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## Nutritional sensitivity of fifth instar prothoracic glands in the Tobacco hornworm, *manduca sexta*

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**NUTRITIONAL SENSITIVITY OF FIFTH INSTAR PROTHORACIC GLANDS  
IN THE TOBACCO HORNWORM, *MANDUCA SEXTA***

A dissertation presented

by

Amy L. Walsh

to

The Department of Biology

In partial fulfillment of requirements for the degree of

Doctor of Philosophy

in the field of

Biology

Northeastern University  
Boston, Massachusetts

November 2009

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**ABSTRACT OF DISSERTATION**

Submitted in partial fulfillment of the requirements  
for the degree of Doctor of Philosophy in Biology  
in the Graduate School of Arts and Sciences of  
Northeastern University, November, 2009

## ABSTRACT

The tobacco hornworm, *Manduca sexta*, has been a useful model for studying insect endocrinology. In *M. sexta* the majority of growth occurs during the fifth (final) larval stage. This growth is concurrent with endocrine changes that lead to a metamorphic molt. Past research has focused on the interplay of three growth hormones: juvenile hormone (JH), prothoracicotropic hormone (PTTH) and ecdysone, in regulating metamorphosis. The prothoracic glands, source of ecdysteroid molting hormones in insects, play a crucial role in coordinating growth. Insulin/insulin-like growth factor (IGF) signaling has been shown to regulate growth and secretion of steroidogenic tissues in both vertebrates and invertebrates, but it had yet to be well-characterized in *M. sexta*.

In this dissertation I examined the role of insulin-directed growth in prothoracic glands during both normal larval development and in response to nutrient deprivation. In particular, I predicted that in response to a reduction in nutrients, the prothoracic glands would stop growing and ecdysone secretion would be reduced. Changes were assessed in actin abundance and in two nutritionally sensitive transcripts, insulin receptor and 4EBP. Actin abundance in prothoracic glands increased in the first four days of this stage, demonstrating significant growth. Actin decreased in glands from starved larvae, indicating a cessation of growth. Both insulin receptor and 4EBP transcript were present during normal larval development. As expected, both insulin receptor and 4EBP transcript increased in response to starvation. This research was the first demonstration of insulin signaling in prothoracic glands of fifth instar *M. sexta* larvae, and revealed nutritional sensitivity of the prothoracic glands.

Finally, ecdysone assays were conducted to determine the impact of nutrition on glandular secretory capacity. Starved larvae were unable to secrete as much ecdysone as fed controls. Injections of insulin were unable to rescue the ability of the glands to secrete high levels of ecdysone. This demonstrated that although insulin signaling was correlated with normal growth of the glands, insulin is not sufficient to impact the ability of the glands to secrete ecdysone in the absence of nutrients.

*This thesis is dedicated to my parents,  
Robert and Angela Walsh,  
whose love and support  
made this dissertation possible.*

## TABLE OF CONTENTS

Abstract	4
Dedication	6
List of Figures	8
Abbreviations	10
Acknowledgements	11
Introduction	14
Chapter 1: Characterization of insulin signaling in prothoracic glands of feeding fifth instar larvae.	30
Chapter 2: Nutritional sensitivity of prothoracic glands during the fifth instar.	55
Chapter 3: Starvation-elicited changes in ecdysone secretion by prothoracic glands of fifth instar larvae.	81
References	106

## LIST OF FIGURES

**Figure 1.** Schematic of hormone levels in the fourth and fifth instars of *Manduca sexta*.

**Figure 2.** Model of insulin signaling and growth.

**Figure 3.** Abundance of actin protein in prothoracic glands in *Manduca sexta* during the fifth larval instar.

**Figure 4.** Abundance of insulin receptor (IR) transcript in *Manduca* prothoracic glands during the feeding stage of the fifth larval instar.

**Figure 5.** The nucleotide and deduced amino acid sequence of *Manduca* initiation factor 4E binding protein (4EBP) homolog (GenBank accession no. EU622640).

**Figure 6.** Abundance of initiation factor 4E binding protein (4EBP) transcript in *Manduca* prothoracic glands during fifth instar.

**Figure 7.** Abundance of non-phospho4EBP and p4EBP protein in prothoracic glands of *Manduca sexta* during the fifth instar.

**Figure 8.** Activation of phosphoproteins by insulin in prothoracic glands during the fifth instar in *Manduca sexta*.

**Figure 9.** Effect of starvation on actin protein abundance in prothoracic glands of larvae starved prior to minimal viable weight (2.5g).

**Figure 10.** Abundance of insulin receptor transcript in prothoracic glands of *Manduca sexta* starved prior to minimal viable weight (2.5g).

**Figure 11.** Abundance of initiation factor 4E binding protein (4EBP) transcript in prothoracic glands of *Manduca sexta* starved prior to minimal viable weight (2.5g).

**Figure 12.** Effect of starvation on 4EBP protein abundance in prothoracic glands of *Manduca sexta* starved prior to minimal viable weight (2.5g).

**Figure 13.** Effect of 30 hour starvation on actin protein abundance in prothoracic glands of *Manduca sexta* larvae starved upon entry into the fifth instar.

**Figure 14.** Abundance of insulin receptor transcript in prothoracic glands of *Manduca sexta* larvae starved for 30 hours upon entry into the fifth instar.

**Figure 15.** Abundance of initiation factor 4E binding protein (4EBP) transcript in prothoracic glands of *Manduca sexta* larvae starved for 30 hours upon entry into the fifth instar.

**Figure 16.** Effect of a 30 hour starvation on initiation factor 4E binding protein (4EBP) protein abundance in prothoracic glands of *Manduca sexta* larvae starved upon entry into the fifth instar.

**Figure 17.** Effects of starvation on *in vitro* ecdysone secretion in *Manduca sexta* larvae.

**Figure 18.** Effects of insulin injection on ecdysone secretion in *Manduca sexta* larvae starved for 48 hours.

**Figure 19.** Effects of insulin injection on 4EBP in starved *Manduca sexta* larvae.

**Figure 20.** Effects of insulin injection on ecdysone secretion in wandering *Manduca sexta* larvae.

**Figure 21.** Effects of 8 hour insulin incubation on ecdysone secretion from prothoracic glands of wandering *Manduca sexta* larvae.

**Figure 22.** Proposed model for insulin involvement in initiation of the metamorphic molt.

## ABBREVIATIONS

cDNA	complementary deoxyribonucleic acid
CA	corpora allata
DNA	deoxyribonucleic acid
EH	eclosion hormone
ERK	extracellular signal-regulated kinase
FOXO	forkhead box-containing protein
IGF	insulin-like growth factor
JH	juvenile hormone
JHE	juvenile hormone esterase
mRNA	messenger ribonucleic acid
PCR	polymerase chain reaction
PI3K	phosphatidylinositol-3-kinase
PKB	protein kinase B
PTEN	phosphatidylinositol-3,4,5-triphosphate 3-phosphatase
PTTH	prothoracicotropic hormone
RNA	ribonucleic acid
TSC	tuberous sclerosis complex
TOR	target of rapamycin
4EBP	initiation factor 4E binding protein
V	fifth instar

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## INTRODUCTION

The tobacco hornworm, *Manduca sexta*, is a popular organism for studying endocrine regulation of growth and metamorphosis in holometabolous insects (Nijhout, 1994). These large insects develop synchronously in the lab and grow well on an artificial diet, making them a good model for investigating questions pertaining to development.

Growth and metamorphosis in insects is regulated by hormones such as ecdysone, juvenile hormone (JH), and prothoracicotropic hormone (PTTH), that have been the subject of intensive study for many years. More recently other hormones, such as insulin, have received attention as regulators of metamorphosis (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005). It is unclear how, specifically, insulin-like hormones interact with classical regulators of insect metamorphosis such as ecdysone. One key to understanding the interaction between insulin driven processes, such as growth, and ecdysone driven processes, such as differentiation, is to be able to understand the regulation of the source of ecdysone, the prothoracic glands.

Of particular interest is the nutritional regulation of prothoracic gland growth. While the endocrine events that lead to metamorphosis are progressing during the final instar, the larva is continuing to feed and grow. Since size increases exponentially during the last larval stage, factors that alter the time of secretion of the first ecdysone peak (which triggers cessation of feeding) can have a large impact on the final size of the larva. Adult insects do not grow, so the size attained by a larva at the time of ecdysone secretion determines the size of the adult insect. Therefore, prothoracic gland growth and

ability to secrete ecdysone are keys to understanding the determination of overall body size. Ultimately, the glands should be able to translate environmental cues like changes in nutritional state into intrinsic cues like the secretion of ecdysone. This dissertation is directed toward understanding the means by which this occurs, with particular emphasis on the role played by insulin signaling.

## **The Classical Hormonal Control of Metamorphosis**

### *Hormonal regulation of molting and metamorphosis*

In holometabolous insects there are three stages of post-embryonic development: the larval stage, pupal stage and adult stage. The number of larval molts varies among species. The period of time between successive molts is called an instar. In the larval stage, insects are reproductively immature and grow exponentially.

Metamorphosis is the transition from larval life through the pupal stage to produce an adult insect. This includes both molting and a switch from larval gene expression to pupal gene expression and subsequent development. The key driver of metamorphosis is ecdysone, the steroid hormone responsible for orchestrating the molting process.

In insects, a pulse of ecdysone is responsible for the molt into the next stage, whether the next stage is another larval stage, the pupal stage or the adult stage. Ecdysone is primarily synthesized and released from the prothoracic glands. The form of ecdysone secreted can vary among species. For example in some insects, a prohormone, ecdysone is released from the prothoracic glands, while in *M. sexta*, a pre-prohormone is

released (3-dehydroecdysone) from the prothoracic glands. 3-dehydroecdysone is readily converted in the hemolymph to the prohormone ecdysone, and is finally converted to the active form, 20-hydroxyecdysone (20-HE), in target tissues (Nijhout, 1994).

A second hormone, juvenile hormone (JH) is responsible for the nature of a given molt (Figure 1). JH is a terpenoid that is synthesized and released by the corpora allata which are located near the insect brain. JH is a “status quo” hormone which maintains preexisting types of gene expression and hence produces larval-larval molts (Williams, 1953). Absolute levels of JH alone do not dictate the effect of JH on the larva; the presence or absence of JH during tissue-specific windows of sensitivity determines its impact. During larval-larval molts, JH levels are high at the onset of ecdysone secretion that stimulates the molt.

A third hormone, prothoracicotropic hormone (PTTH) is responsible for timing the release of ecdysone. PTTH is synthesized in the brain and released from the corpora allata. PTTH stimulates the synthesis and secretion of ecdysone (Bollenbacher et al., 1979; Bollenbacher et al., 1981). The release of PTTH is dependent upon the time of day (Truman, 1972; Truman and Riddiford, 1974). For example, in the fourth larval instar, PTTH secretion is allowed during a 10 hour window that occurs from about 1.5 hours before lights off to about 8.5 hours after lights off, under a 12L:12D photoperiod (Truman, 1972). Every molting cycle, whether it is a larval, pupal or an adult molt, begins with the secretion of PTTH.

