A study on the midgut hormone and its intermediate target hormones in the queen blow fly Phormia Regina (Diptera: Calliphoridae).

A STUDY ON THE MIDGUT HORMONE AND ITS INTERMEDIATE TARGET HORMONES IN THE QUEEN BLOW FLY *PHORMIA REGINA* (DIPTERA: CALLIPHORIDAE)

A Thesis Presented

by

HEPING LIN

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DEDICATION

To my wife Yanqun Li

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CHAPTER I

LITERATURE REVIEW

Introduction

Our laboratory has extensively studied the interactions among nutrition, endocrine and physiology on the reproductive development of the black blow fly, *Phormia regina* (Liu *et al.*, 1988; Yin *et al.*, 1989; Zou *et al.*, 1989; Yin and Stoffolano, 1990; Yin *et al.*, 1990; Yin *et al.*, 1993; Yin *et al.*, 1994; Qin *et al.*, 1995; Tu *et al.*, 2001, 2002). Results suggest that a protein meal triggers the release of midgut hormone, which activates a neuroendocrine cascade leading to oogenesis (Yin *et al.*, 1994). A concise description of the working hypothesis of this midgut hormone is shown in Figure 1.1, but many details including direct evidence of its targets, its receptors and its intermediate messengers, are still lacking.

In this chapter, I first reviewed the history of midgut hormone discovery, function and biochemical properties. I then talked about the neuropeptidal controlled biosynthesis of juvenile hormones (JHs) and ecdysteroid. Also included here is a discussion of the documented roles of insulin-like peptides and insulin-like receptor in insects as related to the function of the midgut hormone in *Phormia regina*.

Discovery of Midgut Hormone in *Phormia regina*

In vertebrates, gut endocrine cells and gut hormones have long been studied. More than 100 different hormonally active peptides are produced in the gastrointestinal tract

which belong to seven families. These include gastrin, secretin, pancreatic polypeptide, epidermal growth factor, tachykinin and somatostatin families, which makes gut the largest endocrine organ in the body (Rehfeld, 1998).

It is not surprising that the insect gut has also been known as a rich endocrine source (Sehnal and Zitnan, 1990). Ultrastructural and/or immunological evidence indicates that both gut endocrine cells and gut hormones exist in a wide spectrum of insects such as *Calliphora erythrocephala* (De Priester, 1971), *Bombyx mori* (Kobayashi, 1971), *Aedes aegypti* (Hecker, 1977; Brown *et al.*, 1985), *Periplaneta americana* (Nishiitsutsuji-Uwo and Endo, 1981), *Calliphora vomitoria* (Duve and Thorpe, 1982), and *Helicoverpa zea* (Crim *et al.*, 1992). In contrast to the mammalian situation, the function of most insect gut hormones or hormone-like substances remains unknown.

Nevertheless, in a few cases, insect gut hormones have been isolated/purified and/or bioassayed for specific activities. An insulin-like peptide has been partially purified from both the brain and the midgut of *Tenebrio molitor* (Teller *et al.*, 1983). Eight years after that, a prothoracicotropic peptide was identified from the hindguts of *Ostrinia nubilalis* and *Lymantria dispar* (Gelman *et al.*, 1991).

Studies of the relationship between nutrition and endocrine on *Phormia regina* suggest that a protein meal triggers the release of a midgut hormone, which travels through the hemolymph to reach the brain where it activates a neuroendocrine cascade leading to oogenesis (Yin *et al.*, 1994). Results indicated that this hormone is released

from the midgut to reach the brain (i.e., the target) between 4 to 8h after the onset of the liver meal. The brain then initiates a neuroendocrine cascade leading to the onset and completion of oogenesis. High activity of this hormone is detected in extract prepared from midguts of liver-fed females at 6h after the onset of the liver meal. The midgut hormone is heat resistant at 100°C up to 4 minutes and remains active after exposure to 80% methanol. Its estimated M_r resides between 10 kDa and 20 kDa. This is the first hormone identified from an insect midgut that connects nutrition, the neuroendocrine system and oogenesis. Results of neck-ligation and decerebration experiments, and the histological study on the volume changes of median neurosecretory type-A cells in *Phormia regina* after a liver meal, suggest that median neurosecretory type-A cells are one of the targets of this midgut hormone (Yin *et al.*, 1993; Tu, 2000). A working hypothesis of the mechanism on the action of the midgut hormone that explains the regulation of oogenesis has been proposed (Figure 1.1). Briefly, after a liver meal, a midgut message activates the brain neurosecretory cells to stimulate the corpus allatum to produce JHs, which are required to prime the previtellogenic ovaries and the fat bodies to become "competent" (Yin *et al.*, 1994). Another set of brain neurosecretions stimulates the ovaries to produce ecdysteroids, which in turn stimulate the "competent" fat bodies to produce vitellogenin to be taken up by the "competent" ovaries to make vitellin (Yin *et al.*, 1993). The possible neuropeptide targets of the midgut hormone that control the biosynthesis of JHs and ecdysteroids are introduced below.

Biosynthesis of JHs Controlled by Neuropeptides

JHs are synthesized in the endocrine gland, corpus allatum (CA). They regulate insect metamorphosis, caste determination, behavior, polymorphism, diapause, vitellogenin synthesis, ovarian development, as well as various aspects of metabolism associated with these processes (see review by Nijhout, 1998).

JH synthesis is regulated by many factors which include neuropeptides, biogenic amines and sex peptides (Gäde *et al.*, 1997; Gilbert *et al.*, 2000; Chiang *et al.*, 2002). There are two groups of neuropeptides controlling the synthesis of JH: allatotropins (ATs) or allatostatins (ASTs) which are secreted from the brain neurosecretory cells (Khan, 1988). ATs stimulate JH biosynthesis while ASTs inhibit JH biosynthesis. So far, one AT and several ASTs have been isolated from several different insect orders.

AST was first isolated from *Diploptera punctata* (Woodhead and Stay, 1989). Since then, four ASTs from *Blattela germanica* (Blg-AST) (Bellés *et al.*, 1994), seven from *Diploptera punctata* (Dip-AST) (Woodhead *et al.*, 1989; Pratt *et al.*, 1991; Donley *et al.*, 1993; Woodhead *et al.*, 1994), two from *Periplaneta americana* (Pea-AST) (Stay *et al.*, 1994; Weaver *et al.*, 1994), six from *Gryllus bimaculatus* (Grb-AST) (Lorenz *et al.*, 1995a, 1995b), one from *Manduca sexta* (Mas-AST) (Kramer *et al.*, 1991), and three from *Apis mellifera* (Apm-AST) (Gäde *et al.*, 1997) have been identified. In addition, the genes, structure-activity relations, receptors, distribution, and metabolism of some these ASTs have been studied in some species. (For recent reviews, see Stay *et al.*, 1994; Gäde *et al.*, 1997; and Weaver *et al.*, 1998).

In contrast to the extensive studies of the ASTs, only a limited amount of research has been focused on the ATs. Mas-AT, the only AT isolated, was obtained from pharate adult heads of *Manduca. sexta* (Kataoka *et al.*, 1989). This amidated tridecapeptide, with a sequence of G-F-K-N-V-M-M-T-A-R-G-F-NH_2 activates JH synthesis of adult *M. sexta*. The Mas-AT gene was cloned in *Manduca sexta* (Taylor *et al.*, 1996), in *Pseudaletia unipuncta* (Truesdell *et al.*, 2000) and in *Spodoptera frugiperda* (Abel-latief *et al.*, 2003). In the last larval instar of *Galleria mellonella*, a partially purified 20 kDa brain polypeptide stimulated JH biosynthesis in CA *in vitro* (Bogus and Scheller, 1994). In *Phormia regina*, substances positive to Mas-AT antiserum were observed in the lateral neurosecretory cells and the brain region between the median neurosecretory cells and the oesophageal foramen. *In vitro* CA incubation studies show that Mas-AT antiserum can only partially reduce the synthesis of JH, thus suggesting that in *Phormia regina* there may be allatotropic factors other than Mas-AT (Tu *et al.*, 2001).

Neuropeptidal Control of Ecdysteroid Biosynthesis

Ecdysteroids are produced in the prothoracic gland of insects, they play an important role in the molting of larval (nymphal) and pupal stages, its direct action on the epidermal cells causes them to undergo apolysis, cell division, digestion of the old cuticle, and secretion of a new cuticle. In adult insects, the gonads are the primary source of ecdysteroids which play an important role in the control of gonadal

maturation. Ecdysteroids also play a role in embryonic development. [see recent review by Nijhout (1998)].

The prothoracicotropic hormones (PTTHs) of *Manduca sexta* and *Bombyx mori* have received considerable attention. Before the adult stage, PTTH from the brain regulates ecdysteroid synthesis (Bollenbacher *et al.*, 1979). In *Manduca sexta*, PTTHs are produced by lateral neurosecretory cells of group III and exist in two molecular forms: big PTTH with an approximate M_r of 28,500 Da, and small PTTH with a M_r of about 7 kDa (Bollenbacher and Granger, 1985; Westbrook *et al.*, 1993). There are also more than one PTTHs in *Bombyx mori*. A small PTTH of about 5 kDa has been isolated from the brains of adult moths, while a much larger PTTH, of about 22 kDa, has been isolated from pupal brains (Yamazaki and Kobayashi, 1969; Ishizaki and Ichikawa, 1967; Nagasawa *et al.*, 1990). Later, the smaller PTTH was renamed bombyxin, which is not considered as a true PTTH but as an insulin-like molecule because it shows considerable homology in the primary amino acid sequence with the insulin-family peptides. In addition, the A- and B-chains of bombyxin have been shown to link with disulfide bonds in exactly the same way as in insulin (Nagasawa, *et al.*, 1988) and the insulin-like tertiary structure of bombyxin has been predicted by comprehensive computer analysis (Jhoti, *et al.*, 1987). Furthermore, the genomic DNA sequence coding for bombyxin shows high homology to the corresponding genomic DNA sequences of the insulin-family members and, the overall organization of the preprobombyxin gene is the same as in preproinsulin genes (Iwami *et al.*, 1989).

In addition to PTTH and bombyxin regulation of ecdysteroid production, other peptides have also been identified to have ecdysteroidogenic properties. For example, in the yellow fever mosquito, *Aedes aegypti,* the ingestion of a blood meal stimulates release of gonadotropic neurohormones from median neurosecretory cells in the brain for up to 12 hours post-ingestion (Lea, 1967, 1972). These neurohormones stimulate the ovaries to secrete ecdysteroids and are referred to as "ovary ecdysteroidogenic hormones" (OEHs) (Matsumoto, *et al.*, 1989). The gene of one of these OEHs, OEH I, was sequenced, and is the first steroidogenic gonadotropin to be identified for invertebrates (Brown, *et al.*, 1998). Whole-mount immunocytochemistry using OEH I antiserum revealed an extensive distribution of immuno-positive cells in larvae and adults of *Aedes aegypti* and *Anopheles gambiae* (Brown and Cao, 2001). Immuno-positive median neurosecretory cells were found in brains of larvae and adult *Aedes aegypti.* In *Anopheles gambia,* there are more immunoreactive lateral neurosecretory cells than in *Aedes aegypti.* In both species, immuno-positive axons from these cells extended out of the brain through the neurohemal organ associated with the aorta and branched extensively along the midgut. Immuno-positive endocrine cells were also observed in larval and adult midguts of both species. The markedly similar localizations of immuno-positive cells in larvae and adults of two distantly related species indicate that OEH I, or a homolog, is conserved within this group of Diptera and likely has stage- and sex- specific functions.

