1st abdominal ganglion with a pair of reactive neurons. A third, unpaired reactive cell is also observed (arrowhead). (c) An abdominal ganglion with a pair of reactive neurons associated with a neurohemal organ (arrowhead). (d) TAG showing varicosities (arrow). Bar 0.1 mm
Figure 10. Fluorescent micrographs showing the immunolocalization of PBAN-like peptides in *An. stephensi* larvae CNS. (a) SEG showing three groups of reactive neurons. (arrowheads) (b) Thoracic ganglia with a pair of reactive neurons in the first thoracic ganglion (arrow) and axons extending the length of the VNC (arrowhead). (c) An abdominal ganglion with a pair of reactive neurons associated with a neurohemal organ (arrowhead). (d) TAG showing varicosities (arrow). *Bar* 0.1 mm
Figure 11. Fluorescent micrographs showing the immunolocalization of PBAN-like peptides in *Cx. Pipiens* larvae CNS. (a) SEG showing three groups of reactive neurons (arrowheads) and an axon leaving the SEG and terminating in the corpora cardiaca (arrow). (b) Thoracic ganglia showing a pair of reactive cells in the first thoracic ganglion (arrow) and varicosities (arrowhead). (c) An abdominal ganglion with a pair of reactive neurons associated with a neurohemal organ (arrowhead). *Bar* 0.1 mm.
Figure 12. Fluorescent micrographs showing the immunolocalization of PBAN-like peptides in brain and SEG of 4th instar *Ar. subalbatus*. (a) Arrows point to two pairs of reactive neurons in the brain. Three groups of reactive neurons are seen in the SEG (arrowheads) corresponding to the mandibular, maxillary and labial neuromeres. (b) View of the corpora cardiaca (arrows) and the three groups of reactive neurons in the SEG (arrowheads). *Bar* 0.1 mm
Figure 13. Fluorescent micrographs showing the immunolocalization of PBAN-like peptides in CNS of 4th instar *Ar. subalbatus*. (a) View of CNS starting with the SEG and ending with TAG showing a pair of reactive neurons located at the ventral mid-line in abdominal ganglia 2-7. (b) View of thoracic ganglia with a pair of reactive neurons at the ventral mid-line of the first thoracic ganglion (arrow) and varicosities (arrowhead). (c) View of a neurohemal organ (arrow) associated with a pair of reactive neurons in an abdominal ganglion and axons (arrowhead) which originated in the SEG. (d) The axons originating in the SEG travel through the VNC and terminate in varicosities found in the TAG (arrowhead). *Bar* 0.1 mm
Figure 14. Fluorescent micrographs showing the immunolocalization of PBAN-like peptides in *Tx. Amboinensis* larvae CNS. (a) View of abdominal ganglia showing pairs of neurons in ganglia 2-7. Single unpaired neurons (arrow heads) were detected in ganglia 2, 4 and 5. Neurohemal organs (arrow) are associated with the paired neurons. (b) The SEG of this individual was flipped showing the posterior portion of the SEG being closest to the brain. Two pairs of reactive neurons are shown in the brain (arrows) and three groups of reactive neurons (arrow heads) in the SEG. *Bar* 0.1 mm
**Figure 15.** Fluorescent micrographs showing the immunolocalization of PBAN-like peptides in adult female *Ae. aegypti* CNS. (a) Brain and SEG showing a pair of immunoreactive neurons in the protocerebrum (arrows) and immunoreactive axons in the circumesophageal connectives (arrowhead). (b) SEG showing three groups of immunoreactive neurons (arrowheads). (c) Thoracic ganglia showing varicosities throughout and a pair of reactive neurons (arrow). *Bar* 0.1 mm
Figure 16. Fluorescent micrographs showing the immunolocalization of PBAN-like peptides in adult female *Ae. triseriatus* CNS. (a) View of thoracic and abdominal ganglia with varicosities in thoracic ganglia (arrow) and a pair of reactive neurons in all abdominal ganglia. (b) An abdominal ganglion showing a neurohemal organ (arrow) associated with a pair of reactive neurons. (c) Terminal abdominal ganglion showing neurohemal organ (arrow) associated with a pair of reactive neurons and varicosities (arrowhead) associated with axon terminals, which originated in the SEG. *Bar* 0.1 mm
Figure 17. Fluorescent micrographs showing the immunolocalization of PBAN-like peptides in adult female *Tx amboinensis* CNS. SEG with three groups of neurons (arrow heads) and brain with two groups of neurons (star) and a pair of neurons (arrows) in the protocerebrum. *Bar* 0.1 mm
CHAPTER 4. General conclusions