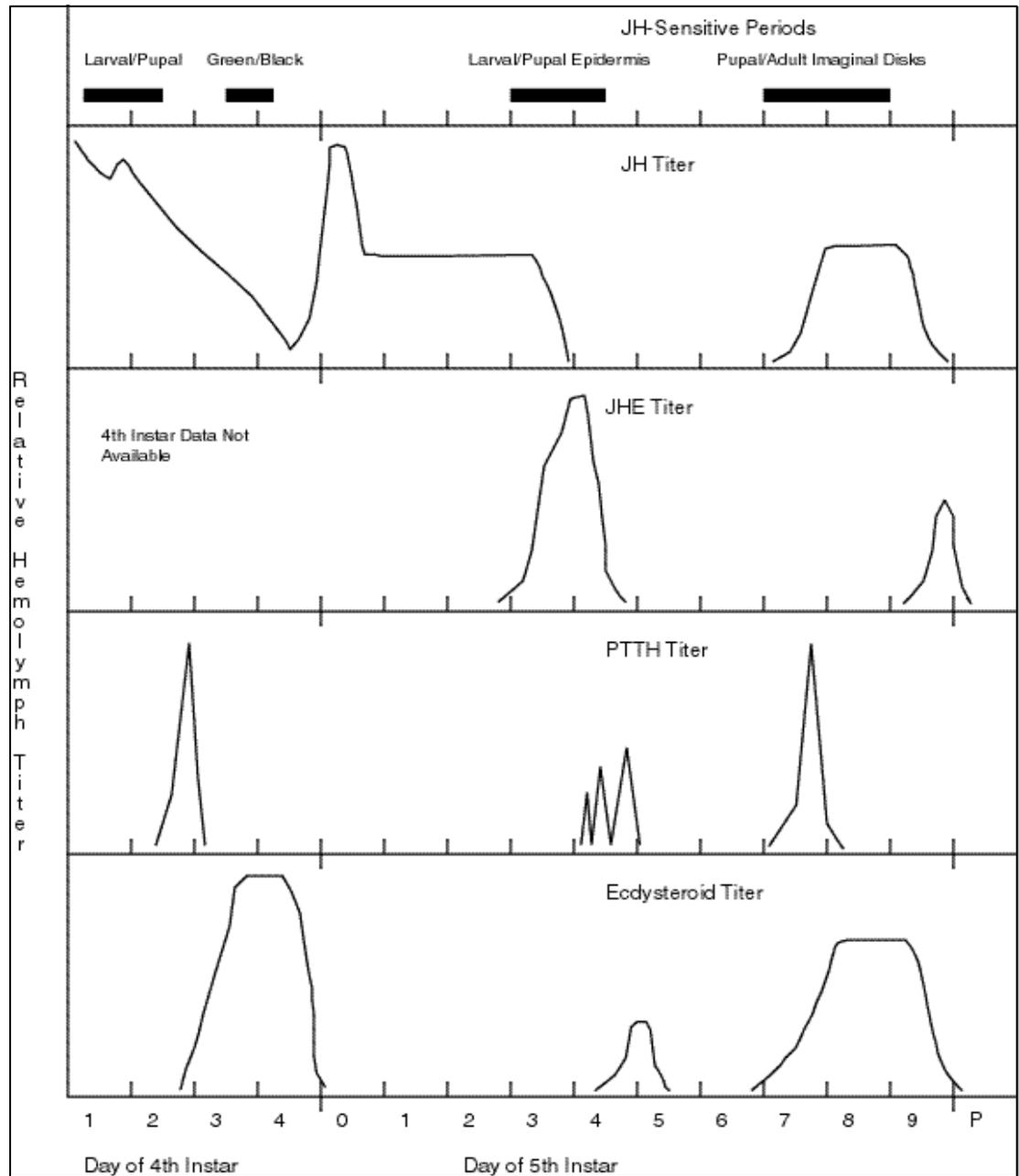
A well-characterized interplay of these three hormones in the last (fifth) larval stage of *M. sexta* leads to the metamorphic molt (Figure 1). Upon ecdysis into the fifth instar there is a high level of JH that inhibits prothoracic glands and prevents the release

of PTTH (Nijhout and Williams, 1974b; Bollenbacher et al., 1981). By the second day of the fifth larval stage there is a decline in JH (Wolfgang and Riddiford, 1986; Baker et al., 1987). The decline in JH is caused by both a lack of production of JH by the corpora allata and an increase in the enzyme responsible for catabolizing the hormone, JH esterase (JHE) (Vince and Gilbert, 1977). The decrease in JH titer elicits changes that are characteristic of a larval-pupal molt, including expression of the Broad transcription factor that specifies pupal cuticle whenever it is expressed (Zhou and Riddiford, 2002). Also, the reduction in JH is permissive of the release of PTTH at the next photogate. Once secreted, PTTH stimulates the prothoracic glands to secrete the first peak of ecdysone (Bollenbacher et al., 1981). The first peak of ecdysone occurs on the fourth day of the fifth instar. This is called the commitment peak and causes the larva to cease feeding (Nijhout and Williams, 1974b; Rountree and Bollenbacher, 1986). The commitment peak also initiates prepupal behavior that includes a massive gut purge, exposure of the dorsal vein and wandering behavior that allows the larva to find a suitable substrate in which to pupate (Nijhout and Williams, 1974b; Rountree and Bollenbacher, 1986). At this time there is an increase in the transcription of pupal transcription factors (Riddiford and Kiely, 1981; Riddiford, 1982, 1985).

Later in the instar there is a second pulse of PTTH that precedes a second ecdysone peak. The second pulse of ecdysone occurs on the seventh day of the fifth instar. This is called the prepupal peak and is responsible for the actual molt, including the separation of epidermal cells from the old cuticle (apolysis) and the subsequent growth of a new cuticle. At this time JH levels are high again to prevent premature expression of adult characters (Nijhout, 1994). This is also when pupal transcription

factors are activated and pupal gene products begin to be expressed (Kiely and Riddiford, 1985; Riddiford, 1985).

About 48 hours after pupal ecdysis PTTH is secreted again (Nijhout, 1994). This is followed by a long period of ecdysone secretion that is necessary for the complex processes of adult development and occurs in the absence of JH. The culmination of metamorphosis occurs when the pupa emerges as an adult. The process by which the adult emerges is called eclosion. Similar to larval ecdysis, eclosion behavior is controlled by time of day and driven by additional endocrine factors such as eclosion hormone (EH) (Truman, 1971; Kim et al., 2004).



**Figure 1.** Schematic of hormone levels in the fourth and fifth instars of *Manduca sexta*. The JH-sensitive periods are indicated by black bars above the JH titer. Abbreviations: JH= juvenile hormone, JHE= juvenile hormone esterase, PTTH= prothoracicotropic hormone. Redrawn from Nijhout (1994). JHE activity redrawn from Vince and Gilbert (1977). For further explanation, see text.

### *Triggering metamorphosis*

Environmental and physiological cues determine when metamorphic hormones are released (Nijhout, 1999). These cues allow for metamorphosis to occur in a timely and developmentally appropriate manner. In order to begin metamorphosis, the larva is faced with two decisions. It must decide which instar is the final instar, and when in the final instar to initiate a metamorphic molt. These decisions, which ultimately lead to metamorphosis, are based on three checkpoints. The first checkpoint is called threshold size. Threshold size determines that the next molt will be the final larval molt. In *M. sexta* this occurs during the fourth instar, and is seen experimentally by measuring the width of the head capsule (Nijhout, 1975). A larva must have a head capsule width greater than 5.1 mm to ensure the next molt will be the final instar (Nijhout, 1975). If the head capsule is smaller than 5.1 mm, then the larva will molt into an additional (supernumerary) instar (Nijhout, 1975). This is an absolute size which is not dependent on the developmental history of the larva, therefore the larva continues to grow and molt until it reaches a certain threshold size (Nijhout, 1975). The mechanism by which size is assessed is not known.

A second checkpoint is called minimal viable weight. The minimal viable weight is the minimum weight at which larvae can successfully complete metamorphosis. For *M. sexta* the minimal viable weight is about 3g and is usually attained on the first day of the fifth instar (Nijhout, 1975). Starvation prior to 3g results in a prolonged larval stage and many of the larvae molt into non-viable larval-pupal intermediates (Nijhout, 1975).

The final and most important checkpoint is called critical weight (Nijhout and Williams, 1974a, 1974b). Critical weight is the weight at which no further growth is necessary for a normal time course to metamorphosis. Larvae starved between minimal viable weight and critical weight show a delay in molting, while larvae starved at or above critical weight metamorphose at the same time as larvae that continue to feed normally. Critical weight marks significant endocrine and behavioral changes that ensure the larva will pupate at the next molt (Nijhout and Williams, 1974a). For example, critical weight is a key factor in stimulating PTTH release through a reduction in JH secretion (Nijhout and Williams, 1974b; Nijhout, 1981). In some insects critical weight is assessed through the physical size of the larva. For example in Hemiptera, *Oncopeltus fasciatus* and *Rhodnius prolixus*, abdominal stretch after a meal stimulates stretch receptors that trigger ecdysone secretion most likely through effects on PTTH release (Nijhout, 1979, 1981; Chiang and Davey, 1988). This is not the case for all insects. In *M. sexta*, critical weight occurs when the larvae achieve a weight of 6g, yet stretch alone does not stimulate a molt (Nijhout and Williams, 1974a; D'Amico et al., 2001). While it is known that acquisition of critical weight is correlated with the release of ecdysone, the mechanism of how the larva assesses critical weight remains unknown (Nijhout and Williams, 1974a; D'Amico et al., 2001).

## Regulation of growth and metamorphosis due to the insulin pathway

### *Insulin-like proteins in insects*

In addition to the triumvirate of metamorphosis-regulating hormones described above —PTTH, ecdysone, and JH— insulin has been found to play an important role. The insulin-like hormone in lepidopterans is called bombyxin. Bombyxin is a neurosecretory hormone that like insulin is a dimer with A and B chains connected by disulfide bonds. A substantial body of work in the silkworm, *Bombyx mori*, has identified a minimum of 38 bombyxin genes (Kondo et al., 1996). This protein was first identified in *B. mori* and has been found ubiquitously in Lepidoptera (Nagasawa et al., 1986). For example, homologs to bombyxin have been identified in several lepidopteran species including *Samia cynthia*, *Agrius singulatus*, and *Precis coenia*. It is thought to be the ligand for the lepidopteran insulin receptor (Wu and Brown, 2006).