Recently, a peptide (Bom-PTSP) with a prothoracicostatic effect is reported in *Bombyx mori* (Hua *et al.*, 1999). This peptide inhibites PTTH stimulated ecdysteroidogenesis by the prothoracic gland at both the spinning and feeding stages of *Bombyx mori.* It has the same sequence as Mas-MIP-I, a myoinhibitory peptide previously isolated from the ventral nerve cord of *Manduca sexta* by Blackburn *et al.*, (1995) and is highly homologous with the N-terminal portion of vertebrate peptides of the galanin family.

Insulin-Like Peptides and Insulin Receptor in Insect

Another group of molecules that I believe that they may be involved in the midgut hormone regulation of oogenesis acting via the juvenile hormone and/or ecdysteroid pathways in *Phormia regina* are insulin-like peptides and their corresponding receptors. Peptide and gene sequences for insulin-like peptides (ILPs) are known for only a few insect species belonging to two orders, Orthoptera (Hetru *et al.*, 1991; Kromer-Metzger and Lagueux, 1994) and Lepidoptera (Ishizaki and Suzuki, 1994; Yoshida *et al.*, 1998). These peptides clearly belong to the insulin superfamily as surmised by their protein and gene structures (Smit *et al.*, 1998; Chan and Steiner, 2000). These genes all encode contiguous B, C, and A chains that are processed into an active peptide with the excision of the C chain and a dimerization of A and B chains. There is one intrachain disulfide bond for A and two interchain disulfide bonds between the A and B. However, Diptera provided us with the first evidence for the existence and/or function of

insulin-like factors with studies on: *Drosophila melanogaster* (Seecof and Dewhurst 1974; Meneses *et al.*, 1975; Gorczyca *et al.*, 1993; Zitnan *et al.*, 1993); *Calliphora erythrocephala* (Normann, 1975; Duve, 1978) *Phormia regina* (Chen and Friedman, 1977). Based on the *Drosophila melanogaster* genome information, an insulin- like peptide was been used to make an antiserum. This antiserum was used to probe tissues of *Drosophila melanogaster*. There are two clusters of median neurosecretory cells in female *Drosophila melanogaster* brain and two clusters of lateral neurosecretory cells in female *Aedes aegypti* brain immono-positive to this antiserum (Cao and Brown, 2001). These specific localizations substantiate the existence of an endogenous insulin-like peptide in *Drosophila melanogaster* and suggest that a similar peptide is present in the distantly related mosquitoes.

The functions of insulin and insulin-like peptides in insect are also documented. As indicated before, bombyxins, which are insulin-like peptides, stimulate ecdysteroidogenesis in silkworm larvae. In *Aedes aegypti*, porcine insulin also can activate the ovary to produce ecdysteroids through a conserved signaling cascade as in mammalian cells (Graf, *et al.*, 1997).

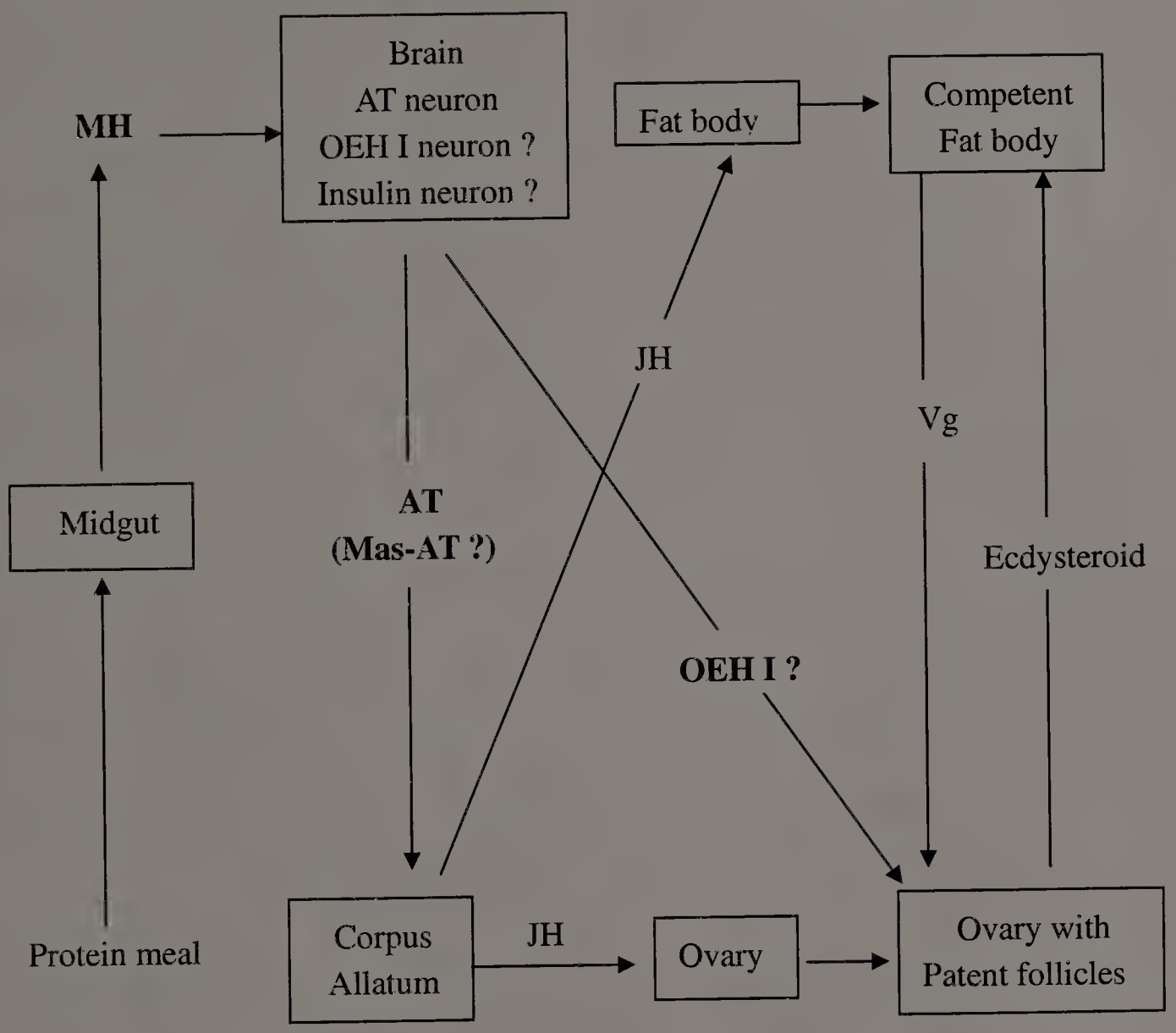
In contrast to the extensive studies of insulin-like peptides in insects, insulin receptors have been identified only in two dipteran species, *Drosophila melanogaster* (Ruan *et al.*, 1995; Chen *et al.*, 1996), and *Aedes aegypti* (Graf *et al.*, 1997). These receptors show strikingly high homology to the human insulin receptor, exhibiting the same $\alpha_2\beta_2$ subunit structures and also containing a ligand-activated tyrosine kinase in

its cytoplasmic domain. Mutation studies show that this insulin receptor regulates the growth of *Drosophila melanogaster* (Chen *et al.*, 1996). Mutation of this receptor (*InR*) results in slower growth in this fly. More interestingly, the *InR* mutant extends longevity up to 85% more than the wild type. This extended life may be brought back to normal life-expectancy by treating the long-lived mutant with a JH analog, thus suggesting that in *Drosophila melanogaster*, a failure of insulin-like ligands increases the life-span through retardation of growth or inactivation of specific endocrine tissue such as corpus allatum (Tatar *et al.*, 2001).

Among many unknown elements in the working mechanism of the *Phormia regina* midgut hormone, I studied a migut peptide antiserum, which was raised on the partial sequence of this midgut hormone. Meanwhile, I tried to evaluate if the two possible steps downstream to the midgut hormone were somehow related to the *Aedes aegypti* OEH I and *Drosophila* insulin receptor. An *Aedes aegypti* OEH I antiserum (gift from Dr. Mark Brown, University of Georgia,) and a *Drosophila melanogaster* insulin receptor antiserum (gift from Dr. Robert S. Garofalo, Pfizer Global Research and Development) were used for this evaluation. In addition, I tried to identify the substance which was immunopositive to the Mas-AT antiserum. The results of these studies hopefully will further improve the current general understanding of the endocrine control of *Phormia regina* oogenesis by the diet and midgut hormone (Chapman, 1998).

Figure 1.1. A model summarizing the working mechanism of midgut hormone leading to oogenesis in *Phormia regina* (adapted from Yin *et al.*, 1994). The events in bold font indicate the areas of focus in this thesis.

AT: allatotropin; JH: juvenile hormone; Mas-AT: *Manduca sexta* allatotropin; MH: midgut hormone; OEH I: ovary ecdysteroidogenic hormone I; Vg: vitellogenin.


Brain
AT neuron
OEH I neuron ?
Insulin neuron ?
MH
Fat body
Competent
Fat body
JH
Vg
Midgut
AT
(Mas-AT ?)
Ecdysteroid
OEH I ?
Protein meal
Corpus
Allatum
JH
Ovary
Ovary with
Patent follicles

CHAPTER II

A STUDY USING AN ANTISERUM AGAINST A MIDGUT PEPTIDE

Introduction

The insect gut has been known as a rich endocrine source (Sehnal and Zitnan, 1990). Ultrastructural and/or immunological evidence indicates that both gut endocrine cells and gut hormones exist in a wide spectrum of insects, such as *Calliphora erythrocephala* (De Priester, 1971), *Bombyx mori* (Kobayashi, 1971), *Aedes aegypti* (Hecker, 1977; Brown *et al.*, 1985), *Periplaneta americana* (Nishiitsutsuji-Uwo and Endo, 1981), *Calliphora vomitoria* (Duve and Thorpe, 1982), and *Helicoverpa zea* (Crim *et al.*, 1992). However, the function of most insect gut hormones or hormone-like substances remains unknown.

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Studies of the relationship between nutrition and endocrine on *Phormia regina* suggest that a protein meal triggers the release of a midgut hormone, which travels through the hemolymph to reach the brain where it activates a neuroendocrine cascade leading to oogenesis (Yin *et al.*, 1994). Results indicated that this hormone is released from the midgut to reach the brain (i.e., the target) between 4 to 8h after the onset of the liver meal. The brain then initiates a neuroendocrine cascade leading to the onset and completion of oogenesis.

In our attempt to unravel the working mechanism of the midgut hormone in *Phormia regina*, the first step is to characterize this hormone. A purification scheme was developed using methanol precipitation, Rotofor™ preparative isoelectric focusing, and C18 reverse-phase HPLC to purify one of the midgut factors. Isoelectric focusing analysis revealed two factors (e.g. MHs) with isoelectric point (pI) of pH 6.75 and 4.25 (MH-6.75 and MH-4.25) (Yin *et al.*, unpublished). An analysis of methanolic midgut extracts using ultrafiltration membrane devices with different molecular mass cut-off filters revealed that the bulk of midgut hormone activity exists between 10 and 20 kDa (Yin *et al.*, 1994). After the C18 reverse-phase HPLC purification step, the pI 6.75 (i.e., MH-6.75) fraction was determined to have the following putative amino acid sequence: LGRVGQKQQTDKDLRDAIDQ (Yin *et al.*, unpublished, named as MH-6.75). An antiserum (MH-6.75 antiserum) was raised against this peptide sequence by using a synthetic, multiple-antigenic-peptide that contains a branching heptalysine core and eight MH-6.75 molecules (method invented by Tam, 1988). This antiserum was used to test: 1) if it can partially neutralize the effect of midgut hormone. 2) if there is some substance in the midgut cells which is immuno-reactive to this antiserum. and 3) if the M_r of the substance is consistent to the M_r range of 10 to 20 kDa estimated earlier.