Neuropeptides are essential neurohormones found in most animals and regulate a variety of physiological and behavioral events. Pyrokinin/PBAN (pheromone-biosynthesis-activating-neuropeptide) represents a large family of insect neuropeptides characterized by a common C-terminal pentapeptide, FXPRLeamide, which is the active core (Raina and G. Kempe, 1992). The first peptide in this family was identified from the cockroach, *Leucophaea maderae*, as a myotropin (Holman et al., 1986), and various other myotropins were identified in other insect orders (Nachman et al., 1986). Research in cockroaches also suggested the pyrokinin/PBAN family of peptides function as hormones as they were found in neurohemal organs (Predel et al., 1999). A variety of physiological functions for this family of peptides have been described. These include stimulation of pheromone biosynthesis in female moths (Raina et al., 1989), induction of melanization in moth larvae (Matsumoto et al., 1990), induction of embryonic diapause in the silk moth (Suwan et al., 1994), stimulation of visceral muscle contraction in cockroaches (Predel and Nachman, 2001), and termination of pupal diapause development in heliothine moths (Xu and Denlinger, 2003). A highly conserved five amino acid C-terminal sequence has been identified as the active core required for physiological functions (Nachman et al., 1986). The pyrokinin/PBAN peptide family is cross-reactive among species and physiological functions and is expected to be distributed throughout class Insecta.

The pyrokinin/PBAN family of peptides has been found in a variety of insect species including Dipterans such as the fruit flies *Drosophila melanogaster* (Choi et al., 2001) and *Anastrepha suspensa* (Teal, 1998), and the blow fly *Protophormia terraenovae*.
(Shiga et al., 2000). Acceleration of pupariation formation and cuticular tanning has been shown to be affected by pyrokinin/PBAN peptides in fleshflies, Diptera: Sarcophagidae (Nachman et al., 2006; Zdarek et al., 1997; Zdarek et al., 1998). The identification and characterization of pyrokinin/PBAN peptides in mosquitoes will lead to further understanding of the physiology of the Culicidae family and may lead to novel methods for control of the important viral and protozoan disease vectors.

In the present study, whole-mount immunocytochemical techniques have verified the presence of pyrokinin/PBAN peptides in the central nervous system of Ae. aegypti, Ae triseriatus, An. Stephensi, Ar. subalbatus, Cx. pipiens, Oc. caspius, and Tox. amboinensis. PBAN-like immunoreactivity was observed in the CNS of fourth instar larvae and adults of mosquitoes using an antiserum generated against the C-terminus of H. zea PBAN (Ma and Roelofs, 1995; Ma et al., 1996). Both life stages displayed a similar staining pattern, which included three groups of neurons in the SEG, two groups of neurons in the dorsolateral portion of each protocerebrum and seven pairs of neurons in the ventral nervous system. The paired neurons in the abdominal ganglia appear to send their axons to a neurohemal organ associated with each ganglion. Two pairs of axons were also observed originating in the SEG and terminating in the TAG.