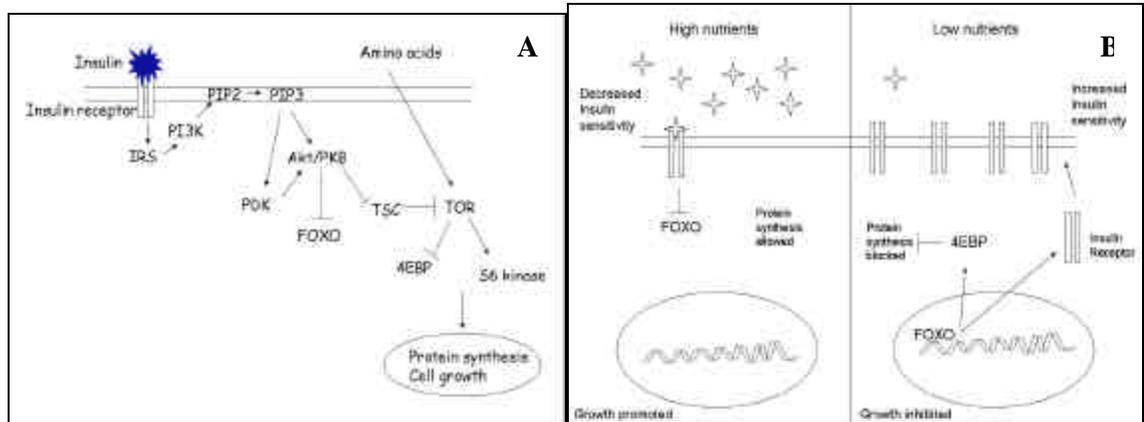
Insulin-like proteins are found in other insects as well. Seven insulin-like proteins have been identified in *D. melanogaster*, the *Drosophila* insulin-like proteins (Dilps) (Brogiolo et al., 2001). Dilps 1-5 are more conserved relative to vertebrate insulin-like proteins than Dilp-6 and Dilp-7, but all are structurally similar to the insulin-like superfamily of proteins. Individual Dilps are found in different tissues and not all appear to be neurosecretory (Brogiolo et al., 2001). In the African malaria mosquito, *Anopheles gambiae*, there are five putative insulin-like peptides (Riehle et al., 2002) and in the yellow fever mosquito, *Aedes aegypti*, there are eight putative insulin-like peptides at least three of which are produced in the brain (Riehle et al., 2006).

### *Insulin signaling pathway in insects*

The cellular pathway by which insulin-like hormones act in insects has been characterized in detail for *D. melanogaster*, and is similar to how insulin functions in vertebrates (Garofalo, 2002). The signaling proteins in this pathway affect cellular metabolism, growth and survival. The insulin receptor is a heterotetrameric protein that has two extracellular  $\alpha$  subunits (120 kD) and two transmembrane  $\beta$  subunits (90 kD) (Luo et al., 1999). Insects only have a single insulin-like receptor that is responsible for modulating both growth and nutritional cues. Binding of the hormone to its receptor creates a binding domain for downstream signaling molecules (Hubbard et al., 1994) (Figure 2). An example of one signaling molecule is insulin receptor substrate (IRS), which binds to the receptor and is then responsible for the regulation of many growth related pathways (White and Yenush, 1998). In vertebrates, one IRS-mediated changes pathway involves activation of MAP kinase and stimulates cell division. It has not yet been deduced whether this process is stimulated by insulin in insects. A second pathway involves the activation of phosphatidylinositol 3-OH kinase (PI3K). PI3K-mediated changes will be the focus of the present study as these changes are well described in some insect models.

PI3K leads to the phosphorylation of the phosphatidylinositol-dependent kinase (PDK), and protein kinase B (PKB/Akt) (Avruch, 1998). PKB/Akt can then phosphorylate a number of other downstream proteins such as transcription factors including forkhead box-containing protein (FOXO), and tuberous sclerosis complex (TSC), which in their unphosphorylated form inhibit growth or promote cell death (Rhodes and White, 2002). For example, in its unphosphorylated state, TSC also inhibits

a growth regulating kinase, target of rapamycin (TOR) (Hay and Sonenberg, 2004). TOR is a nutrition sensor that is also activated indirectly by the presence of amino acids, and can regulate translation by phosphorylating both initiation factor 4E binding protein (4EBP) and a kinase that activates ribosomal protein S6 (p70S6K).



**Figure 2.** *Model of insulin signaling and growth.* **A.** A simplified model of insulin/IGF signaling and pathway interactions. See text for details. **B.** Model of effects of insulin on growth due to nutrient deprivation. In larvae fed *ad libitum*, insulin binds to the insulin receptor resulting in the phosphorylation of FOXO. This allows protein synthesis to occur, thus promoting cellular growth. When larvae are starved, FOXO is able to enter the nucleus and serve as a transcription factor for both insulin receptor and 4EBP. An increase of insulin receptor protein results in an increase of insulin sensitivity. 4EBP prevents the translation of proteins and inhibits growth.

4EBP is a negative regulator of growth that works by inhibiting the translation of proteins. Translation initiation of most eukaryotic mRNAs requires the binding of eIF4F, a protein complex made up of eIF4A, eIF4G and eIF4E to the 5' cap structure (Gingras et al., 1999). eIF4E is highly regulated by repressor proteins called eIF4E-binding proteins (4EBPs), which compete with eIF4G for the same binding site on eIF4E (Haghighat et al., 1995; Mader et al., 1995). In flies, 4EBP can be deactivated in

response to insulin signaling through direct phosphorylation by Akt, in addition to phosphorylation by TOR (Miron et al., 2001; Miron et al., 2003).

### *Insulin signaling and growth in insects*

Insulin signaling has been shown to have an impact on growth, aging and metabolism in both vertebrates and invertebrates. In insects much of the work has been done in *D. melanogaster*, while less is known about the impact of insulin signaling on growth in *M. sexta*. In *D. melanogaster*, genetic manipulations that cause alterations in the insulin receptor and other insulin pathway elements have been shown to have a profound effect on development. Several studies have directly implicated the insulin signaling pathway as a regulator of growth and body size. For example, mutations in the insulin receptor homolog in flies result in embryonic or larval lethal phenotypes (Chen et al., 1996). If PI3K is mutated so that it cannot interact with IRS proteins (known as Chico in *D. melanogaster*), growth is reduced (Oldham et al., 2002). In contrast, when the genes that encode *Drosophila* insulin-like peptides (Dilps) are overexpressed, the flies show increased body size (Brogiolo et al., 2001). Furthermore, the overexpression of dFOXO, which is normally repressed through phosphorylation by PKB/Akt, decreases organ size and increases relative amounts of transcript of both *Drosophila* insulin receptor and 4EBP (Puig et al., 2003).

There appears to be an inverse relationship between insulin-directed tissue growth and overall body size in the prothoracic glands of *D. melanogaster*. Genetic alterations to enhance PI3K signaling in prothoracic glands of *D. melanogaster* cause the glands to grow to a size that is larger than normal (Caldwell et al., 2005; Colombani et

al., 2005; Mirth et al., 2005). Paradoxically, the larvae with these larger glands produce adult flies that are smaller in size (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005). The cause of the smaller adult size seems to be due to the larger glands secreting ecdysone earlier than they normally would (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005). Conversely, when PI3K signaling is genetically modified to be down-regulated in the prothoracic glands, the adult flies are larger in size (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005). This is likely due to the prothoracic glands secreting ecdysone later than they normally would, so the larvae are able to grow for a longer period of time. Taken together, these results imply that gland size is in some way an assessment of critical size. It makes sense that a tissue that is responsible for secreting ecdysone would be instrumental in regulating body size.

It is likely that insulin-directed growth in prothoracic glands occurs in response to nutrient status. Secretion of insulin-like hormones in insects, as in vertebrates, is nutritionally regulated. For example, if *B. mori* are starved, bombyxin levels decrease, while injection of glucose stimulates bombyxin secretion (Satake et al., 1997; Masumura et al., 1999). When *M. sexta* are starved, their hemolymph carbohydrate levels decrease significantly in a short amount of time (Dahlman, 1973). These fluctuations in nutritional state also cause changes in the growth of target tissues. In *Precis coenia*, the wing discs stop growing soon after the larva is removed from food (Miner et al., 2000). The growth of these starved discs is rescued when the discs are placed in media containing bombyxin and ecdysone (Nijhout and Grunert, 2002; Nijhout et al., 2007). Insulin secretion is also nutritionally regulated in Diptera. For example, mRNAs of insulin-like peptides increase in response to a diet of sucrose and amino acids in *Anopheles gambiae* (Arsic and Guerin,

2008). Additionally, in Diptera, transcript levels of some insulin signaling pathway members have been shown to change in response to alterations in nutritional state. For example when flies are starved there is an increase in the transcript of both insulin receptor and 4EBP (Puig and Tjian, 2006). Therefore insulin signaling is able to translate the environmental cue of nutritional state into appropriate alterations of growth in target tissues.

The role of insulin signaling in coordinating growth and nutrition makes it a likely candidate to assess critical weight in *Manduca sexta*. Research in *M. sexta* has provided much of our understanding of the endocrine control of molting and metamorphosis due to the ability to rear larvae on artificial diet, their large size and developmental synchrony. However, little is known about the involvement of insulin signaling in *M. sexta* growth and metamorphosis. The main focus of my work is to explore the nutritional sensitivity of the prothoracic glands. I investigate the effect of starvation on growth in the glands, as proposed by Figure 2B, and the impact of nutrient deprivation on ecdysone secretion. This will provide clues regarding the potential ability of the prothoracic glands to assess critical weight. Understanding the nutritional sensitivity of prothoracic glands in *M. sexta* gives insight into molecular mechanisms underlying decisions to undergo metamorphosis, in an organism in which the endocrinology underlying metamorphosis is well-described. Unlike previous work with *D. melanogaster*, study of prothoracic gland sensitivity to nutrition in *M. sexta* can be accompanied by direct measurement of ecdysone titers.

In Chapter 1, I describe insulin signaling during the feeding portion of the fifth larval stage in prothoracic glands of *M. sexta*. The majority of prothoracic gland growth

occurs in the first portion of the fifth instar (Sedlak et al., 1983; Smith and Pasquarello, 1989; Hanton et al., 1993; Lee et al., 1995). In order to ultimately evaluate changes in insulin signaling due to starvation, it was first important to characterize insulin signaling during normal glandular growth. To accomplish this, two known nutrient-sensitive transcripts, insulin receptor transcript, and 4EBP transcript, were quantified. Also it was confirmed that insulin signaling pathway members were present at this time. It was hypothesized that concomitant with periods of growth, insulin receptor transcript would be present in relatively high amounts. Conversely, because of its role as a negative regulator of growth, 4EBP transcript levels were expected to be relatively low. I found that insulin receptor and insulin signaling pathway members including 4EBP were present, and changed in a manner in keeping with postulated involvement in growth.

Next, I explored the effect of starvation on insulin receptor transcript and 4EBP transcript to determine the nutritional sensitivity of the prothoracic glands at varying times during development (Chapter 2). It was hypothesized that insulin receptor and 4EBP transcript would increase upon starvation. Furthermore it was thought that the glands would be most sensitive to changes in nutritional state prior to the attainment of minimal viable weight. I found that in response to starvation, prothoracic glands stopped growing. Insulin receptor and 4EBP transcripts increased in response to nutrient deprivation. This implied that insulin signaling is nutritionally responsive in the prothoracic glands. However in contrast to expectations, larvae starved prior to minimal viable weight were not as sensitive to changes in nutritional status as larvae starved upon entry into the fifth instar.