Materials and Methods

Animals

Phormia regina was reared and maintained as previously described (Stoffolano, 1974; Zou *et al.*, 1989; Yin *et al.*, 1994). Mature larvae were allowed to crawl out of the medium and into sand to pupariate. Puparia were collected daily and allowed to emerge

in screened cages. Flies emerging within 8h were sexed and each sex placed in the same age group or cohort (hour 0, day 1). All flies were kept under a 16h light, 8h dark photoregime at 28±2°C, 50% relative humidity. During the first 3 days after emergence, a 4.3% sucrose solution was provided to all flies. To prepare for liver feeding, sucrose solution was taken away and the flies were starved overnight for 18h to ensure an empty crop (Yin *et al.*, 1994). Flies were then fed for one hour with finely chopped beef liver, *ad libitum*, at 72h of adulthood, to provide adequate protein. The sucrose solution was again provided to all flies following the liver meal.

To better synchronize the physiology of *Phormia regina*, only flies weighing equal or above 55mg right after liver feeding were used (Yin *et al.*, 1994).

Canton-S strain of *Drosophila melanogaster*, as a gift from Dr. John Nambu, Dept. of Biology, UMass, was maintained on a corn-meal based artificial diet as described by Roberts (1986).

Midgut Dissection and Extraction

Midguts from properly selected, liver-fed females were dissected out in *Phormia* saline (Chen and Friedman, 1975) at 6h after the onset of the liver meal. The criteria for the fly selection were previously reported (Yin *et al.*, 1994). After cleaning the food debris (*i.e.*, gut content) from the gut lumen, each cleaned midgut epithelium was placed in ice-chilled 80% MeOH. For each one hundred cleaned midguts, one ml of 80% MeOH was used. Many of these 100-midgut batches were stored at -20°C before processing later. For each batch of 100-midgut, one more ml of ice-cold 80% MeOH was added before the guts and methanol were transferred into a glass Wheaton Porter-

Elvehjem tissue grinder equipped with a PTFE pestle. Homogenization was achieved after more than 40 strokes and 10 seconds of ultrasonication. Supernatant was obtained after spinning the homogenate at 10,500 X g (Eppendorf 5415D) for 20 min at room temperature. The supernatant was then dried by a SpeedVac SC210A concentrator (Savant). To remove lipid, each dried brain extract sample was resuspended in 50 µl of distilled water and thoroughly mixed with 150 µl of a chloroform:methanol solution (2:1 by volume) by sonication. After a thorough mixing, the sample was centrifuged at 1,000 X g for 2 min, its supernatant was saved and dried by Speed Vac again, and stored at –20 °C for future use.

Neutralization of MH-6.75, *In Vivo*

The neutralization procedure followed that of Tu (2000). MH-6.75 antiserum was used to neutralize the MH-6.75 in *Phormia regina*, *in vivo*. Flies injected with normal rabbit serum (NRS) diluted 4X with *Phormia* saline served as controls. The 4X dilution used here was the highest concentration tested with little detrimental effect on flies and was used for further experimentation. A 4X diluted MH-6.75 antiserum (1 µl) was then injected into the liver-fed flies 2h post-liver feeding to neutralize MH-6.75, *in vivo*. Control flies were injected with 4X diluted NRS. Follicle development was examined at 48h post-injection. Three trials with 8 to 10 flies each were run for each experiment. Ovaries were dissected in *Phormia* saline (Chen and Friedman, 1975) and the primary follicles staged according to the scheme of Adams (1974) used for *Musca domestica*. Primary follicles, prior to the presence of visible opaque materials in oocytes, were considered pre-vitellogenic and classified as stages 1 to 3. Vitellogenic follicles were

categorized as stages 4 to 9, while fully mature, chorionated eggs were considered stage 10 (Figure 2.1.A).

Immunohistochemistry

Flies were anesthetized by CO_2 and kept on ice before dissection. Sugar- and liver-fed *Phormia regina* were injected with 4% (W/V) paraformaldehyde in sodium phosphate buffer (0.1M, pH 7.4) and holes were cut into the body, and then the flies were soaked in the paraformaldehyde solution overnight before dissection. Such tissues as brain, thoracicoabdominal ganglion, CC-CA, thoracic muscle, foregut, midgut and Malpighian tubules were dissected in *Phormia regina* saline solution for whole-mount preparations.

Whole-mount preparations were processed using a previously published method (Davis, 1987). Briefly, the tissues were washed thoroughly 5 times (one hour each time) in phosphate buffered saline plus 0.5% Triton X-100 (PBST). The tissues were then blocked in 10% normal goat serum (NGS) in PBST for 1h before soaking in the MH-6.75 antiserum (1:100 dilution in 10% NGS/PBST) for three days at 4°C without agitation. After being treated with the primary antiserum, the tissues were washed 5 times (1h each) in PBST, blocked a second time in 10% NGS/PBST for 1h. Tissues were then treated with rhodamine (TRITC)-conjugated goat anti-rabbit IgG solution (Jackson ImmunoResearch Laboratories, Inc.) (1:200 dilution in 10% NGS/PBST) in the dark overnight (about 18h). Tissues were then washed 3 times with PBST (30 min each) in the dark before clearing in a glycerol series (40, 60 and 80% glycerol in carbonate/bicarbonate buffer, 1h in each glycerol solution). All steps described here done at room temperature with agitation unless indicated otherwise. To confirm the specificity

of the primary antibody, tissues were treated as above with antiserum preabsorbed with the antigenic peptide (2 μg of the antigenic peptide per 1 μl of MH-6.75 antiserum overnight at 4°C). Another set of tissues from sugar-fed flies treated as the same way was considered as the control. Preparations were mounted between a microscopic slide and a cover glass in Vectashield H-1000 (Vector Laboratories, Inc) mounting medium. Preparations were observed and photographed using a Nikon Eclipse E-600 microscope equipped with a cooled-CCD camera located at the Central Microscope Facility of the University of Massachusetts, Amherst. Micrographs were recorded using Spot 32. The digitized images were then edited using Photoshop (V. 5.0).

SDS-PAGE and Western Blot

SDS (sodium dodecyl sulfate) slab polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot were performed under non-reducing conditions on a Hoefer Mighty Small™ unit (cat. # SE 220/225). The protocols to run SDS-PAGE and Western Blot were those from *Short Protocols in Molecular Biology* (Ausubel *et al.*, 1995) with some modifications. SDS-PAGE was carried out with a 22% acrylamide/bis-acrylamide separating gel [in 10 ml gel solution, there were 3.67 ml of 60% acrylamide/1.6% bis-acrylamide solution, 3.33 ml of 3 M Tris buffer (pH 8.45), 1.06 ml of glycerol and 3.41 ml of distilled water]. The stacking gel is 6% acrylamide/bis-acrylamide [in 6 ml of gel solution, there were 0.625 ml of 60% acrylamide/1.6% bis-acrylamide solution, 1.55 ml of 3 M Tris buffer (pH 8.45) and 4.075 ml of distilled water]. Proteins equivalent to 12.5 midguts were dissolved in 24 μl water with 6 μl of 5 X SDS sample loading buffer (6% 1 M Tris buffer, pH 6.8; 25% glycerol; 0.2% SDS; 0.1% bromophenol blue; 66% distilled water) and heat-treated in water bath (100°C) for 5 min before loaded into the well. The

running buffer for the anode is 0.2 M Tris buffer (pH 8.9), and the running buffer for the cathode is 0.1 M Tris buffer with 0.1 M tricine and 1% SDS. The gel was running at 4°C with constant current 15 mA until the indicator dye almost reached the bottom of the gel.

After electrophoresis, the protein was transferred to a piece of nitrocellulose membrane (NitroBind, 0.22μm pore size) at 4°C at constant 80 voltage for an hour in a Hoefer transfer unit. Before transfer, the membrane was soaked in transfer buffer (0.025 M Tris with 0.2 M glycine) for 10 min to make sure that it was completely wet. After the transfer, the membrane was rinsed with distilled water twice to remove gel debris and transfer buffer components. The membrane was then incubated in a blocking solution (gelatin/carbowax in TBS) overnight at 4°C followed by three washes (5 min each) with phosphate buffered saline (pH 7.4) with 0.1% Tween 20 (PBST). A 10 ml diluted MH-6.75 antiserum [1:200 (v/v)] in PBST, was used to probe the membrane with midgut proteins for two hours at 37°C. After this probe was removed by rinsing three times (5 min each) with PBST, the membrane was incubated in 10 ml (at 1:1,000 dilution) of a horseradish peroxidase conjugated goat anti-rabbit IgG (HRP-IgG) solution (Jackson ImmunoResearch 111-035-003) for an hour at 37°C. After washed three times with PBST to remove the non-specifically bound secondary antibody, the membrane was soaked in 15 ml of a chromogen solution containing 3,3'-diaminobenzidine tetrahydrochloride (DAB), and urea peroxide from a Sigma Fast™ kit (Sigma D-4418) for 30 min to develop color. The membrane was air-dried and the results were recorded with a Visioneer OneTouch 8900 USB scanner (8900 USB).

A Comparative Study Using *Drosophila melanogaster*

After discoverying that the three *Phormia regina* midgut proteins reacted positively to the MH-6.75 antiserum, I proceeded to check if similar proteins may also exist in *Drosophila melanogaster*. The whole body extracts of *Drosophila melanogaster* were prepared from flies at 2, 3, and 4 days of adulthood without separating the sexes. Each batch of 50 flies were homogenized in the 80% methanol in a glass Wheaton Porter-Elvehjem tissue grinder equipped with a PTFE pestle. After homogenization, the homogenate was ultrasonicated to make sure the cells were completely broken and then centrifuged at 5,200 X g at room temperature for 60 min. The supernatant was saved and dried by using a Speed Vac SC210A (Savant) instrument after the lipid in each sample was removed. To remove lipid, each dried brain extract sample was resuspended in 50 µl of distilled water before thoroughly mixed with 150 µl of a chloroform:methanol solution (2:1 by volume) by sonication. After well mixed, the sample was centrifuged at 1,000 X g for 2 min, its supernatant was saved and dried by Speed Vac again, before stored at –20°C for future use. Western blot analyses of the *Drosophila* whole body samples were conducted in the same way as described for midgut samples from *Phormia regina.*

Data Analysis

A two sample *t*-test was used to compare the neutralization effect of MH-6.75 antiserum on the follicle development. The data were analyzed by using Minitab 1.2.

Results

Effect of Neutralization of MH-6.75, *in Vivo*

The neutralization by MH-6.75 antiserum *in vivo* was to lower the activity of MH-6.75 to affect its stimulation of oogenesis, which was estimated by judging the status of follicle development. In control flies injected with normal rabbit serum, the ovarian follicles developed to an average of stage 6.32±0.6 (average±standard of mean) 48h later. In contrast, the follicle development of MH-6.75 antiserum injected flies reached only to stage 5.20±0.51 that is more than one stage delayed which is statistically significant (two sample *t*-test, p=0.0064) (Table 2.1). The difference of follicle development between stage 5 and 6 was shown in Figure 2.1.B.