Current results show three groups of neurons in the SEG with PBAN-like immunoreactivity in 4th instar larval and adult mosquitoes, which correspond to the mandibular, maxillary and labial neuromeres. These results are similar to those previously reported from moths (Choi et al., 2004; Davis et al., 1996; Duportets et al., 1998; Kingan et al., 1992; Ma and Roelofs, 1995; Ma et al., 1996; Sato et al., 1994; Wei et al., 2008), Orthoptera (Tips et al., 1993), Drosophila (Choi et al., 2001) and Solenopsis
Axons arising from the SEG neuromeres project to the corpora cardiaca, a paired neurohemal organ that is thought to release PBAN-like peptides. The maxillary group of immunoreactive neurons is thought to send axons down the VNC, terminating in the TAG (Choi et al., 2001; Kingan et al., 1992), while the mandibular neuromeres have been associated with a set of neuritis forming an arch-like process in the protocerebrum (Choi et al., 2001; Davis et al., 1996; Shiga et al., 2000). A group of neurons was detected in the median bundle of the brain and a pair of neurons was identified in the lateral protocerebrum of Culicidae, which is comparable to results known from adult blowfly, *Protophormia terraenovae* (Shiga et al., 2000).

The VNC of *Drosophila* and moths contains one to three pairs of neurons with PBAN-like peptides (Choi et al., 2001; Davis et al., 1996; Ma and Roelofs, 1995; Sato et al., 1994). In Culicidae, however, we have observed seven pairs of immunoreactive neurons; which are more similar to those from *Solenopsis* (Choi et al., 2009) and Orthoptera (Predel and Eckert, 2000; Schoofs et al., 1992). The abdominal ganglia contain several groups of neurons that project axons anteriorly to a neurohemal organ. Varicosities were detected throughout the ventral ganglia of both life stages of Culicidae, showing similar results to adult *D. Melanogaster* (Choi et al., 2001). It has been proposed that this may indicate a possible communication with other neurons in the CNS. While some differences have been found in the distribution of pyrokinin/PBAN-like immunoreactive neurons in Orthoptera, Lepidoptera, Hymenoptera and Diptera, the general patterns have been similar across orders.

Previously unreported in other insects, a third unpaird reactive neuron in the abdominal ganglia was observed in some individual larval mosquitoes. Dorsal unpaird
median neurons (DUM neurons) are located on the dorsal surface of the thoracic and abdominal ganglia of insects (Hoyle et al., 1974; Plotnikova, 1969). DUM neurons are octopaminergic and have not been shown to produce pyrokinin/PBAN peptides. Further studies would be necessary to determine if the unpaired reactive neuron is a DUM neuron, and if there is cross-reactivity occurring between the primary antibody and peptides within the DUM neuron or if these neurons are producing peptides in the pyrokinin/PBAN family of peptides.

In *Drosophila*, immunoreactivity has been found in the SEG and ventral ganglia. To date, two genes produce pyrokinin/PBAN-like peptides, hug (PK-2) and CAPA (PK-1) (Choi et al., 2001). PK-1 is putatively expressed in the SEG and PK-2 in the ventral ganglia (Melcher and Pankratz, 2005). This same two gene expression pattern for pyrokinin/PBAN-like peptides has been proposed for *Solenopsis* (Choi et al., 2009), *Manduca sexta* (Loi et al., 2004), *Bombyx mori* (Roller et al., 2008) and Orthoptera (Predel and Eckert, 2000; Schoofs et al., 1992). A search of the *Ae. aegypti* and *An. gambiae* genomes (Nene et al., 2007; Riehle et al., 2002) indicates two genes putatively coding for peptides in the pyrokinin/PBAN family of peptides.

The role of pyrokinin/PBAN-like peptides has been proposed for several other insect species. In fleshflies, pyrokinin/PBAN peptides have been shown to accelerate pupariation formation. As the Culicidae life cycle does not include the use of a puparium, pyrokinin/PBAN would have a different physiological role. In fleshflies pyrokinin/PBAN peptides were shown to accelerate cuticular tanning and in moth larvae melanization. This could also be a role of pyrokinin/PBAN peptides in larva, pupa, and young adult mosquitoes. Since pyrokinin/PBAN peptides were found older adult mosquitoes, these
peptides would not be involved in cuticular tanning or melanization process, but could be involved in melanization of parasites associated with the immune responses. In silkmoths, pyrokinin/PBAN peptides have been shown to induce embryonic diapause. This physiological role has not been studied in other species and may explain the presence of the peptide in older mosquitoes as many species diapause in the egg stage.