Finally, the effect of starvation on the ability of the prothoracic glands to secrete ecdysone was assessed (Chapter 3). Also the ability of insulin to impact ecdysone secretion was determined. It was hypothesized that the ability of the glands to secrete ecdysone would be reduced upon starvation, delaying metamorphosis. Further, it was hypothesized that this decreased secretory capacity would be rescued by the injection of insulin into starved larvae. I found that there was indeed a decrease in ecdysone secretion due to starvation. However, this decrease was not rescued by insulin-injection. I also assessed the effect of insulin on the ability of the prothoracic glands to secrete ecdysone later in the instar. The effects of insulin on ecdysone secretion were tested both *in vivo* and *in vitro* in larvae from the fifth day of the last larval stage. In both cases, I found that there was no effect of insulin on ecdysone secretion in post wandering larvae. Taken together this data indicated that secretory capacity of the prothoracic glands is impacted by nutritional deprivation. Additionally, it appeared that insulin does not directly stimulate ecdysone secretion in the absence of nutrients.

## **CHAPTER ONE**

Characterization of insulin signaling in prothoracic glands of feeding fifth instar larvae

## INTRODUCTION

A fundamental question that remains unanswered is whether or not the secretory activity of prothoracic glands is nutritionally responsive, and if so, how. An ideal model in which to address these questions is *Manduca sexta* due to their large size and developmental synchrony. The first step is to demonstrate that fifth instar prothoracic glands of *M. sexta* are growing and that this growth coincides with factors that regulate both growth and nutrition, such as the insulin signaling pathway.

Insulin-directed growth is seen in both vertebrates and invertebrates. In vertebrates, insulin and insulin-like growth factors (IGFs) have been extensively studied and shown to impact growth in a variety of tissues. For example, brain, muscle, bone, cartilage, pancreas, ovary, skin and fat tissue have all been identified as targets for IGF-directed growth (Dupont and Holzenberger, 2003). Additionally, IGFs are involved in both embryonic and postnatal growth and have been shown to fluctuate throughout development (Dupont and Holzenberger, 2003). IGFs have even been implicated in the determination of body size as demonstrated through mutations in mice that disrupt IGF-2 resulting in significantly smaller mice (DeChiara et al., 1990). IGFs function through IGF receptors, insulin receptor and hybrid receptors. These receptors have a profound impact on growth and development in diverse tissue types. For example, a constitutive knock-out of IGF-1 receptor in mice results in brain growth retardation (Liu et al., 1993). Additionally, muscle-specific inactivation of IGF-1 receptor results in hypoplasia in skeletal muscle of mice early in development (Fernandez et al., 2002).

A great deal of research has been done on insulin-directed growth in *Drosophila melanogaster*. The insulin receptor has been shown to have an integral role in growth as well as in the assessment of nutritional state. It has been shown that the insulin receptor is a positive regulator of growth (Chen et al., 1996; Brogiolo et al., 2001). Insulin receptor transcription has also been shown to be nutritionally regulated. For example, in times of nutrient deprivation insulin receptor mRNA levels have been shown to increase dramatically along with 4EBP which is regulated by both TOR and Akt (Puig and Tjian, 2006). 4EBP itself has been shown to have an important role in growth regulation. Specifically, over-expression of 4EBP in flies can inhibit growth (Miron et al., 2001). Interestingly, the size of the larvae is not affected in mutants lacking 4EBP activity, but this mutation does make them more sensitive to starvation (Teleman et al., 2005; Tettweiler et al., 2005).

Growth of the prothoracic glands in *M. sexta* has been correlated with ecdysone secretion during the last larval stage (Sedlak et al., 1983; Smith and Pasquarello, 1989; Lee et al., 1995). Previous research has revealed that the growth of the prothoracic glands in *M. sexta* appears to be due in large part to a change in cell size, while it is unclear if there is an increase in cell number. The protein content of the glands increases significantly by the second day and reaches its peak by the third day of the fifth instar (Sedlak et al., 1983; Smith and Pasquarello, 1989; Hanton et al., 1993; Lee et al., 1995). The increase in protein causes an increase in both volume and diameter of the cells (Hanton et al., 1993). The change in protein content is due in part to a change in the types of proteins synthesized by the glands (Lee et al., 1995). Concurrent with the increase in protein is an increase in DNA synthesis that occurs on the third day of the

instar, but it remains unclear if this increase coincides with an increase in cell number (Lee et al., 1995). Molecular mechanisms that underlie the increase in size of prothoracic glands are poorly understood. It is likely that insulin signaling plays some role in the regulation of growth.

In order to assess whether insulin signaling occurs during prothoracic gland growth, the first step was to confirm that insulin receptor and insulin signaling pathway members were present during normal larval growth. This was accomplished by looking at the transcripts for insulin receptor and 4EBP. Due to its role as a positive regulator of growth, it was predicted that insulin receptor transcript would be highest early in the fifth instar when growth is most rapid. As a negative regulator, it was hypothesized that 4EBP transcript would be relatively low throughout the instar, particularly during times of rapid growth. As transcript levels do not always accurately reflect the quantity of translated protein, protein levels of 4EBP were also assayed, as was 4EBP phosphorylation. When 4EBP is phosphorylated it can no longer prevent the translation of proteins. Therefore, if the 4EBP protein present, it was expected that most would be phosphorylated when the glands were growing. Finally, it was anticipated that insulin signaling pathway proteins such as Akt and FOXO would be present in growing glands. Hence, the ability of insulin to activate (enhance the phosphorylation of) insulin signaling pathway proteins was verified *in vitro* in order to assess the functional state of the pathway.

## MATERIALS AND METHODS

### *Animals*

*Manduca sexta* eggs were obtained from Carolina Biological Supply (Burlington, NC). Larvae were reared on artificial diet (Bell and Joachim, 1976) at 25°C on a 16:8 light:dark cycle. The larvae were placed in individual cups from the third larval stage. Feeding fifth (final) instar larvae were placed on fresh diet at the time of ecdysis (day 0). Prothoracic glands for quantitative real time PCR were removed under lepidopteran saline (Riddiford et al., 1979), and rinsed quickly in Grace's medium before being flash-frozen and stored at -80° C. Glands used for Western blots were removed under lepidopteran saline, rinsed quickly in Grace's medium, placed in 2x SDS sample buffer and incubated for 3 minutes at 90°C.

### *Identification of Manduca 4EBP gene*

For the cloning of *Manduca* initiation factor 4E binding protein (4EBP) gene, total RNA was extracted from day 4 fifth instar (V4) wing discs using SV total RNA isolation system (Promega) according to the kit protocol, and quantified spectrophotometrically. First strand cDNA was reverse transcribed using a Superscript III First Strand Synthesis System (Invitrogen) and oligo(dT)<sub>17</sub> primer. To design degenerate primers against 4EBP, conserved regions in the following insects were compared: *Drosophila melanogaster* (GenBank accession no. 24581447), *Anopheles gambiae* (GenBank accession no. 58390448), *Bombyx mori* (GenBank accession no. 114050837), *Tribolium castaneum*

(GenBank accession no. 91089709), *Apis mellifera* (GenBank accession no. 110759149), and *Glossina morsitans morsitans* (GenBank accession no. 83595296). Sequences for degenerate primers were as follows: forward 1 5'-ATGTC(A/C)GC(T/G)TCACC(C/T)A(C/T)CG-3', forward 2 5'-CTCCACCACTCCTGGAGG(C/T)A-3', reverse 1 5'- CCTCCAGGAGTGGTGGAGTA-3', and reverse 2 5'- ATCCA(T/G)(T/G)(C/T)(T/G)GAAS(T/G)(T/G)TTGGTG-3'. Pairs of degenerate primers were used to amplify cDNA from *Manduca* wing discs. Samples were heated to 94 °C for 5 min followed by 35 amplification cycles (94 °C for 30 s, 53 °C for 45 s and 72 °C for 30 s), and a final 7 min extension period at 72 °C. Products were separated on 3% agarose gels, purified using the Qiaquick gel extraction kit (Qiagen), and sent out for sequencing (University of Maine DNA Sequencing Facility). The fragment sequences were aligned using ClustalW (Thompson et al., 2002). Gene specific primers were then used to amplify 5'- and 3'-ends by the Generacer RACE-PCR kit (Invitrogen) according to kit protocol including nested PCR. Gene specific primer sequences were as follows: forward 5'-CACAGACCCTTCGCAGATGCCTGAT-3', nested forward 5'- GGAGGCACTGTTTACTCCACCACTCCTG-3', reverse 5'-TCAGGCATCTGCGAAGGGTCTGTGAT-3', reverse nested 5'-ATGGCTTGGCTGTGGGTCGCTTG-3' and were used in conjunction with primers provided in the kit. The amplified products were subjected to gel electrophoresis, purified and sent out for sequencing (University of Maine DNA Sequencing Facility). Clone sequences were aligned to form contigs spanning the 5'- and 3'-translated region.

### *Quantitative real time PCR (qPCR) analysis of gene expression*

Gene expression of *Manduca IR* and *4EBP* was analyzed by qPCR using a real-time sequence detection system (Applied Biosystems 7000). Prothoracic glands were pooled from five to fifteen individuals depending on developmental stage. Stages included the end of the fourth instar at the time of head capsule slippage and each of the first five days of the fifth instar. Total RNA was collected from samples using SV Total RNA isolation kit (Promega). RNA was tested spectrophotometrically for purity and concentration, and run on a formaldehyde gel to ensure quality. 100 ng of each RNA sample was reversed transcribed using gene specific primers in a Superscript III First Strand Synthesis System (Invitrogen) according to manufacturer's protocol. Primers for qPCR analysis were designed using Primer Express (Applied Biosystems). The *Manduca* insulin receptor sequence, against which primers were designed, was obtained from the partial gene sequence of Stefan Girgenrath (Ph.D. thesis, 1999). A complete *Manduca* insulin receptor sequence was subsequently published by Koyama et al (GenBank accession no FJ169469) and matched to the partial sequence used (Koyama et al., 2008). Primer sequences were as follows:

*IR* forward 5'- GCGCCGCCGACCAT-3',

*IR* reverse 5'-AGTATTTTGGGCTTGTTGTAGCTGTT-3',

*4EBP* forward 5'-AAACCCACCGAAGTGTTCA-3',

*4EBP* reverse 5'-CCTGCACTGGTGTGGATTGT-3'.