Localization of MH-6.75 in the Midgut

Because the highest biological activity was found in the midgut of 6h after a liver meal (Yin *et al.*, 1994), all the tested tissues (brain, thoracicoabdominal ganglion, CC-CA, thoracic muscle, foregut, midgut and Malpighian tubules) were collected from 6h post liver-fed female *Phormia reigna*. Among all tested tissues, positive responses were identified only in the midgut. The immunoreactive substances appeared to concentrate around the nucleus of the cell (Figure 2.2.A). Among all our midgut preparations these cells appeared to be only in the anterior half. When present, the distribution was not even, the positive cells appeared as patches in the gut epithelium

Characterization of Immunoreactive Substances to MH-6.75 Antiserum in the Midgut of *Phormia regina* and *Drosophila melanogaster*

Western blot analyses were carried out to characterize the substances in the midgut extract that are immunoreactive to MH-6.75 antiserum. Three immuno-positive midgut substances were found, their M_r were ca. 19, 20 and 62 kDa, respectively (Figure 2.3.A).

In the *Drosophila* whole body extracts, there was only one immuno-positive substance with M_r of about 40 kDa (Figure 2.3.B). Whether this substance was from the midgut remain to be determined.

Discussion

Anautogenous species like *Phormia regina* require a protein meal to support oogenesis (Stoffolano *et al.*, 1992; Yin and Stoffolano, 1990, 1997; Yin *et al.*, 1993). In addition to serving as a raw materials source, the protein meal activates the brain, which then triggers the entire neuroendocrine cascade leading to oogenesis in this fly (Yin *et al.*, 1994). Previous ultrastructual study has suggested that between 4h and 8h after a liver meal, the midgut of *Phormia regina* releases some secretions into the hemolymph, concurrently there is a drastic reduction in the number of secretory granules in midgut closed secretory cells (Stoffolano *et al.*, 1989).

When the timing of the release of midgut secretory granules is considered together with the temporal patterns of other protein meal triggered events, it is apparent that these granules would most likely activate the brain to begin the endocrine cascade leading to completion of oogenesis. Thus, a midgut hormone was proposed (Yin *et al.*, 1994; more details in the Chapter I). The following events take place after the release of midgut secretory granules: 1. morphometric changes of protocerebral neurosecretory cells (Tu, 2000), 2. changes of the effect of protocerebroectomy on oogenesis (Yin *et al.*, 1994), 3. rise and fall of the hemolymph ecdysteroid titer (Yin *et al.*, 1990), 4. activation of the corpus allatum to synthesize JHs (Zou *et al.*, 1989), and 5. vitellogenin biosynthesis and uptake (Yin *et al.*, 1989). More recently, a Mas-AT or Mas-AT related substance is

found to play a considerable role in the juvenile hormone biosynthesis in *Phormia regina* (Tu *et al.*, 2001). In this thesis, I also demonstrated that mosquito OEH I, *Drosophila* insulin receptor may be involved in the midgut hormone initiated endocrine regulation of oogenesis in *Phormia regina.*

Neutralization experiments slowed oogenesis significantly. The delay of one developmental stage from 6.32 to 5.20 was detected. At stages 5, 6, and 7, the average sizes of the terminal oocytes are 0.12 X 0.17, 0.27 X 0.50, and 0.24 X 0.61 mm, respectively. Since MH-6.75 does not represent the entire midgut hormone activity, slowed but not stopped oocyte development was consistent with my expectation.

At this moment, although I have found three midgut proteins reacting positively to the MH-6.75 antiserum, their chemical structures and functions remain to be determined. After determining their structures and synthesizing them through chemical and/or genetic engineering means, bioassays should be conducted to verify their brain and oogenesis stimulating effects. My current finding represents the beginning of the midgut hormone story. To have a more comprehensive understanding of these three newly discovered midgut proteins, they should be purified in adequate quantities to allow determination of their amino acid sequences to facilitate subsequent gene cloning. Once specific obligonucleotide probes are available, *in situ* hybridization experiments may be conducted to prove or disprove that those midgut cells, showing the massive release of granules at 4 to 8h after a liver meal, indeed contain the mRNAs specific for the three midgut proteins identified in this thesis.

The presence of one protein band reactive to the MH-6.75 antiserum in the whole body extract of *Drosphila melanogaster* is extremely interesting. It showed that the

distribution of MH-6.75-related substances is inter-specific and a possibility of identifying the gene after obtaining a partial amino acid sequence of the immunoreactive *Drosophila* protein by using the recently available FlyBase (1996).

Table 2.1. The effect of neutralization of MH-6.75 by its antiserum, *in vivo*, on the ovarian follicle development.

Treatment	Number of flies	Stage of follicle development Mean±SEM[3]
Normal rabbit serum[1] injection	28	6.32±0.60*
MH-6.75 antiserum[2] injection	56	5.20±0.51*

1) Flies were injected with 1 µl diluted normal serum (1:3), 2h after a liver meal.

2) Flies were injected with 1 µl diluted antiserum at (1:3), 2h after a liver meal.

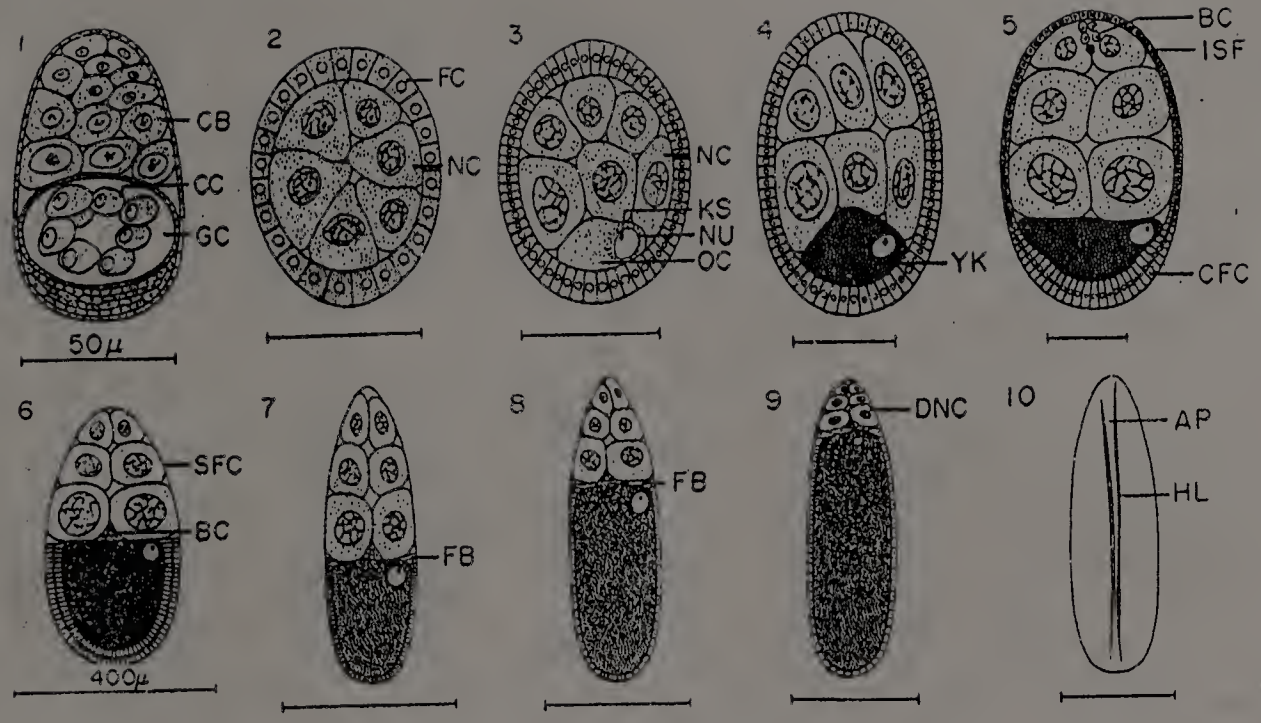
3) SEM means standard error of mean.

4) Two sample *t*-test, p=0.0064, * means significant difference.

Figure 2.1. A. Diagram of the 10 stages of oogenesis in the housefly *Musca domestica*. Nos. 1 to 9 are cross-sections of the follicles, whereas No. 10 shows the egg surface. The scale bar in the upper row is 50 μ and 400 μ in the lower row (adapted from Adams, 1974). **B.** Diagram shows the stage 5 and 6 of oogenesis based on the same scale.

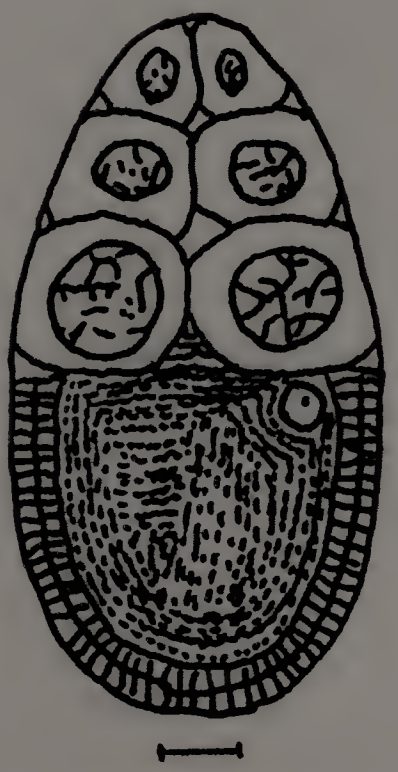
AP: aeropyle; BC: boarder cells; CB: cystocyte; CFC: columnar follicle cell; DNC: degenerating nurse cell; FB: follicle cell bridge; GC: germarial cyst; HL: hatching line; ISF: incipient squamous follicle cells; NC: nurse cell; NU: nucleus; OC: oocyte; SFC: squamous follicle cell; YK: yolk.

A



B

Stage 5



Stage 6

Figure 2.2. **A,** Localization of MH-6.75 at the anterior part of midgut of female *Phormia regina* at 6h post liver-feeding, the arrow points to the location of MH-6.75 in one of the immuno-positive cells to MH-6.75 antiserum. **B**, Sugar-fed control showing no immunoreactive signal. The scale stands for 25 μm.