In conclusion, for the first time we have demonstrated pyrokinin/PBAN peptides in the CNS of Culicidae. Localization of immunoreactive neurons indicates that the peptides are present in the CNS. These peptides may act within the CNS and may be released into the hemolymph to act at peripheral sites. Results from Culicidae are similar to previously reported insect species, but the physiological roles of these peptides in Culicidae remain to be determined.

**Future Research**

- **Are there life stage differences in pyrokinin/PBAN expression?**

  Based on our results, a difference was seen between *Ae. aegypti* larvae and the larvae of the other species. The first abdominal ganglia in *Ae. aegypti* contained a pair of immunoreactive cells that was not present in the first abdominal ganglia of the other species. Life stage differences have been shown in *Manduca sexta* (Loi and Tublitz, 2004). In *M. sexta*, the number of CAPA immunoreactive cells in 1st instars was 54 and declined to 14 midway through adult development. Differences were seen between *M. sexta* larval stages, which may explain the differences seen in our results. To determine if there are life stage
differences, immunocytochemistry would need to be done on the CNS of all
instars, pupae, and adults.

- **Use RNA isolation and PCR to determine localization of pyrokinin/PBAN genes
  and respective receptors.**

  A search of the *Ae. aegypti* and *An. gambiae* genomes (Nene et al., 2007;
Mongin et al., 2004; Riehle et al., 2002) indicates there are two possible genes
that encode peptides that belong to the pyrokinin/PBAN family of peptides
(Table 1 and Table 2). In *D. melanogaster*, pyrokinin/PBAN peptides are known
to be located on two different genes, the *capa* gene (Kean et al., 2002) and the
*hugin* gene (Meng et al., 2002). The *capa* gene potentially encodes three
nueropeptides: capa-1, capa-2, and capa-3, with capa-3 being a member of the
pyrokinin/PBAN family of peptides. The *hugin* gene is postulated to code for two
nueropeptides, an ecdysis-triggering hormone-like peptide and a pyrokinin-like
peptide.

  Pyrokinin/PBAN receptors have been characterized in *H. zea* (Choi et al.,
2003), *B. mori* (Hull et al., 2004), *P. xylostella* (Lee and Boo unpublished data),
*H. armigera* (Rafaeli et al., 2007), *H. virescens* (Kim et al., 2008), *S. littoralis*
(Zheng et al., 2007), *S. exigua* (Cheng et al., 2007 unpublished date), along with
*D. melanogaster* (Park et al., 2002). The receptor has been recognized as a G-
protein coupled receptor (Rafaeli and Gileadi, 1996a; Rafaeli and Gileadi, 1996b).
Two receptors have been predicted based on the genome sequence of *Ae. aegypti
and An. gambiae* (Table 3).
A preliminary study was conducted by isolating RNA from whole body female *Ae. aegypti* using SV Total RNA Isolation System (Promega). cDNA was synthesized using GoScript™ Reverse Transcription System (Promega). Primers were designed based on the sequences for *Ae. aegypti* (Tables 1-3) and are shown in Table 4. PCR was done and the products were run on a 3% agarose gel (Figure 1). The PCR products were at the desired sizes showing promise the designed primers work and will need to be verified by sequencing the PCR products. Future research will include isolating RNA from the head, thorax, abdomen, and abdominal VNC from female *Ae. aegypti* followed by cDNA synthesis and PCR with the PBAN and CAPA primers to determine the location of these two transcripts. RNA will also be isolated from various tissues of female *Ae. aegypti*, such as salivary glands, ovaries, midgut and fat body, followed by cDNA synthesis and PCR with the PK-1 and PK-2 primers to determine the location of the receptors.