PCR reactions were performed using SYBR green mastermix (Applied Biosystems). *IR* PCR reactions contained 0.1  $\mu$ M of each primer. *4EBP* PCR reactions contained 0.9  $\mu$ M

of each primer. qPCR was performed in a final volume of 25  $\mu$ l. All quantitative reactions were subjected to 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15s, and 59°C for 1 min. Melting curve analysis was applied to all reactions to ensure homogeneity of the reaction product. In addition, products were subjected to gel electrophoresis to check product size for each primer pair. Potential contamination was assessed by including non-reverse transcribed total RNA (genomic DNA contamination) and non-template controls. No products were observed in these reactions. Dilution curves generated by serial dilutions of cDNA were used to calculate amplification efficiencies. Transcript levels of the target genes were normalized to the *Manduca* ribosomal gene *rpL17A* after correcting for differences in amplification efficiency (Pfaffl, 2001). Primer sequences for the ribosomal gene were as follows: forward 5'-TCCGCATCTCACTGGGTCT-3' and reverse 5'-CACGGCAATCACATACAGGTT-3'(Rewitz et al., 2006). Data was analyzed using a relative efficiency method described by Pfaffl (Pfaffl, 2001).

#### *Western blot analysis*

The prothoracic glands were dissected in pairs, placed in 20  $\mu$ L of 2X SDS sample buffer, and incubated at 90°C for three to five minutes. The samples were then run on a 10-20% SDS-PAGE gradient gel (Bio-rad) to separate the proteins (Laemmli, 1970). They were transferred from gels to nitrocellulose membranes at 4°C. Membranes were then blocked in 3% BSA for one hour, rinsed quickly (10-15 minutes total) and placed on primary antibody overnight. Primary antibodies included: anti-non-phospho4EBP, anti-p4EBP, anti-phosphoFOXO, antiphospho-Akt, anti-ERK and anti-actin (all antibodies

were obtained from Cell Signaling). Blots were rinsed again and placed on secondary anti-rabbit antibody with attached HRP (Cell Signaling) for 75 minutes. They were treated with Western blotting chemiluminescence reagents (Pierce ECL Western blotting substrate, Thermo Scientific). The blots were exposed on blue sensitive autoradiographic film (Marsh Bio Products) and developed (Kodak GBX fixer and developer). Blots were scanned and analyzed using ImageJ (Abramoff et al., 2004). Protein bands were normalized to actin for loading control where appropriate.

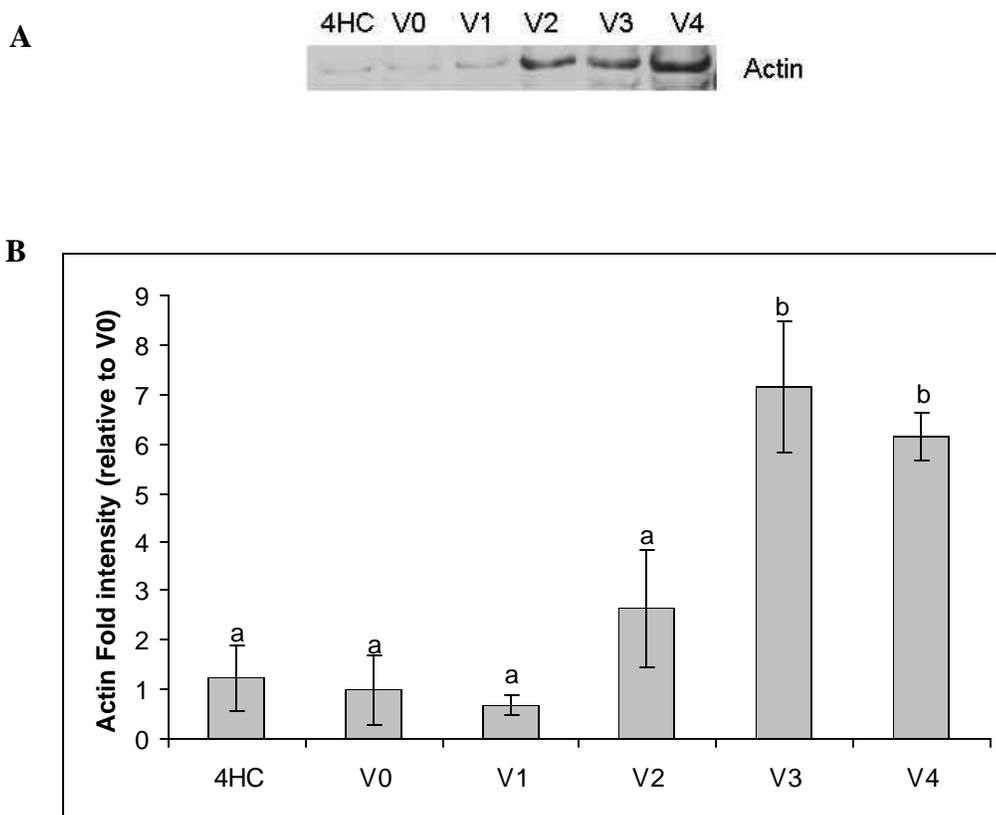
### *Statistical analysis*

Gene expression in the prothoracic glands was subjected to statistical analysis using one way ANOVA, with a Dunnett's post hoc test applied to compare specific developmental stages against a reference. Due to small sample sizes for Western blot analysis, the Kruskal-Wallis nonparametric ANOVA test was used to determine statistical significance. A Dunn's post hoc test was applied to compare specific developmental stages against a reference. For the insulin stimulation experiment, a paired *t*-test was applied to determine significance between control and stimulated glands. In all cases,  $p < 0.05$  was used to determine significance. All statistical analysis was performed using InStat v.3.0 (GraphPad Software, 2008).

## RESULTS

### Confirmation of growth in prothoracic glands

Prior to characterizing insulin signaling in the prothoracic glands, I confirmed that significant growth was occurring in these glands during the fifth larval stage. These findings are shown in Figure 3, which illustrates the profile of actin content in prothoracic glands during the feeding stage of the fifth instar. Figure 3B demonstrates that the change in actin content was significant (one way ANOVA,  $F=10.758$ ,  $p=0.0001$ ). In particular, actin content was low upon entry into the fifth instar (V0) increased by V2 and was significantly greater by V3 and V4 (Dunnett Multiple Comparisons test,  $p<0.01$  for both).

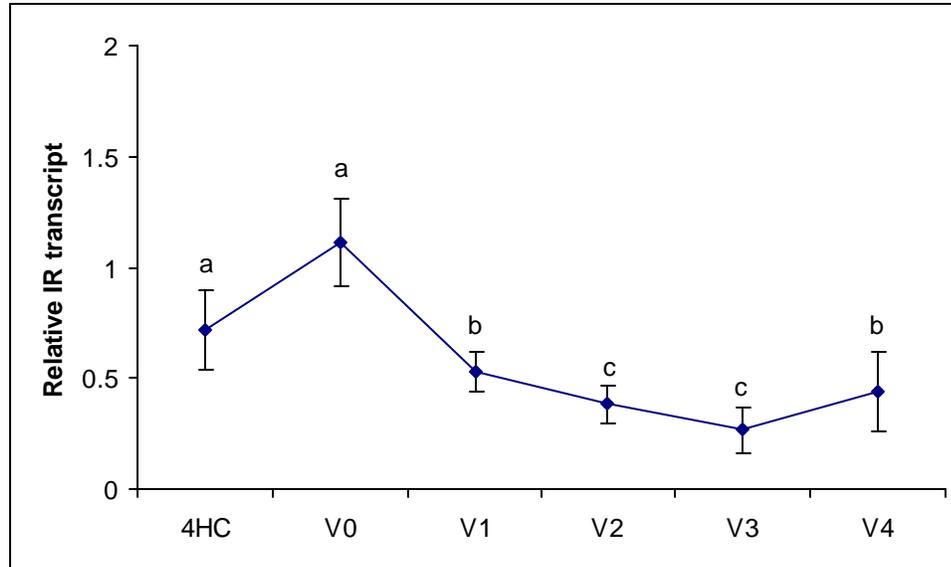


**Figure 3.** Abundance of actin in prothoracic glands during the feeding stage of fifth larval instar in *Manduca sexta*. **A.** Representative Western blot. Each lane represents a pair of glands from the given day of larval development. 4HC indicates fourth instar head capsule slip which occurs just prior to molting into the fifth instar. “V” indicates fifth instar and the subsequent number indicates developmental day. The actin band migrated at the expected size of 45 kD. **B.** Quantification of Western blots. Blots were analyzed using ImageJ (Abramoff et al., 2004). Bars are means of 4 independent experiments  $\pm$  SE. The change in actin protein abundance was significant through development (one way ANOVA,  $F=10.758$ ,  $p=0.0072$ ). There was no significant difference in 4HC, V1 or V2 in comparison to V0 as designated by “a” (Dunnett Multiple Comparison test,  $p>0.05$ ). There was a significant increase in actin content of V3 and V4 in comparison to V0 as designated by “b” (Dunnett Multiple Comparison test,  $p<0.01$  and  $p<0.01$  respectively).

## **Quantification of insulin receptor transcript**

Once growth was established, the involvement of the insulin receptor and insulin signaling in prothoracic glands could be characterized. Relative transcript levels of insulin receptor were assessed during the fifth larval stage. It was expected that higher transcript levels would coincide with times of growth. Figure 4 demonstrates that there was a significant trend in insulin receptor transcript levels during development (one way ANOVA,  $F=4.933$ ,  $p=0.0023$ ). The transcript levels were highest upon entry into this stage. There was a significant decline in transcript by V1 (Dunnett Multiple Comparisons test,  $p<0.05$ ). Insulin receptor transcript continued to significantly decline on V2 and V3 relative to V0 (Dunnett Multiple Comparisons test,  $p<0.01$  and  $p<0.01$  respectively). By V4 there was a slight increase in insulin receptor transcript, but it was still significantly less than V0 (Dunnett Multiple Comparison test,  $p<0.05$ ). The presence of actively transcribed insulin receptor in the prothoracic glands upon entry into the instar suggests that insulin receptor is important to cellular functions during this stage.

In addition to quantifying transcripts of the insulin receptor, an effort was made to quantify insulin receptor protein in the prothoracic glands during the fifth larval stage. Unfortunately, there was no band corresponding to the expected size when antibodies against the phosphorylated form of the insulin receptor were used on Western blots of glands from each day of the instar. This suggests that the phosphorylation of insulin receptor was ephemeral. Unfortunately, we have yet to identify a reliable antibody to identify total, unphosphorylated insulin receptor in *M. sexta* tissues.



**Figure 4.** Abundance of insulin receptor (IR) transcript in *Manduca* prothoracic glands during the feeding stage of the fifth larval instar. RNA was collected from the prothoracic glands of 5-15 larvae for each sample. Samples include glands of head capsule slipped larvae (4HC)(n=4), and larvae in the first four days of the fifth larval stage (V0 n=7, V1 n=7, V2 n=5, V3 n=6, V4 n=5). 100 ng of RNA from each pooled sample were reverse transcribed with gene specific primers. cDNA was analyzed using quantitative real time PCR and calculated using the Pfaffl method where the mean level of the V0 transcript was set to 1 and samples were normalized to the *Manduca* ribosomal protein *rpL17A* (Pfaffl, 2001; Rewitz et al., 2006). Bars are means  $\pm$  SE. The trend in transcript was significant during development (one way ANOVA,  $F=4.933$ ,  $p=0.0023$ ). There was no significant difference in transcript levels between 4HC and V0 as designated by “a” (Dunnett Multiple Comparisons test,  $p>0.05$ ). There was a significant decline in transcript by V1 as designated by “b” (Dunnett Multiple Comparisons test,  $p<0.05$ ). This significant decline in transcript relative to V0 continued in V2 and V3 as designated by “c” (Dunnett Multiple Comparisons test,  $p<0.01$  and  $p<0.01$  respectively). There was a small increase in transcript by V4 but it remained significantly less than V0 (Dunnett Multiple Comparisons test,  $p<0.05$ ).