A

B

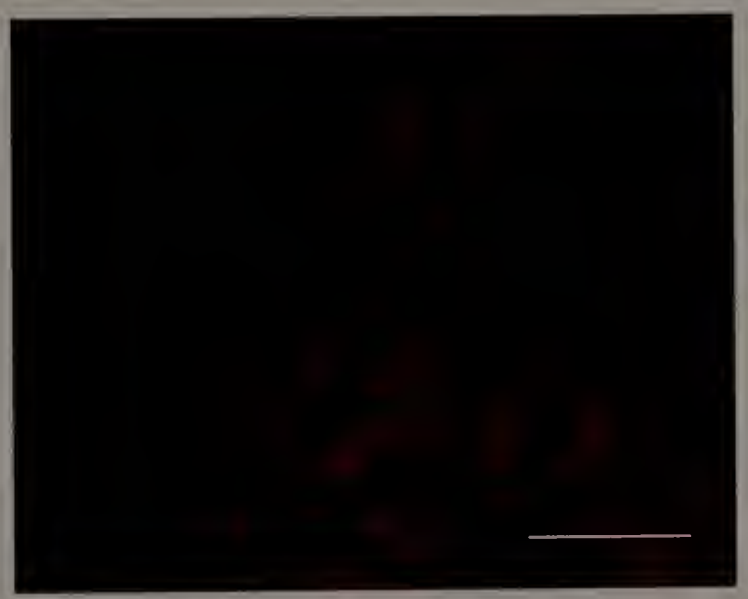
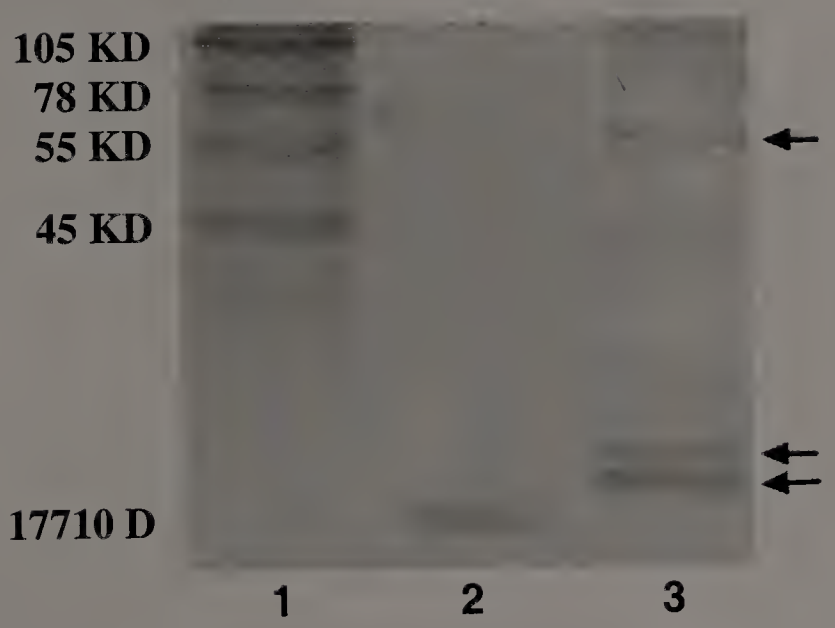
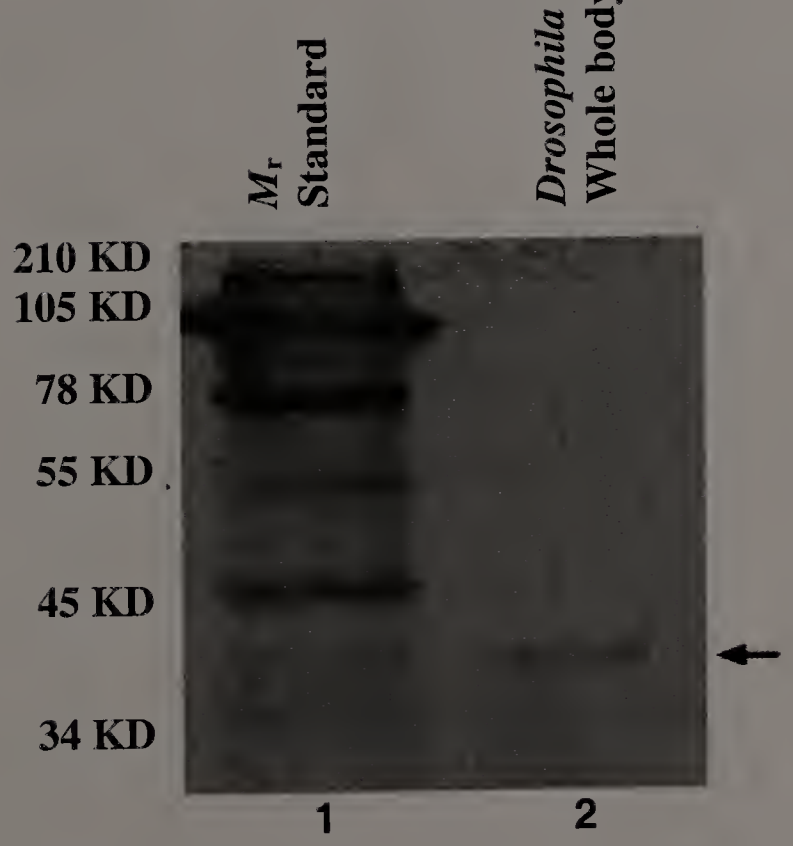


Figure 2.3. Western blot analyses of the extracts of midgut of female *Phormia regina* at 6h post liver-fed (**A**) and *Drosophila* whole body extracts (**B**), probed by MH-6.75 antiserum, the arrows point to substances immuno-positive to MH-6.75 antiserum. **A.** Lane1: 3 µl of M_r standard; Lane 2: 18 ng of MH-6.75-MAP; Lane 3: 12.5 equivalent midguts of *Phormia regina*. **B.** Lane 1: 3 µl of M_r standard; Lane 2: 50 equivalent of *Drosophila* whole body.

A


M_r Standard
MH-6.75-MAP
Midgut
105 KD
78 KD
55 KD
45 KD
17710 D
1
2
3

B


M_r Standard
Drosophila Whole bod
210 KD
105 KD
78 KD
55 KD
45 KD
34 KD
1
2

CHAPTER III

TWO POSSIBLE LINKS IN THE PATHWAY OF MIDGUT HORMONE REGULATED OOGENESIS

Introduction

Studies of the relation between nutrition and endocrine on *Phormia regina* suggest that a protein meal triggers the release of a midgut hormone, which travels through the hemolymph to reach the brain and activates a neuroendocrine cascade leading to oogenesis (Yin *et al.*, 1994). The working hypothesis of the role of this midgut hormone is reviewed in the Chapter 1, but many factors in the pathway remain to be defined, such as its targets, and secondary messengers.

In the yellow fever mosquito, *Aedes aegypti*, the ingestion of a blood meal leads to the release of hormones from median neurosecretory cells in the brain to stimulate the ovaries to secrete ecdysteroids (Lea, 1967, 1972). These hormones were later named as "ovary ecdysteroidogenic hormones" (OEHs) by Matsumoto *et al.* (1989). One of these OEHs, OEH I, was isolated and identified as the first steroidogenic gonadotropin from invertebrates (Brown *et al.*, 1998). Since this hormone is triggered by a protein meal in the yellow fever mosquito leading to oogenesis, the possible existence of a similar hormone or hormone family in another dipteran, namely, *Phormia regina* was explored in this thesis. I was also inspired by the discovery of an insulin-like peptide and insulin-like receptor in *Drosophila melanogaster* (Ruan *et al.*, 1995; Chen *et al.*, 1996; Graf *et al.*, 1997) and *Aedes aegypti* (Cao *et al.*, 2001) and the correlation of insulin-like receptor and the production of ecdysteroids in *Aedes aegypti* (Graf *et al.*, 1997). I postulated that there

might be an insulin-like peptide involved in the hormone pathway of regulation of oogenesis beginning at the release of midgut hormone in *Phormia regina*.

Materials and Methods

Animals

Maintenance of *Phormia regina* colony, liver-feeding procedure, and the method used to weigh the liver-fed flies followed the procedures described in Chapter II.

To better synchronize the physiology of *Phormia regina*, only flies weighing about 55mg right after liver feeding were used (Yin *et al.*, 1994).

Immunohistochemistry

The Immunocytochemistry analyses were conducted in the same way as described in Chapter II with some modification. *Aedes aegypti* OEH I antiserum was used here at 1:500 dilution in 10% NGS/PBST (a kind gift from M. R. Brown, Department of Entomology, University of Georgia, Athen, GA). *Drosophila melanogaster* insulin receptor antiserum was used here at 1:100 dilution in 10%NGS/PBST (from Robert S. Garofalo, Pfizer Global Research and Development, Groton, CT). Control whole mount immunohistochemical preparations for OEH I antiserum were prepared in the absence of the antiserum. Control preparations for *Drosophila* insulin receptor antiserum were prepared in the absence of the antiserum or in the presence of a preabsorbed *Drosophila* insulin receptor antiserum (2 μg of the antigenic peptide per 1 μl of *Drosophila* insulin receptor antiserum overnight at 4°C).

Preparations were observed and photographed using a confocal microscope (MRV600 Krypton-Argon, Bio-Rad), using exciter wavelength of 568 nm and GR2 emission filter block located at the Central Microscope Facility of the University of Massachusetts, Amherst. Microimages were recorded with COMOS (Bio-Rad). The digitized images were edited by using Photoshop (V.5.0)

Results

Localization of *Aedes aegypti* OEH I immunoreactive cells in *Phormia regina*

I began my examination with flies collected at 0h (i.e., sugar-fed flies) and 4h (liver-fed, 4h) after the onset of the liver-meal. Positive responses were only observed in the cephalic nervous system. However, the details of the distribution and cell number of these responsive neurons were not identical between sexes. Regardless of sexes and dietary regimens (e.g., sugar- or liver-fed), there were always one pair of median ($MNC_{OEH\ I}$) and one pair of lateral neurosecretory cells ($LNC_{OEH\ I}$) responding to OEH I antiserum (Figure 3.1.A). The $MNC_{OEH\ I}$, like other MNC reported in numerous articles, were located in the typical regions flanking the median furrow of the pars intercerebralis. Each of the two $LNC_{OEH\ I}$ was located in a position that is very close to the optical lobe and rather deep in protocerebrum at nearly 1/3 to ½ the way between its dorsal and ventral aspects. Unexpectedly, these two lateral neurons were not always found at the similar depth of field under the confocal focusing manipulation. In other words, the location may not be similar to that of other reported LNC, which may contain hormones other than OEH. However, an obvious difference was observed between sexes, in addition to the $MNC_{OEH\ I}$ and $LNC_{OEH\ I}$. Males always had 4 more pairs of positive cells arranged in

2 quite straight, dorsal-ventral lines, which were located on each side of the esophageal foramen and half way to the inner edges of the optical lobes (Figure 3.1.B). These males specific cells were located very close to the posterior brain surface. The dorsal most cell pair of these cells were located more dorsal than the dorsal aspect of the oesophageal foramen, while the ventral most cell pair were located at slightly more dorsal or about the same level of the dorsal aspect of the foramen. These OEH I antiserum responsive, male specific, near the foramen neurosecretory cells ($MSNFNC_{OEH\ I}$) were not reported in *Phormia regina* before (Figure 3.1.B).

Because of the lack of difference in both the number and distribution of OEH I positive neurons between sugar-fed (liver-fed, 0h) and liver-fed (4h) flies, I decided to probe the female cephalic nervous system at 8, 16, 20, 22, 23, 24, 26, 28, 36, 48 and 72h after the liver-meal. This time period covered the entire first ovarian cycle (up to 48h after the liver meal) and 1 full day thereafter. This more detailed study uncovered additional locations in the suboesophageal ganglion of cells that contained neurons positive to the anti-OEH I antiserum. At each side of the median line of the suboesophageal ganglion, two large adjacent neurons ($SOGNC_{OEH\ I}$) were prominently stained immunohistologically (Figure 3.1.C). Moreover, the presence of this specific immunoreactivity showed a discrete temporal pattern. Only the suboesophageal ganglia of flies removed at 16, 23, 28, 36, and 72h after their liver-meal contained OEH-I positive neurons. In all the females examined, the number and location of $MNC_{OEH\ I}$ and $LNC_{OEH\ I}$ remained the same. Table 1 summarizes the results of the time course study in females. A similar study was done on males at 4 and 16h after the liver-meal and revealed no positive neurons in the suboesophageal ganglion.