- **Gene silencing using RNAi to determine physiological function of pyrokinin/PBAN in Culicidae**

  RNA interference (RNAi) is a sequence-specific process to silence genes. Double stranded RNA (dsRNA) is introduced into a cell where it is cut into small interfering RNA (siRNA) of 21-25 nucleotides by Dicer and form a RNA-induced silencing complex (RISC). This leads to the cleavage of homologous transcripts and silencing of the specific gene. In 2000, RNAi was shown to silence genes in *Drosophila* (Hammond et al.) RNAi was also shown to silence genes in...
Culicidae (Blandin et al., 2002; Levashina et al., 2001). RNAi has been used to reveal the functional significance of PBAN-receptor in a male moth (Bober and Rafaeli, 2010). RNAi has been studied in mosquitoes as a way to control virus replication (Blair et al., 2000; Sanchez-Vargas et al., 2009; Sanchez-Vargas et al., 2004; Travanty et al., 2004; Wu et al., 2010). Based on this evidence it is possible to use RNAi against the pyrokinin/PBAN peptides to reveal the function of this family of peptides in mosquitoes.
Table 1. Peptides predicted based on the CAPA gene sequence.

<table>
<thead>
<tr>
<th>Insect</th>
<th>Cap2b-1&amp;2</th>
<th>Cap2b-3</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. melanogaster</em></td>
<td>GANMGlyAPRVRa</td>
<td>ASGLVAAPRVRa</td>
<td>TGPSASSGLMFGRLa</td>
</tr>
<tr>
<td><em>Ae. aegypti</em></td>
<td>PGTGLFAAPRVRa</td>
<td>QGLVPAPRVRa</td>
<td>AGNSGAAPRVRa XP_001650889.1</td>
</tr>
<tr>
<td><em>An. gambiae</em></td>
<td>GPTVGLFAAPRVRa</td>
<td>QGLVPAPRVRa</td>
<td>AGGTTAAPRVRa XP_566030.2</td>
</tr>
<tr>
<td><em>M. sexta</em></td>
<td>DGVLNLAPRVRa</td>
<td>QLYAPRVRa</td>
<td>TEGPSAMFGRLa AAT69684.1</td>
</tr>
<tr>
<td><em>B. mori</em></td>
<td>PDGVLNLAPRVRa</td>
<td>QLYAPRVRa</td>
<td>NEPHDDALLGLDDLPGMNP_001124357.1</td>
</tr>
</tbody>
</table>
Table 2. Peptides based on the PBAN-like gene sequence.

<table>
<thead>
<tr>
<th>Insect</th>
<th>Diapause hormone</th>
<th>PBAN</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. melanogaster</em></td>
<td>missing</td>
<td>QLQNGEPAYRVTTPRLa</td>
<td>missing</td>
</tr>
<tr>
<td>Aedes aegypti</td>
<td>AAAMWFgPRLa</td>
<td>QPLLVPYHSITTPRLa</td>
<td>DASSNENNSRPFFAPRLa</td>
</tr>
<tr>
<td>Anopheles gambiae</td>
<td>AAAMWFgPRLa</td>
<td>QPLLVPYHSTTPRLa</td>
<td>DSSSCHGPRPFSPRLa</td>
</tr>
<tr>
<td>Culex quinquefasciatus</td>
<td>ASAMWFgPRLa</td>
<td>QPLLVPYHSTTPRLa</td>
<td>DSSSCHGPRPFSPRLa</td>
</tr>
<tr>
<td><em>Manduca sexta</em></td>
<td>NDIKDEGDRGAESEGALWFgPRLa</td>
<td>VFTPRLa</td>
<td>missing</td>
</tr>
<tr>
<td><em>Bombyx mori</em></td>
<td>TDKIDDRGAESEGALWFgPRLa</td>
<td>HFTPRLa</td>
<td>SVAKPQTHESELFPRLa</td>
</tr>
</tbody>
</table>
Table 3. Accession numbers for the PVK-receptors, DH-receptors, and PBAN-receptors