## Quantification of initiation factor 4E binding protein (4EBP) transcript and protein

### *Cloning of 4EBP nucleotide sequence*

In order to quantify 4EBP transcript, the nucleotide sequence of 4EBP needed to be determined. First, degenerate primers were designed using gene sequences of other known insect 4EBPs. Degenerate PCR yielded an amplicon of approximately 142bp. Subsequent 3'RACE yielded an amplicon of approximately 400bp using both of the primers provided in the kit as well as gene specific primers designed for nested PCR. The nested PCR primers were designed against the known region of sequence deduced from degenerate PCRs. 5'RACE yielded amplicons that were 200bp in length for both primers provided in the kit and gene specific primers designed for nested PCR. All of the sequences were aligned to reveal the entire coding sequence. A final PCR was performed and sequenced to ensure that each portion of the gene sequence agreed with previous fragments. All portions of the sequence were confirmed by three separate PCR products.

The *M. sexta* nucleotide sequence was seen to be closest to the *Bombyx mori* 4EBP sequence with a 76% identity (GenBank nucleotide accession no DQ443244, E-value: 4e-64). Protein BLAST with the deduced amino acid sequence identified the sequence as a member of the conserved eIF\_4EBP superfamily. Again the closest match was to the *Bombyx mori* 4EBP with an 81% identity (GenBank accession no ABF51333, E-value: 2e-47). Figure 5A shows the complete nucleotide sequence and predicted amino acid sequence. Figure 5B represents an alignment of the predicted *M. sexta* 4EBP amino acid sequence with *B. mori*, *D. melanogaster* and *H. sapiens*. Although lepidopterans (*M. sexta* and *B. mori*) were not closely related to dipterans (*D. melanogaster*) there was

still a high level of homology in 4EBP among the three species. More importantly the phosphorylation sites were conserved in all three species, as well as in mammals.

Phospho-specific antibodies are available for these conserved residues (Figure 5B).

**A**

```
1 atgtcagcgtcacctatcgcgaggcaagcgacccacagccaagcc
  M S A S P I A R Q A T H S Q A
46 atcccgtcgagacgggtgctgatcacagacccttcgcagatgctt
  I P S R R V L I T D P S Q M P
91 gatgtgtactcgagcactcccgaggcactgtttactccaccact
  D V Y S S T P G G T V Y S T T
136 cctggaggtaccaggatagtatacgcggtcattcatgatgtcgc
  P G G T R I V Y D R S F M M S
181 cttagacagtccccgatctcacaacccccaccgaagtgttactg
  L R Q S P I S Q T P P K C S L
226 cctgcagctctgctgaggaatccgccagcaaatgcacaatccaca
  P A A L L R N P P A N A Q S T
271 ccagtgcaggcaccacaaaccttcctctaattcaattgtcttcgac
  P V Q A P K P S S N S I V F D
316 gaatcgcaggagacattcagcatggatctctaa 348
  E S Q E T F S M D L *
```