Localization of immunoreactive cells to the *Drosophila* insulin-like receptor antiserum in *Phormia regina*

The same set of tissues were probed for immunoreactivity against *Drosophila melanogaster* insulin-like receptor antiserum for female flies at 6 and 20h after a liver-meal. Among all the tissues tested, positive responses were identified only in the corpus cardiacum (CC) and its nerves going to the corpus allatum (CA) at both 6 and 30h as tested (Figure 3.1.D). In no case did we observe any positive signals from the CA cells at these two time points. The positive material appeared as aggregates of substances of irregular shapes and varying sizes. We did not see any fluorescence signal depicting an outline of a cell in any of the aggregates suggesting that these aggregates are associated with nervous fibers rather than the perikaryons.

Discussion

It is a long-term goal to define oogenesis regulation by the midgut hormone in *Phormia regina*. There are two possible elements in the regulatory pathway that may be related to OEH I or OEH I-like substance, which controls the ecdysteriodogenesis and to the insulin receptor or insulin receptor-like substance that mediates the insulin signaling pathway controlling juvenile hormone biosynthesis in *Drosophila melanogaster* (Tatar *et al.*, 2001). OEH and insulin functions may also serve as necessary mediators because Zou *et al.* (1989) and Yin and Stoffolano (1990) showed that after the release of the midgut hormone there is a tremendous increase in juvenile hormones and ecdysteroid syntheses in liver-fed *Phormia regina*. Taking advantage of the availability of an *Aedes aegypti* OEH I antiserum and a *Drosophila melanogaster* insulin receptor antiserum, I

found materials reacted to these antisera in the cephalic nervous system of *Phormia regina*.

The neurohormone, OEH I, is originally identified in two to three pairs of MNCs of adult female of *Aedes aegypti* (Brown *et al.*, 1998). There are about 10 median neurosecretory cells (MNCs) stained in larvae and 24 MNCs in adult *Aedes aegypti* and in both sexes to OEH I antiserum (Brown and Cao, 2001). In contrast, the brain of female *Phormia regina* contained one pair of MNCs and one pair of LNCs. Further, it may be prudent to think about the function(s) of OEH in terms other than its role in "ovary ecdysteroidogenesis" because additional OEH-I positive cells exist in male flies. It is tempting to postulate that these OEH I cells may be involved in a testis steroidogenic function. The presence of OEH I or an OEH I-like substance in female *Phormia regina* suggests that it also controls the ovarian production of ecdysteroids. However, this possibility is not so obvious when data is compared. In *Phormia regina*, the titer changes of ecdysteroids in the hemolymph, as well as in the ovary, are triggered by a liver meal and are correlated to vitellogenesis and oogenesis (Yin and Stoffolano, 1990). Subsequently, one would expect to see a series of changes in the brain OEH I cells triggered by the dietary intake of the fly. Our results showed that there is no obvious change in the number, location and immunoreaction of brain OEH I cells between sugar- and liver-fed flies. A loose, but tentative, correlation may exist between the suboesophageal ganglion OEH I cells and the hemolymph ecdysteroid titer changes in the liver-fed, female *Phormia regina*. Data show (Yin and Stoffolano, 1990), before liver-feeding the hemolymph ecdysteroid titer is low (i.e., at less than 5 pg/fly level). The titer changes from 70 to 80 pg/fly at 16h after a liver-meal. A further increase to nearly 100

pg/fly occurs at 24h prior to a steep increase around 250 pg/fly at 36h post a liver-meal. The ecdysteroid titer then decreases steadily to a very low level towards the completion of sequestration of vitellogenin by the developing oocytes 60h post liver meal. Within 12 hours, after chorion development, eggs are laid by mated females. Interestingly, these landmarks of events appear to be punctuated by the presence of the 4 suboesophageal ganglion neurosecretory cells. They appeared at 16, 23, 28, 36, and 72h post-liver meal. The appearances at 16, 23, 28, and 36h apparently links to ecdysteroidogenesis, whereas the appearance at 72h to oviposition. At the moment, we don't know, at least in *Phormia regina*, if the presence of immunoreactive material in a neuron means production and release of OEH I or just storage with little release. In 1986, Brown *et al.* showed that within 6h after a blood meal, the intensity of immunoractivity towards FMRFamide and the number of reactive cells are reduced in the midgut endocrine cells of a mosquito. On the other hand, Jenkins *et al.* (1989) found that twice as many of FMRFamide cells and four times as many of FMRFamide are found in the midgut of the fed compared to the starved corn earworm. A bioassay to confirm the ecdysteroidogenic function of OEH I in *Phormia regina* and a determination of hemolymph OEH I will be needed to better interpret the present immunohistological results.

Our results with sugar-fed female and liver-fed male *Phormia regina* suggest that OEH may have vital functions other than ovary ecdysteroidogenic ones in these two categories of flies. Neuropeptides are known to have more than one major function. For instance, the neuropeptide known as *Manduca sexta* allatotropin has myotropic, midgut ion transportation regulatory, and allatotropic functions (Lee *et al.*, 1998; Veenstra *et al.*, 1994).

The existence of an insulin receptor in insects has received considerable attention, but its gene structure has been only characterized in *Drosophila melanogaster* and *Aedes aegypti* (Ruan *et al.*, 1995; Cao and Brown, 2001; Chen *et al.*, 1996; Graf *et al.*, 1997). In *Drosophila melanogaster*, the insulin receptor is involved in the regulation of its growth (Chen *et al.*, 1996). In *Aedes aegypti*, the insulin receptor is involved in ecdysteroidogenesis (Graf *et al.*, 1997). Immunocytochemistry and *in situ*-hybridization studies showed that this receptor is expressed before a blood meal and mainly in the nurse cells of the developing oocytes. After a blood meal, follicle and nurse cells contain mRNA coding for this receptor. The intensity of expression rises in the follicle cells until they degenerate during chorion production (Helbling and Graf, 1998). Interestingly, ten years earlier, Garofalo and Rosen (1988) showed the presence of *Drosophila* insulin receptor homolog mRNA in nurse cells, and mature oocytes but not in follicle cells. In *Phormia regina*, its specific presence in the region between the CC and CA suggests that the insulin receptor may participate in the regulation of hormone release at the CC and CA level.

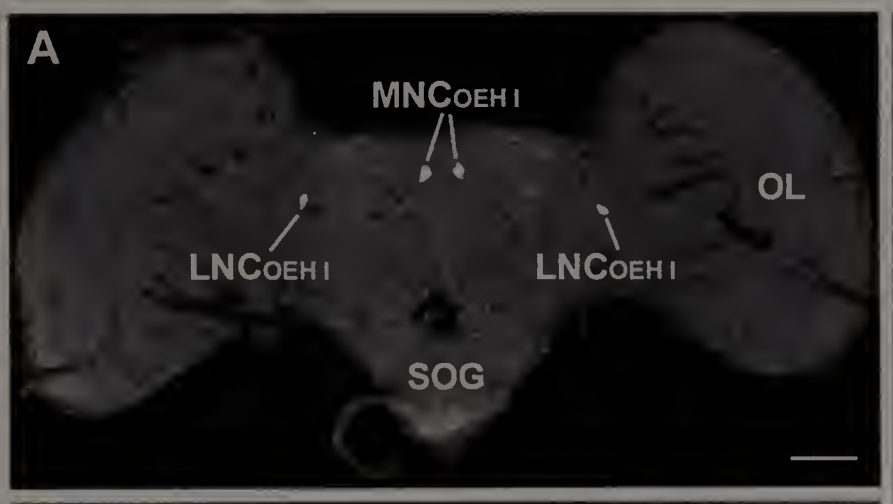
Table 3.1. Immunopositive cells to OEH I antiserum in female *Phormia regina*

Diet&Timing	Brain Median Neurosecretory Cell	Brain Lateral Neurosecretory Cell	Suboesophageal Neurosecretory Cell
Liver-fed, 0h	One pair	One pair	
Liver-fed, 4h	One pair	One pair	
Liver-fed, 8h	One pair	One pair	
Liver-fed, 16h	One pair	One pair	Two pairs
Liver-fed, 20h	One pair	One pair	*
Liver-fed, 22h	One pair	One pair	
Liver-fed, 23h	One pair	One pair	Two pairs
Liver-fed, 24h	One pair	One pair	
Liver-fed, 26h	One pair	One pair	
Liver-fed, 28h	One pair	One pair	Two pairs
Liver-fed, 36h	One pair	One pair	Two pairs
Liver-fed, 48h	One pair	One pair	
Liver-fed, 72h	One pair	One pair	Two pairs

* From six specimens observed, only one contained these 4 cells.

Figure 3.1. Localization of the *Aedes aegypti* ovary ecdysteroidogenic hormone I (OEH I)-like and the *Drosophila melanogaster* insulin receptor-like substances in the neuroendocrine system of *Phormia regina*. **A**. OEH I immunopositive cells in the brain of female; **B**. in the brain of male, the arrows pointing that each $LNC_{OEH\ I}$ is associated with a rather large axonal plexus; **C**. in the suboesophageal ganglion of female *Phormia regina*; **D**. Insulin-receptor immunopositive signals in the CC-CA complex of female *Phormia regina*. The scale bars in A and B are 140 μm, 55 μm in C and D.

CA: corpus allatum; CC: corpus cardiacum; OL: optic lobe; EF: esophageal foramen; $LNC_{OEH\ I}$: OEH I immuno-positive lateral neurosecretory cells; MNC_{OEHI}: OEH I immuno-positive median neurosecretory cells; $MSNFNC_{OEH\ I}$: OEH I immuno-positive male specific cells near foramen neurosecretory cells; PROV: proventriculus; SOG: suboesophageal ganglion; $SOGNC_{OEH\ I}$: suboesophageal ganglionic neurosecretory cells.


A
MNCOEH I
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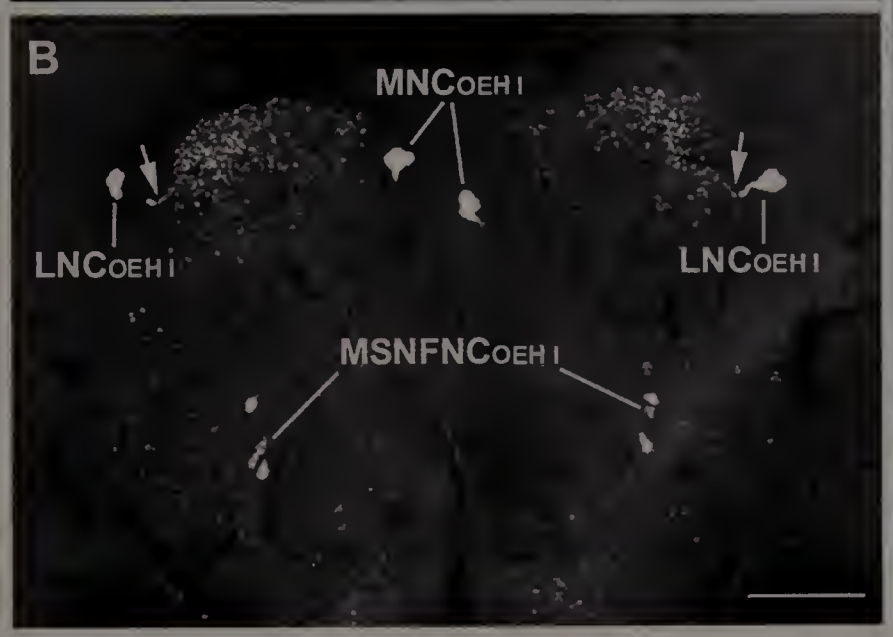
SOG