<table>
<thead>
<tr>
<th></th>
<th>PK-1 or DH-receptor</th>
<th>PK-2 or PBAN-receptor</th>
<th>PVK-receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. melanogaster</em></td>
<td>NP_001014620.1</td>
<td>NP_731788.1</td>
<td>NP_996140.1</td>
</tr>
<tr>
<td><em>Aedes aegypti</em></td>
<td>XP_001662936.1</td>
<td>XP_001657210.1</td>
<td></td>
</tr>
<tr>
<td><em>Anopheles gambiae</em></td>
<td>XP_311184.3</td>
<td>XP_312761.3</td>
<td>XP_312953.3</td>
</tr>
<tr>
<td><em>Culex quinquefasciatus</em></td>
<td>XP_001864163</td>
<td>XP_001861460.1</td>
<td>XP_001865379.1</td>
</tr>
<tr>
<td><em>Bombyx mori</em></td>
<td>NP_001036913.1</td>
<td>NP_001036977.1</td>
<td></td>
</tr>
<tr>
<td><em>Orgyia thyellina</em></td>
<td>BAF36977.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Helicoverpa zea</em></td>
<td></td>
<td>AAP93921.1</td>
<td></td>
</tr>
<tr>
<td><em>Helicoverpa armigera</em></td>
<td></td>
<td>AAW47417.1</td>
<td></td>
</tr>
<tr>
<td><em>Heliothis virescens</em></td>
<td></td>
<td>ABU93812.1</td>
<td></td>
</tr>
<tr>
<td><em>Spodoptera exigua</em></td>
<td></td>
<td>ABY62317.2</td>
<td></td>
</tr>
<tr>
<td><em>Spodoptera littoralis</em></td>
<td></td>
<td>ABD52277.1</td>
<td></td>
</tr>
<tr>
<td><em>Plutella xylostella</em></td>
<td></td>
<td>AAY34744.1</td>
<td></td>
</tr>
</tbody>
</table>
Table 4. Polymerase chain reaction primer pair composition, as designed from proposed sequences, for PBAN, CAPA and two receptor genes in *Ae. aegypti* and the expected size of the respective PCR product.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Predicted Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBAN</td>
<td>CGT CGG GAA AGG ATA GTG AA</td>
<td>CCA ATG GAA ATG GCC TAC TG</td>
<td>473</td>
</tr>
<tr>
<td>CAPA</td>
<td>CCT ACT GGA GTG GAGCGA TG</td>
<td>AAA AGG CGT TCA AAG TCC TC</td>
<td>399</td>
</tr>
<tr>
<td>PK-1</td>
<td>TTC TTC TAC GGA CCC AAA CG</td>
<td>GTT GAT GCA GGT GGA AAG GT</td>
<td>891</td>
</tr>
<tr>
<td>PK-2</td>
<td>GAA ACG GCG AAG TGA TGA AT</td>
<td>TGA TGA TGG ATA CCG TTT GC</td>
<td>700</td>
</tr>
</tbody>
</table>
Figure 1. Gel electrophoresis of the PCR products for PBAN, CAPA and two receptor genes in *Ae. aegypti* as well as a 100 bp ladder (BioExpress) and an actin control.
Literature Cited


Sanchez-Vargas, I., Scott, J.C., Poole-Smith, B.K., Franz, A.W., Barbosa-Solomieu, V., Wilusz, J., Olson, K.E., Blair, C.D., 2009, Dengue virus type 2 infections of Aedes aegypti are modulated by the mosquito's RNA interference pathway. PLoS Pathog 5, e1000299.


ACKNOWLEDGEMENTS

I thank my major professor, Dr. Russell Jurenka, for the opportunity to learn from him as a teacher and a mentor. I especially would like to thank him for giving me the opportunity to come back and finish my degree along with giving me multiple opportunities for teaching. I thank my committee members Drs. Lyric Bartholomay and Dr. Michael Kimber for their guidance and support. Special thanks goes to Dr. Lyric Bartholomay for being a mentor and a friend throughout this entire process.

Thanks goes out to everyone in Dr. Bonning’s lab and Dr. Bartholomay’s lab for their continued support and expertise. I would not have been able to accomplish this project without the mosquito rearing support in Dr. Bartholomay’s lab.

I also thank my parents, Richard and Dee Hellmich, for supporting me in more ways that could ever be mentioned. You have made it possible to follow my dreams wherever they have led me.