**B.**

```
M. sexta MSASPIARQA-THSQAIPSRRVLITDPSQMP-DVYSSTPGGTVYSTTPGGTRIVYDRSFM 58
B. mori MSASPIARQA-THSQSIPRRVLITDPAQMP-DVYSSTPGGTIYSTTPGGTRIVYERSFM 58
D. melanogaster MSASPTARQAITQALPMITRKVVISDPIQMP-EVYSSTPGGTLYSTTPGGTKLIYERAFM 59
H. sapiens MSGGSSCSQTPSRAIPA-TRRVVLGDGVQLPPGDYSTTPGGTLFSTTPGGTRIIYDRKFL 59
**... . *: ::: . :*:*: * *: * **:*:*****:*****:***: * *

M. sexta MSLRQSPISQTPPKCSLPAALLRNPPANA--QSTPVQAPKPSNSIVFDESQE-TFSMDL 115
B. mori LSLRQSPISQTPPQCALPAALLKNPSSVP--NVQPASTQKPRSNSISFDESQE-TFSMDL 115
D. melanogaster KNLRGSPLSQTPP-SNVPSCLLRGTPTPTFRKCVVPTELKQTKSLKIEDQE-QFQLDL 117
H. sapiens MECRNSPVTKTPP-RDLPTIPGVTSPSSDEPPMEASQSHLRNSPEDKRAGGEESQFEMDI 118
. * **:::*** :*: . . . : . . . :* *:::
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**Figure 5.** The nucleotide and deduced amino acid sequence of *Manduca* initiation factor 4E binding protein (4EBP) homolog (GenBank accession no. EU622640). **A.** The amino acid sequence was predicted using the Expert Protein Analysis System (ExPASy)(Gasteiger et al., 2003). **B.** Alignment of predicted amino acid sequence of *Manduca* 4EBP with *Bombyx mori*, *Drosophila melanogaster* and *Homo sapiens*. “\*” indicates that the amino acids in that column were identical. “:” indicates that a conserved substitution has been observed. “.” Indicates that a semi-conserved substitution has been observed. Bold residues indicate phosphorylation sites. Alignment was produced using ClustalW (Thompson et al., 2002).

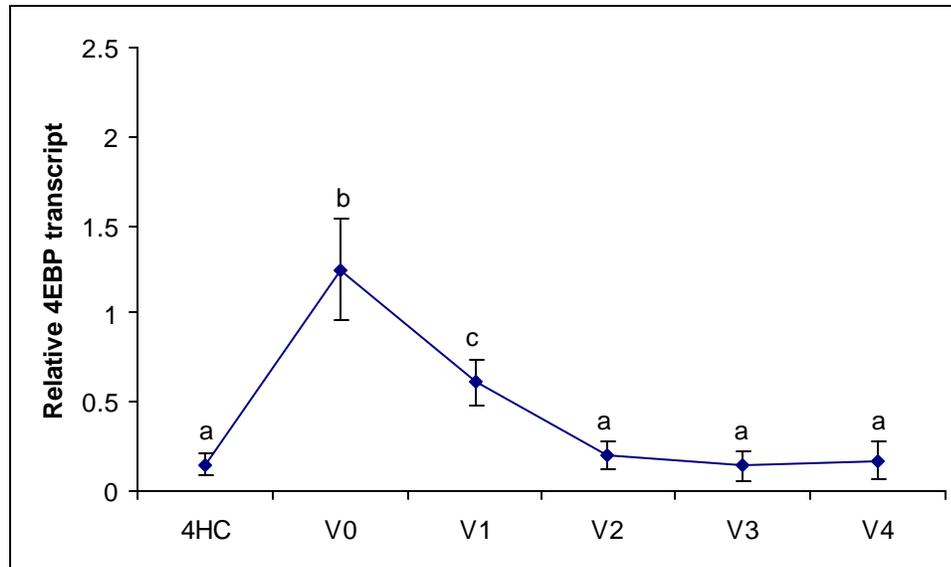
### *Quantification of 4EBP transcript*

Transcript levels of initiation factor 4E binding protein gene (4EBP) in the prothoracic glands were measured (Figure 6). 4EBP is a negative regulator of growth that works by inhibiting protein translation. It was expected that transcript levels would remain low when the glands grow rapidly, i.e. between days 2 and 4 of the fifth larval stage. As shown in Figure 6, the relative transcript levels of 4EBP were highest upon entry into the instar (V0) and decreased significantly by V1 (Dunnett Multiple Comparisons test,  $p < 0.05$ ). 4EBP transcript decreased to a low level by the second day (V2) relative to V0 (Dunnett Multiple Comparisons test,  $p < 0.01$ ) and remained that way for V3 and V4 (Dunnett Multiple Comparisons test,  $p < 0.01$  and  $p < 0.01$  respectively). Thus as expected, a drop in 4EBP transcript coincided with a period of glandular growth.

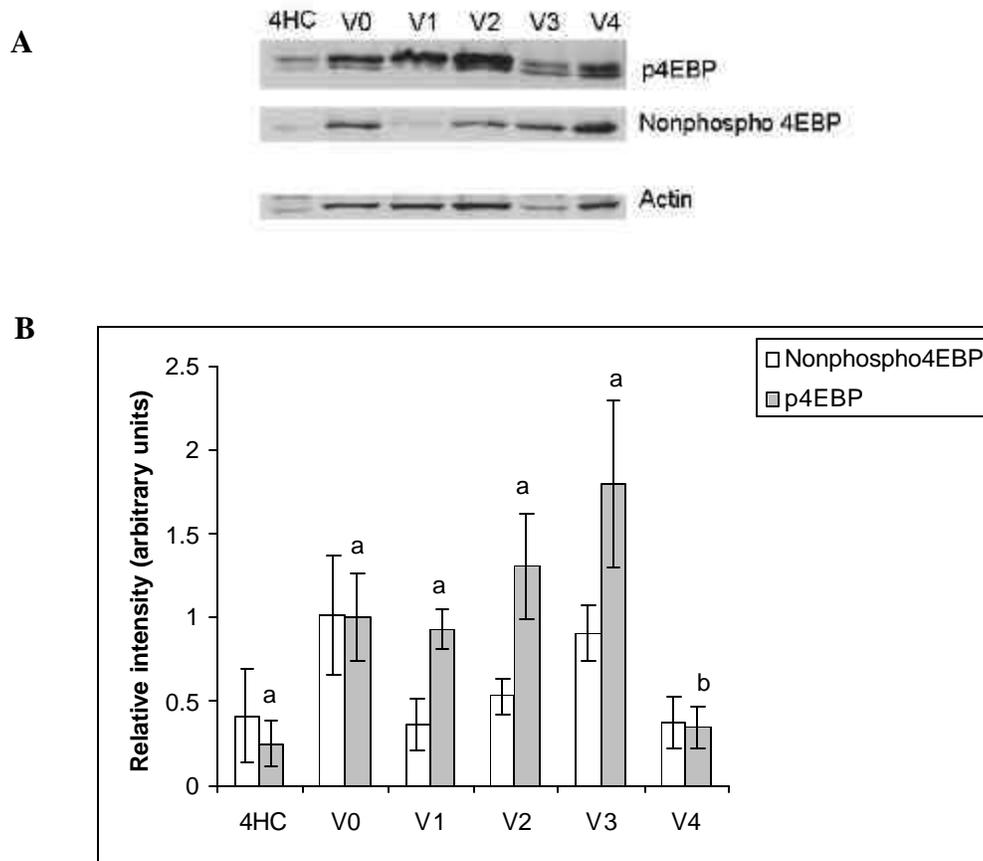
### *Quantification of 4EBP protein*

Levels of 4EBP protein were measured on Western blots using both phospho- and non-phospho4EBP antibodies (Figure 7A). Phosphorylation inhibits 4EBP and permits growth to occur. I thus expected that there would be a significant increase in p4EBP concurrent with periods of growth in the prothoracic glands. As shown in Figure 7B, there was a significant trend in the levels of p4EBP during development (Kruskal-Wallis nonparametric ANOVA,  $KW = 17.203$ ,  $p = 0.0041$ ). p4EBP levels were high upon entry into the fifth larval stage (V0) and remained high until V4 when there was a significant decrease relative to V3 (Dunn's Multiple Comparisons Test,  $p < 0.05$ ). Phosphorylation of 4EBP thus coincided with days of significant growth. As expected, non-phospho4EBP protein levels stayed relatively low throughout the feeding portion of

this instar (Figure 7). There was no a significant trend in the non-phospho4EBP protein levels although levels were highest on V0 when transcript levels were also high (Kruskal-Wallis nonparametric ANOVA, KW=7.015,  $p<0.2196$ ).



**Figure 6.** Abundance of initiation factor 4E binding protein (4EBP) transcript in *Manduca prothoracic* glands during fifth instar. RNA was collected from the prothoracic glands of 5-15 larvae for each sample. Samples included glands from head capsule slipped larvae (4HC)( $n=4$ ), and larvae in the first four days of the fifth larval stage (V0  $n=7$ , V1  $n=7$ , V2  $n=5$ , V3  $n=5$ , V4  $n=5$ ). 100 ng of RNA from each pooled sample were reverse transcribed with gene specific primers. cDNA was analyzed using quantitative real time PCR and calculated using the Pfaffl method where the mean level of the V0 transcript was set to 1 and samples were normalized to the *Manduca* ribosomal protein *rpL17A* (Pfaffl, 2001; Rewitz et al., 2006). Bars are means  $\pm$  SE. Transcript decreased significantly throughout the instar (one way ANOVA,  $F=6.935$ ,  $p=0.0003$ ). 4EBP transcript levels were highest on V0 as designated by “b” and decreased significantly by V1 as designated by “c” (Dunnett Multiple Comparisons test,  $p<0.05$ ). Transcript continued to significantly decrease by V2 to levels similar to 4HC and remain low through V3 and V4 as designated by “a” (Dunnett Multiple Comparisons test,  $p<0.01$  for all).



**Figure 7.** Abundance of non-phospho4EBP and p4EBP protein in prothoracic glands of *Manduca sexta* during the fifth instar. **A.** Representative Western blot. 4HC indicates fourth instar head capsule slip which occurs just prior to molting into the fifth instar. The non-phospho4EBP and p4EBP band corresponds to the expected size of approximately 20 kD. The actin band corresponds to the expected size of 45 kD. **B.** Quantification of Western blots. Blots were analyzed using ImageJ (Abramoff et al., 2004). Bars for non-phospho4EBP represent means of 4 (4HC, V2, V3, V4), 6 (V1) or 7 (V1) independent experiments  $\pm$  SE. Bars for p4EBP represent means of 4 (4HC, V0) or 5 (V1, V2, V3, V4) independent experiments  $\pm$  SE. There was no significant change in non-phospho4EBP protein during development (Kruskal-Wallis nonparametric ANOVA,  $KW=7.015$ ,  $p=0.2196$ ) while p4EBP significantly increased during development (Kruskal-Wallis nonparametric ANOVA,  $KW=17.203$ ,  $p=0.0041$ ). Post-hoc testing showed that p4EBP levels on V4 were significantly lower than on V3 (Dunn's Multiple Comparisons test,  $p<0.05$ ) as indicated by "b" while other days were not significantly different from one another (Dunn's Multiple Comparison's test,  $p>0.05$ ) as indicated by "a".

### **Activation of insulin pathway proteins during development of prothoracic glands**

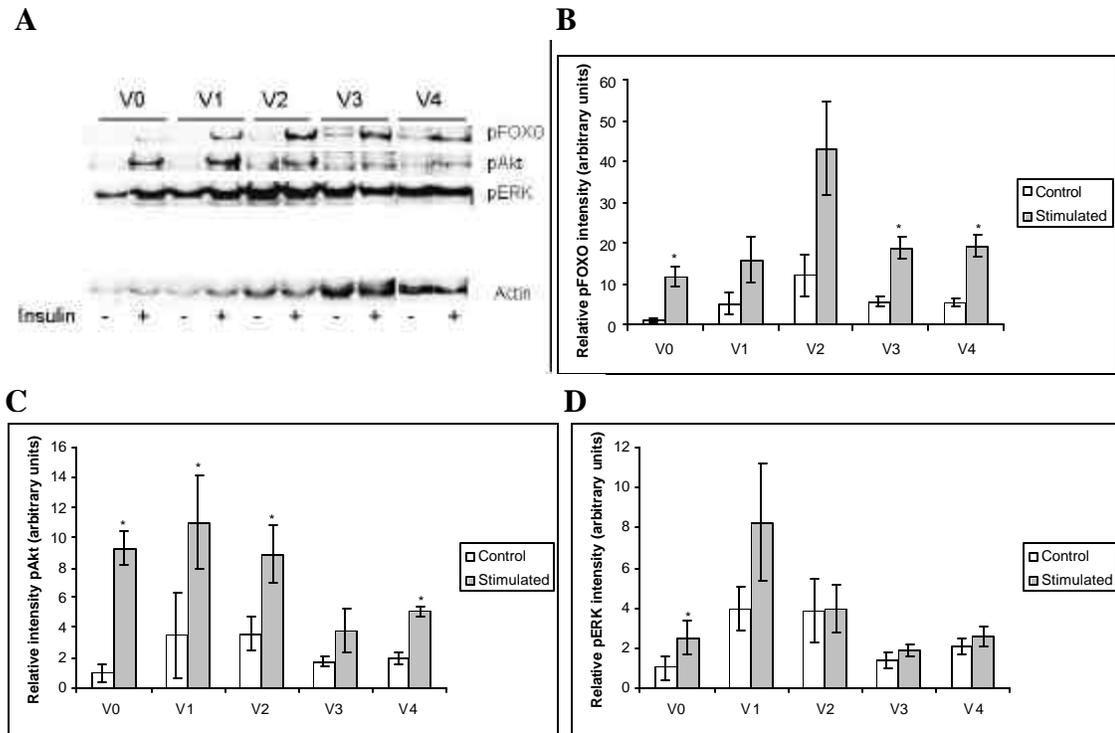
In order to demonstrate that insulin signaling occurs during larval growth, it was of interest to determine levels of activated insulin signaling pathway proteins.

Unfortunately, it was not possible to capture protein activation state *in situ*, as accompanying phosphorylations are relatively short-lived. Instead, I monitored the ability of glands to be activated by insulin *in vitro*. Matched pairs of glands from larvae during the feeding stage were removed and one was incubated with insulin while the other served as an unstimulated control. As shown in Figure 8A, insulin was capable of stimulating a significant increase in phosphorylation in several signaling proteins. For example, there was a significant increase in pFOXO due to insulin stimulation on days V0 (Paired *t*-test,  $t$ -statistic=4.133,  $p=0.0257$ ), V3 (Paired *t*-test,  $t$ -statistic=5.562,  $p=0.0115$ ) and V4 (Paired *t*-test,  $t$ -statistic=5.196,  $p=0.0138$ ) (Figure 8B).

Additionally, there was a significant increase in phosphorylation of PKB/Akt due to insulin stimulation *in vitro* (Figure 8C). In particular there was a significant increase in pAkt on days V0 (Paired *t*-test,  $t$ -statistic=10.308,  $p=0.0019$ ), V1 (Paired *t*-test,  $t$ -statistic=4.816,  $p=0.0171$ ), V2 (Paired *t*-test,  $t$ -statistic=5.106,  $p=0.0145$ ), and V4 (Paired *t*-test,  $t$ -statistic=3.858,  $p=0.0308$ ) (Figure 8C). There was not a significant increase in pAkt due to insulin stimulation on V3 (Paired *t*-test,  $t$ -statistic=1.684,  $p=0.1907$ ) which is likely due to high variability in the stimulated samples.

In wing discs of *M. sexta* it has been shown that insulin does not activate pERK (Nijhout et al., 2007). There was, however, a significant change in pERK due to insulin stimulation of the prothoracic glands on V0 (Paired *t*-test,  $t$ -statistic=5.444,  $p=0.0122$ ) while all the other days were not significantly different from basal levels (Figure 8D).

In addition to looking at insulin signaling pathway proteins, I attempted to capture the phosphorylated state of the insulin receptor in response to insulin stimulation *in vitro*. Unfortunately, the Western blots had a high level of background noise that mostly obscured the protein bands present, making it impossible to quantify the results.



**Figure 8.** Activation of phosphoproteins by insulin in prothoracic glands during the fifth instar in *Manduca sexta*. **A.** Representative Western blot of 4 independent experiments. Each lane represents an individual prothoracic gland. Glands were removed in matched pairs. One gland was incubated in 14  $\mu$ M of insulin (Novorapid) for 30 minutes while its matched control was incubated in medium alone. The protein bands migrated to the expected corresponding sizes (pFOXO 68 kD, pAkt 62 kD, pERK 42 kD and actin 45 kD). There were a few bands of other molecular weights that did not respond to insulin treatment. Therefore these bands were considered nonspecific and are not pictured here. **B.** Quantification of pFOXO Western blots using ImageJ (Abramoff et al., 2004). Bars are means of 4 independent experiments  $\pm$  SE. There was a significant increase in pFOXO due to insulin stimulation on days V0 (Paired *t*-test, *t*-statistic=4.133, *p*=0.0257), V3 (Paired *t*-test, *t*-statistic=5.562, *p*=0.0115) and V4 (Paired *t*-test, *t*-statistic=5.196, *p*=0.0138) as designated by “\*”. Days V1 (Paired *t*-test, *t*-statistic=2.618, *p*=0.0791) and V2 (Paired *t*-test, *t*-statistic=3.169, *p*=0.0505) the increase in pFOXO was not quite significant. **C.** Quantification of pAkt Western blots using ImageJ (Abramoff et al., 2004). Bars are means of 4 independent experiments  $\pm$  SE. There was a significant increase in pAkt due to insulin stimulation on days V0 (Paired *t*-test, *t*-statistic=10.308, *p*=0.0019), V1 (Paired *t*-test, *t*-statistic=4.816, *p*=0.0171), V2 (Paired *t*-test, *t*-statistic=5.106, *p*=0.0145), and V4 (Paired *t*-test, *t*-statistic=3.858, *p*=0.0308) as designated by “\*”. There was not a significant increase in pAkt due to insulin stimulation on V3 (Paired *t*-test, *t*-statistic=1.684, *p*=0.1907). **D.** Quantification of pERK Western blots using ImageJ (Abramoff et al., 2004). Bars are means of 4 independent experiments  $\pm$  SE. There was a significant change in pERK due to insulin stimulation on V0 (Paired *t*-test, *t*-statistic=5.444, *p*=0.0122) as designated by “\*”. V1 (Paired *t*-test, *t*-statistic=1.374, *p*=0.3031), V2 (Paired *t*-test, *t*-statistic=0.3454, *p*=0.7526), V3 (Paired *t*-test, *t*-statistic=0.927, *p*=0.4223), and V4 (Paired *t*-test, *t*-statistic=1.189, *p*=0.3199) showed no significant increase in pERK due to insulin stimulation.

## DISCUSSION

The present study confirms previous findings that prothoracic glands in *M. sexta* increase almost two fold in size during the feeding portion of the fifth instar (Sedlak et al., 1983; Smith and Pasquarello, 1989; Hanton et al., 1993; Lee et al., 1995). Growth of the glands was accompanied by changes in the transcription of insulin receptor and 4EBP. Insulin receptor transcript levels were highest upon entry into the instar. This could be in preparation for the large amount of growth in the glands that occurs during subsequent days. It had been hypothesized that the insulin receptor transcript would remain relatively high throughout periods of rapid growth. Instead, there was a rapid decrease in insulin receptor transcript by the first day of the fifth instar, well before the majority of growth occurs. The decrease in insulin receptor transcript could be due to negative feedback caused by exposure to insulin as larvae feed. In particular, insulin-stimulated phosphorylation of FOXO leads to its exclusion from the nucleus so that it can no longer act as a transcription factor for several gene products including insulin receptor and 4EBP (Puig and Tjian, 2006). A decrease in insulin receptor transcript does not imply that the insulin receptor is no longer necessary, but more likely that there was enough of the protein present to sustain observed growth of the glands. Unfortunately, total insulin receptor protein amounts could not be determined.