B
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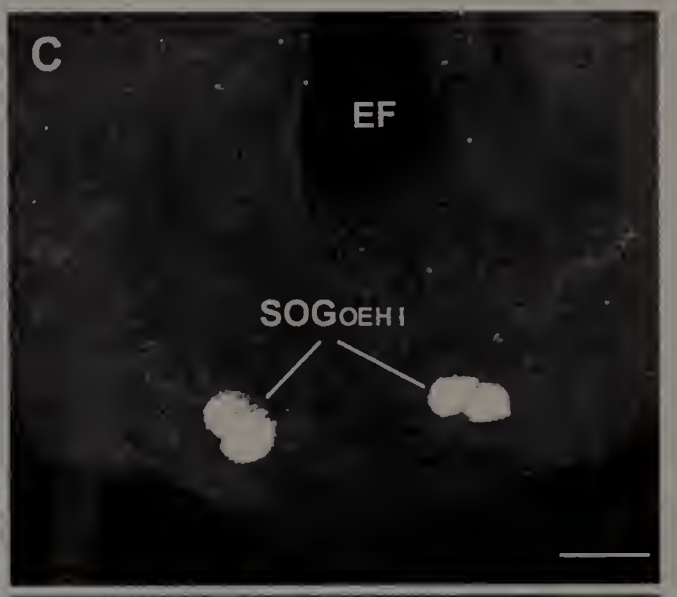
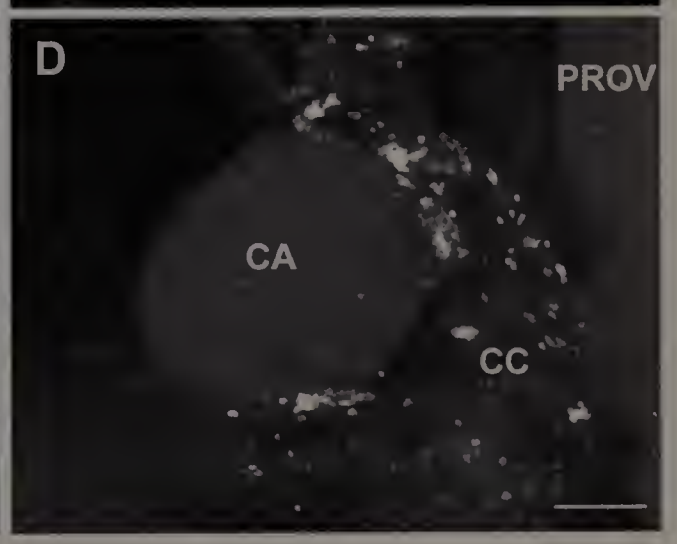

C
EF
SOGOEH I


D
PROV
CA
CC

CHAPTER IV

EXTRACTION AND CHARACTERIZATION OF MATERIALS IN ADULT *PHORMIA REGINA* RESPONSIVE TO *MANDUCA SEXTA* ALLATOTROPIN (MAS-AT) ANTISERUM

Introduction

Juvenile Hormones (JHs) play important roles in regulating insect metamorphosis, caste determination, behavior, polymorphism, diapause, vitellogenin synthesis, ovarian development, as well as various aspects of metabolism associated with these processes and have been viewed as the most versatile insect hormones (Nijhout, 1998).

JH synthesis is regulated by many factors, which include neuropeptides, biogenic amines and sex peptides (Gäde *et al.*, 1997; Gilbert *et al.*, 2000; Chiang *et al.*, 2002). There are two groups of neuropeptides controlling the synthesis of JH: allatotropins (ATs) or allatostatins (ASTs) which are secreted from specific cells such as the brain neurosecretory cells (Khan, 1988; Stay, 2000). ATs stimulate JH biosynthesis, ASTs inhibit JH biosynthesis. In contrast to the extensive studies of the ASTs, only a limited amount of research has been focused on the ATs. Mas-AT, the only AT isolated, was obtained from pharate adult heads of *Manduca sexta* (Kataoka *et al.*, 1989). This amidated tridecapeptide, with a sequence of NH_2-GFKNVMMTARGF-NH_2, activates JH synthesis of adult *Manduca sexta*. In *Phormia regina*, substances positive to Mas-AT antiserum were observed in the lateral neurosecretory cells and neurons that are located in the region between the median neurosecretory cells and the oesophageal foramen (Tu *et al.*, 2001). *In vitro* CA incubation studies show that Mas-AT antiserum can only partially reduce the synthesis of JH, thus suggest that in *Phormia regina*, there may be an

allatotropic factor other than Mas-AT (Tu *et al.*, 2002). In this chapter, I characterized the substances that are immuno-positive to the Mas-AT antiserum in *Phormia regina*.

Materials and Methods

Animals

Maintenance of *Phormia regina* colony, liver-feeding procedure, and the method used to weigh the liver-fed flies, and maintenance of the Canton-S strain of *Drosophila melanogaster* followed the procedures described in Chapter II.

Protocerebrum Extracts of *Phormia regina*

The protocerebrum extracts were prepared from flies at 8h post liver meal. Protocerebra were obtained by cutting the head capsules horizontally at the level of the dorsal aspect of the neck, which is just above the optical lobe by using a sharp pair of scissors. These brain tissues were separated from the head capsule and stored immediately in 80% methanol as batches of 60 brain equivalents. Each batch was homogenized in 80% methanol in a glass Wheaton Porter-Elvehjem tissue grinder equipped with a PTFE pestle. After homogenization, the homogenate was sonicated by ultrasonication to make sure the cells are completely broken and then centrifuged at 5,200 X g at room temperature for 60 min. The supernatant was saved and dried by using a Speed Vac SC210A (Savant) instrument after the lipid in each sample was removed. To remove lipid, each dried brain extract sample was suspended in 50 µl of distilled water before thoroughly mixed with 150 µl of a chloroform:methanol solution (2:1 by volume) by sonication. After well mixed, the sample was centrifuged at 1,000 X g for 2 min, its supernatant saved and dried by Speed Vac again, before stored at –20°C for future use.

Hemolymph Collection and Extraction

Hemolymph collection and extraction followed essentially the procedures adapted from Tu (2000). The flies were anesthetized with CO_2 in a vial and then chilled on crushed ice at 6, 7, 8, 9 and 10h after the onset of a liver meal. The immobilized flies were placed under the dissecting microscope individually. The antennae were pulled off at the bases and the bodies squeezed until drops of the hemolymph appeared at the antennal base for collection into a 10 µl capillary tube. The volume of hemolymph drops was recorded and then was blown into a 6 X 50 mm Kimble glass tube. A crystal of 1-phenol-2-thiourea was added to the hemolymph to inhibit tyrosinase activity, therefore preventing hemolymph blackening. An equal volume of 100% methanol was added to the pooled hemolymph immediately after each collection to make a 50% methanol solution. At the end of each collection, an equal volume of chloroform was added to the 50% methanol solution to obtain a solvent ratio of chloroform:methanol:hemolymph of 2:1:1 (v/v). After sonication for 10 min, the mixture was centrifuged at 5200 X g for 30 min. The supernatant was dried by using a Speed Vac instrumentand kept at –20°C for future use.

Whole Body Extraction of *Drosophila melanogaster*

The whole body extracts of *Drosophila melanogaster* were prepared in the same way as described in Chapter II

SDS-PAGE and Western Blot

SDS (sodium dodecyl sulphate) slab polyacrylamide gel electrophoresis (SDS-PAGE) and the Western Blot were performed in the same way as described in Chapter II but with different amounts of samples. I used 7.5 equivalent of brain and hemolymph

proteins to do the Western blot analyses. Mas-AT antiserum (CY16; Tu *et al.*, 2001) solution (1:200 dilution) was used to probe the membrane with brain and proteins for two hours at 37°C. For the hemolymph proteins, the antiserum dilution is 1:100. The concentration of secondary antibody is different to that used in Chapter II, which I used here as 1:1000 dilution for brain protein and 1:500 dilution for hemolymph protein.

Results

Mas-AT Related Protein in *Phormia regina*

Because the highest allatotropic activity in *Phormia regina* was found in the brains 8h after the liver meal, the brain extract from flies 8h after being liver-fed was used to do Western blot. I first used Mas-AT antiserum to probe 7.5 equivalent of brains protein, which was the substance recognized by Mas-AT antiserum with proximate M_r of 45 kDa (Figure 4.1.A). Interestingly, in the 7.5 flies equivalent of hemolymph protein, there were two bands observed on the membrane with M_r about 12 kDa and 20 kDa. These two proteins existed in the hemolymph at 6, 7, 8, 9 and 10h after the liver meal (Figure 4.1.B).

Mas-AT Related Protein in *Drosophila melanogaster*

It was demonstrated that the Mas-AT immuno-positive cells are present in the larval and adult central nervous system of *Drosophila malanogaster* (Zitnan *et al.*, 1993). As a comparison, I used Mas-AT antiserum to probe *Drosophila malanogaster*. Extracts were prepared from the whole body of flies with mixed ages of 2, 3, and 4 days after emergence. There were three proteins immuno-positive to the antiserum in an extract

equivalent to 50 flies. The M_r of these proteins were ca. 78, 55 and 40 kDa, respectively (Figure 4.2).

Discussion

In *Phormia regina*, the formation and release of vitellogenin by the fat body and the uptake of vitellogenin by the developing oocyte are regulated by JHs (Stoffolano *et al.*, 1992). Studies demonstrated that brains of female *Phormia regina* contain and release allatotropic factor(s) after a liver meal. An allatotropic effect was observed in the brain extracts prepared from 8h post liver meal donors (Yin *et al.*, 1993; Tu *et al.*, 2002), and the decerebration experiment indicated that the brain is involved in regulating oogenesis from 6h to 11h after a liver meal (Yin *et al.*, 1994). The chemistry of allatotropic factor(s) in dipterans remains unclear, although the moth allatotropin, Mas-AT, is an amidated tridecapeptide, which was isolated from heads of pharate adult *Manduca sexta* and showed an allatotropic activity (Kataoka *et al.*, 1989). In addition to the allatotropic function, myotropic and midgut ion transportation regulatory functions are later discovered for this peptide (Lee *et al.*, 1998; Veenstra *et al.*, 1994). Synthetic Mas-AT significantly stimulates *in vitro* JH biosynthesis by the corpus allatum of adult, sugar-fed *Phormia regina* to 2.64 fold that of controls (Tu *et al.*, 2001). Immunohistological studies show that, in *Phormia regina*, substances positive to Mas-AT antiserum were observed in some lateral neurosecretory cells and in neurons that are located in the region between the median neurosecretory cells and the oesophageal foramen (Tu *et al.*, 2001). Because the highest allatotropic activity was found in the brains of females 8h after the liver meal, the brain extract from these females was used to do Western-blotting. The goal was to find

out the approximate size of these substances, whether they are authentic Mas-AT, Mas-AT like, or just Mas-AT related. Now I know that they are Mas-AT related because they are not authentic Mas-AT and their M_r are too large to be considered Mas-AT like. I have also shown that there are two Mas-AT related substances exit in the hemolymph.

In *Manduca sexta*, Taylor *et al.*(1996) found that the Mas-AT gene is expressed as at least three mRNA isoforms that differ from each other by alternative splicing. Horodyski *et al.* (2001) demonstrated that the Mas-AT transcripts are alternatively spliced in a tissue-specific manner in the pharate adult. The pharate adult brain contains predominantly Mas-AT mRNA isoform I and a low level of mRNA II, and a very low level of mRNA III. But the nerve cord contains predominantly Mas-AT mRNA isoform II and a low level of isoform I. The frontal ganglion only contains the isoform I. Three propeptides are predicted translation products of these three Mas-AT mRNAs. The M_r of these three propeptides varies from 14,410 Da to 22,440 Da. Thus, it was not a surprise to find that there were three Mas-AT related proteins in *Drosophila melanogater*, and a brain peptide or protein that was immunoreactive to Mas-AT antiserum that had a M_r of about 42 kDa in *Phormia regina*. Interestingly, there were two different proteins with M_r of about 12k Da and 20k Da in the hemolymph of *Phormia regina*. The relationship between these brain and hemolymph proteins remains unclear.

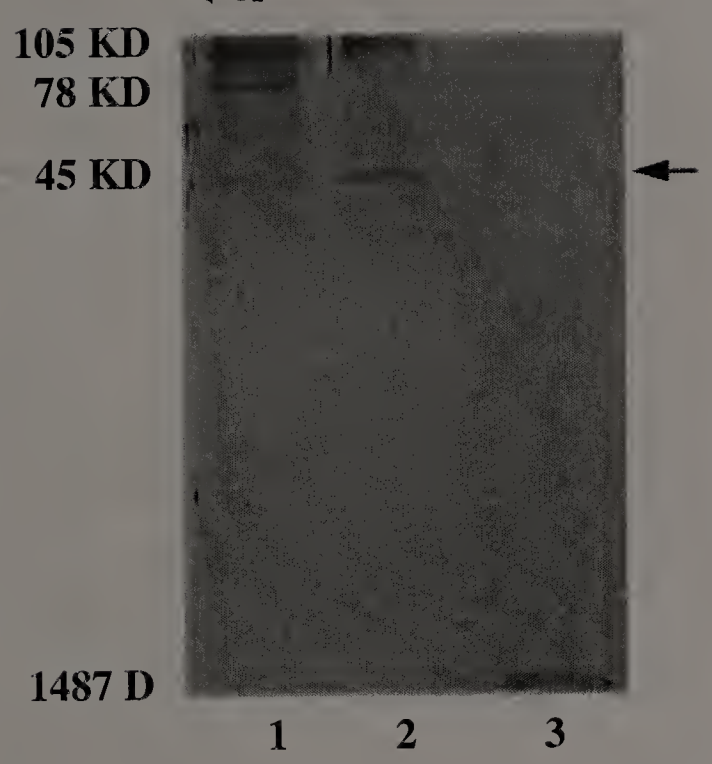
It is evident from this study and from the isolation of Mas-AT like peptides from *Locusta migratoria* (Paemen *et al.*, 1991), *Leptinotarsa decemlineata* (Spittaels *et al.*, 1996), and from *Spodoptera frugiperda* (Abdel-latief *et al.*, 2003) that the Mas-AT related proteins are present in a wide variety of insect orders. Some of the functions of

these proteins may be conserved among many insect species, or they may be specific to a particular order.

Because there are Mas-AT immuno-positive cells found in the central nervous system of *Drosophila melanogaster* (Zitnan *et al.*, 1993) and there are three substances identified in my Western blot analysis, I searched the *Drosophila* genome database (Adams *et al.*, 2000). My search failed to find any sequence to match the Mas-AT gene. It is possible that the genomic sequences of the Mas-AT and its related substances in *Drosophila* are missing in the current version of the published database.

Figure 4.1. Western blot analyses of the extracts of female *Phormia regina* brain prepared at 8h post liver-fed (**A**), and hemolymph at 6, 7, 8, 9, and 10h post liver-fed (**B**) when probed by an anti-Mas-AT antiserum; **A.** Lane 1: 3 µl of molecular mass marker; Lane 2: 7.5 equivalent of fly brains; and Lane 3: 20 ng of synthetic Mas-AT. **B.** Lane 1: 3 µl of molecular mass marker. Lanes 2-6: 7.5 equivalent of hemolymph proteins. The arrows point to the proteins that are immuno-positive to the Mas-AT antiserum.

A


M_r Standrds
Brain
Synthetic Mas-AT
105 KD
78 KD
45 KD
1487 D
1
2
3

B

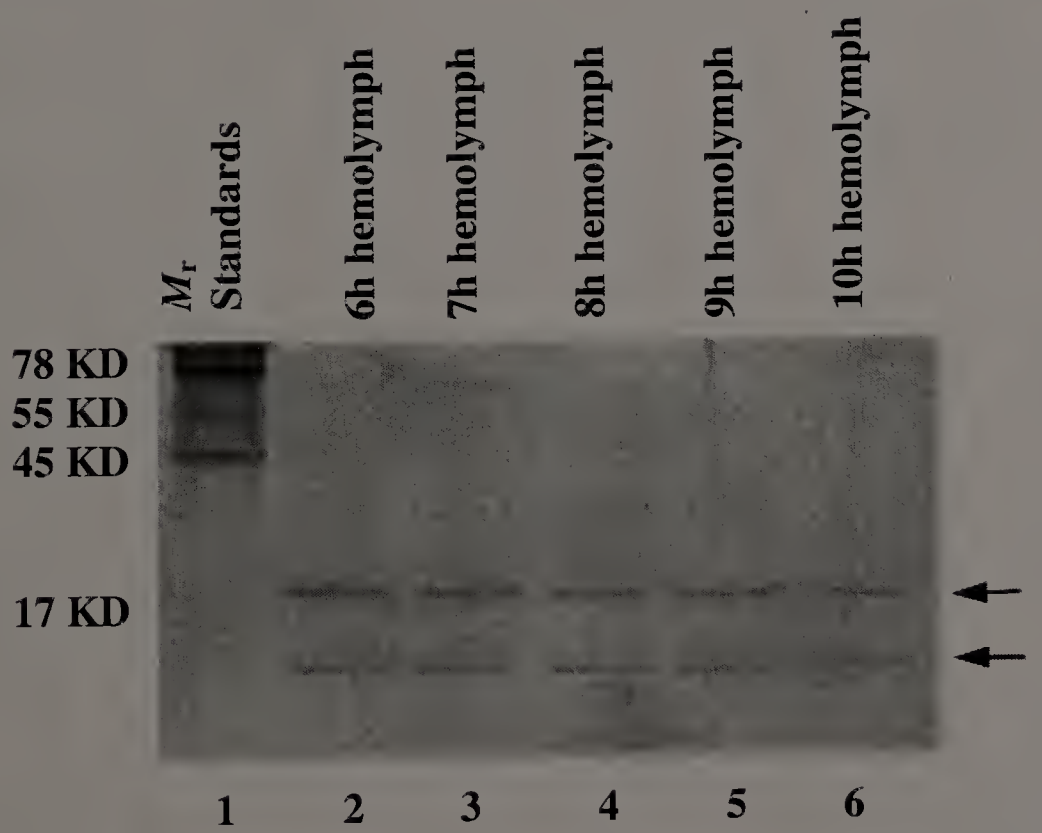

M_r Standards
6h hemolymph
7h hemolymph
8h hemolymph
9h hemolymph
10h hemolymph
78 KD
55 KD
45 KD
17 KD
1
2
3
4
5
6

Figure 4.2. Western blot analyses of the whole body extracts of *Drosophila malanogaster* prepared from 2, 3, 4 days old flies after emergency. Lane 1: 3 μl of M_r marker; Lane 2: 50 equivalents of whole flies. The arrows point to the proteins that are immuno-positive to the anti-Mas-AT antiserum.


M_r
Standard
Drosophila
Whole body
210 KD
78 KD
55 KD
45 KD
34 KD
1
2

CHAPTER V

GENERAL DISCUSSION

Midgut hormone (MH) of *Phormia regina* is the first insect gut hormone discovered with a gonadotropic effect (Yin *et al.*, 1994). Its structure remains unkown, and its down stream targets also remains unclear. My thesis attempted to help unravel some of these unkowns,

Previous data suggested that there were two midgut factors with different pIs at about 6.75 and 4.25 (MH-6.75, MH-4.25) (Yin *et al.*, unpublished). Neutralization by MH-6.75 antiserum experiments slowed oogenesis significantly. A delay of one developmental stage from 6.32 to 5.20 was detected. Since MH-6.75 does not represent the entire midgut hormone activity, significantly slowed, but not stopped, oocyte development was consistent with my expectation. Immunocytochemistry results showed that some immuno-positive substances to this MH antiserum in the anterior part of the midgut--a result that was expected from the bioassay data of midgut extracts (Yin *et al.*, 1994). At this moment, although I have found three midgut proteins reacting positively to the MH-6.75 antiserum, their chemical structures and functions remain to be determined. The presence of a single MH-6.75 related substance in *Drosophila melanogaster* suggested that the distribution of MH-6.75-related substances is inter-specific and offered a possibility of identifying the gene from FlyBase (1996) once obtaining a partial amino acid sequence of the immunoreactive *Drosophila* protein was obtained.

To study the downstream targets of the MH, I used the *Aedes aegypti* OEH I and *Drosophila* insulin receptor antisera to established the existence of OEH I and an insulin receptor in *Phormia regina*. My finding that some OEH I- and insulin receptor-related compounds in the neuroendocrine system of *Phormia regina* suggests that future work should focus on the ovarian ecdysteroidogenic effect of OEH I and *Drosophila* insulin receptor-like materials in *Phormia regina*.

Indeed, a bioassay confirming the ecdysteroidogenic function of OEH I in *Phormia regina* and a determination of its hemolymph-borne nature will be helpful in interpreting the present immunohistological results. In addition, the results of sugar-fed female and liver-fed male *Phormia regina* suggest that OEH I may have vital functions other than an ovary ecdysteroidogenic effect in these flies at different physiological states. The specific presence of *Drosophila* insulin receptor immuno-positive signals in the region between the CC and CA of female *Phormia regina* suggests that the insulin receptor may participate in the modulation of hormone action or the regulation of hormone release at the CC and CA level.

Previous studies showed that Mas-AT may serve as a brain hormone that controls the biosynthesis of JHs in *Phormia regina* (Tu *et al*., 2001). In my study, there was one Mas-AT related protein in the brain and two additional immuno-positive substances in the hemolymph of *Phormia regina*. The relationship between these brain and hemolymph proteins remains unclear. Regardless of their relationship, it is clear that the genetic and biochemistry of the production of these proteins are rather complicated. Further, the presence of different Mas-AT related proteins in *Drosophila melanogaster*

and *Phormia regina* suggest that additional efforts to unravel other Mas-AT related proteins in other insect species should be taken.

I fully realize that a research project focused on immunohistology and immunoblot studies could not produce a complete a story for the OEH I, insulin receptor, and Mas-AT in *Phormia regina.* To better understand the problem at hand, one should combine biology, chemistry, proteomics, molecular biology and genetics to push our knowledge to the next level. A possible next step is to purify these immuno-positive substances with immnunoaffinity chromatography (e.g., Dynabeads technology), which is to initiate a series of experiments that should lead to a fuller comprehension of how diet may control oogenesis in *Phormia regina* through its endocrine/neuroendocrine system. This system features at least the midgut hormone, allatotropin, insulin-like hormone, ovary ecdysteroidogenic hormone, juvenile hormones and ecdysteroids. Neural modulators/hormones such as biogenic amines, octopamine, amphetamine, serotonin, dromyosuppressin and FMRFamide may also play accessory roles.

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