In contrast to the insulin receptor, 4EBP is a negative regulator of growth and its expression was anticipated to decrease. In flies and mammals, activation of the insulin pathway decreases the transcription of 4EBP and increases the inactivation of 4EBP through phosphorylation by PKB/Akt (Miron et al., 2001; Puig et al., 2003). Therefore,

if the insulin signaling pathway was activated during prothoracic gland development as expected, then transcript amounts of 4EBP would be relatively low and the 4EBP protein should be phosphorylated. Transcript levels of 4EBP were highest upon entry into the fifth instar (V0) while every other day has very low amounts. Presumably, the reduction of 4EBP would allow for the majority of growth in prothoracic glands to occur in the glands on the second and third day of the instar.

As for protein levels of 4EBP, non-phospho4EBP protein levels remain relatively low. The trend in transcript levels of 4EBP was similar to the trend in amounts of non-phospho4EBP. The highest levels of phosphorylated 4EBP coincided with the times of greatest growth including, the second and third day of the instar. It is possible that phosphorylation of 4EBP occurs through insulin signaling in *M. sexta*, as is seen in *D. melanogaster* (Miron et al., 2001; Puig et al., 2003). If that was the case then it would imply that insulin signaling was occurring during growth of the prothoracic glands.

In order to confirm that the prothoracic glands are indeed insulin-responsive, glands from each day of development were incubated with insulin. The insulin dramatically increased phosphorylation of PKB/Akt and FOXO. PKB/Akt positively regulates growth when phosphorylated, while FOXO is a negative regulator of growth that is inhibited upon phosphorylation. Taken together, these results show that the signaling proteins were not only present in growing glands, but were also capable of being stimulated by insulin.

In summary, as with other insects, growing tissues of *M. sexta* have the capacity to be regulated by insulin. Insulin receptor transcript was actively transcribed during the fifth larval stage. Presumably, its presence is important for both growth and the

assessment of nutritional status. Further, phosphorylated levels of 4EBP protein are high while transcript levels are low. Additionally, insulin pathway proteins are present and phosphorylated in response to insulin stimulation. This data serves as a context for future work that will focus on the nutritional sensitivity of the prothoracic glands. Insulin receptor and 4EBP transcripts have been shown to fluctuate in response to changes in nutrients. Looking at the nutritional sensitivity of the prothoracic glands will indicate the ability of the glands to translate environmental cues into changes in growth and ultimately secretory capacity.

## **CHAPTER TWO**

Nutritional sensitivity of prothoracic glands during the fifth instar.

## INTRODUCTION

Understanding the nutritional sensitivity of the prothoracic glands in *Manduca sexta* is of particular interest because it will give some insight into the possible role of the glands in the determination of overall body size. It is important to understand the ability of the glands to translate biochemical nutritional cues, like alterations in insulin receptor transcript and 4EBP, into changes in growth. Changes in glandular growth could ultimately affect the secretory capacity of the glands during crucial times of development.

Nutrition has been shown to have a major impact on the growth of vertebrates and invertebrates alike. This occurs mainly through the insulin signaling pathway and the target of rapamycin (TOR) (Hietakangas and Cohen, 2009). Systemic regulation of growth occurs through insulin signaling while TOR signaling affects cellular growth mainly through the regulation of protein biosynthesis (Hietakangas and Cohen, 2009). Short term effects of TOR on protein synthesis include the activation of initiation factors and the inhibition of factors which normally serve to prevent translation of proteins, such as 4EBPs. Both insulin receptor and 4EBP transcript levels are sensitive to changes in nutritional state (Puig and Tjian, 2006). Therefore, nutritional sensitivity of prothoracic glands can be assessed through alterations in the transcription of these two growth regulators.

In *D. melanogaster* genetic manipulations of the insulin signaling pathway, specifically in larval prothoracic glands, have yielded some interesting results. It was shown that changes in insulin signaling in prothoracic glands can alter the final size of the adult flies (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005). Genetic

alterations to enhance PI3K signaling in prothoracic glands of *D. melanogaster* cause the glands to grow to a size that is larger than normal (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005). The adult flies with enlarged prothoracic glands end up being smaller in size (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005). Conversely, when PI3K signaling is genetically down-regulated in prothoracic glands, the glands themselves are smaller, but the adult flies are much larger (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005). This implies that the prothoracic glands are responsible for determining critical weight that causes the cessation of feeding and sets final body size.

It remains unclear to what extent prothoracic glands are nutritionally sensitive during normal development. Insulin receptor transcript and 4EBP transcript have been shown to be present during normal larval development in the glands (Chapter 1). Therefore these transcripts were chosen to reflect the effects of nutrient deprivation on insulin signaling in the prothoracic glands of *M. sexta*. If prothoracic glands are indeed the assessor of critical weight through insulin signaling then growing glands should be susceptible to changes in nutrient status.

Insulin receptor transcript levels have been shown to increase in response to starvation (Chen et al., 1996; Tettweiler et al., 2005; Puig and Tjian, 2006). 4EBP is a negative regulator of growth and as such has been shown to be concomitantly up-regulated in response to starvation (Tettweiler et al., 2005; Puig and Tjian, 2006). Insulin receptor transcript increases in order to maximize nutrient uptake in the event that nutrients become available again, whereas 4EBP transcript increases in order to prevent translation of proteins thereby inhibiting growth. In the present study larvae were starved

both upon entry to the fifth instar and at minimal viable weight. For both time points transcript levels of both insulin receptor and 4EBP were quantified. It was expected that in *M. sexta* when larvae were starved there would be an increase in both insulin receptor and 4EBP transcript levels. This change in 4EBP transcript levels was also expected to be reflected in 4EBP protein levels in prothoracic glands.

The majority of growth of the prothoracic glands occurs between minimum viable weight that occurs on the first day of the fifth instar (V1) and critical weight that occurs two days later (V3) (Sedlak et al., 1983; Smith and Pasquarello, 1989; Hanton et al., 1993; Smith, 1995). Therefore, it was hypothesized that the time of greatest nutritional sensitivity would correspond roughly with the attainment of minimal viable weight. It would follow then that starvation would cause an increase in insulin receptor and 4EBP transcript, indicative of nutritionally-mediated changes in the insulin pathway between days V1 and V3.

## MATERIALS AND METHODS

### *Animals*

Larvae were reared to the fifth stage as previously described. All starved larvae were fed a non-nutritive 3% agar block for moisture (Jones et al., 1981). Larvae starved prior to ecdysis into the fifth instar were identified at head capsule slippage (24-36 hours pre-ecdysis). Larvae were monitored to ensure ecdysis occurred the next day or they were eliminated from the study. Larvae starved at minimal viable weight were taken off normal diet in the morning of day 1 (2-3g). Prothoracic glands for quantitative real time PCR were removed under lepidopteran saline (Riddiford et al., 1979), rinsed quickly in Grace's medium before being flash-frozen and stored at -80° C. Glands used for Western blots were removed under lepidopteran saline, rinsed quickly in Grace's medium, placed in 2x SDS sample buffer and boiled for 3 minutes at 90°C.

### *Quantitative real time PCR analysis of gene expression*

Gene expression of *Manduca IR* and *4EBP* was analyzed by qPCR using a real-time sequence detection system (Applied Biosystems 7000). Prothoracic glands dissected from 8 to 30 individuals were pooled from starved, weight matched and age matched larvae. The wide range in gland number was to ensure enough material was in each sample to obtain a measurable amount of RNA. Total RNA was collected and tested for purity as previously described. 100 ng of each sample of RNA was reversed transcribed using gene specific primers in a Superscript III First-strand RT kit according to manufacturer's protocol (Invitrogen). Primers for qPCR analysis were the same as

previously described. PCR reactions were performed using SYBR green mastermix (Applied Biosystems). qPCR was performed in a final volume of 25  $\mu$ l. All quantitative reactions were run as previously described. Transcript levels of the target genes were normalized to the *Manduca* ribosomal gene *rpL17A* after correcting for differences in amplification efficiency. Data was analyzed using a relative quantification method as described by Pfaffl (Pfaffl, 2001).

#### *Western blot analysis*

The prothoracic glands were dissected in pairs, placed in 20  $\mu$ L of 2X SDS sample buffer. Samples were run on gels and transferred as previously described. Primary antibodies included: anti-non-phospho4EBP, anti-phosphoFOXO, antiphospho-Akt, and anti-actin (all antibodies were obtained from Cell Signaling). Blots were prepped and developed as previously described. All blots were analyzed using ImageJ (Abramoff et al., 2004). Samples were normalized to actin and compared to a reference sample.

### *Statistical Analysis*

Gene expression in the prothoracic glands was subjected to statistical analysis using an one way ANOVA after determining if data were normally distributed. The Tukey-Kramer Multiple Comparisons post hoc test was applied to compare columns. Due to small sample sizes for Western blot analysis, the Kruskal-Wallis nonparametric ANOVA was used to determine statistical significance. A Dunn's Multiple Comparisons post hoc test was applied to compare columns. In both cases,  $p < 0.05$  was used to determine significance. All statistical analysis was performed using InStat v.3.0 (GraphPad Software, 2008).

## RESULTS

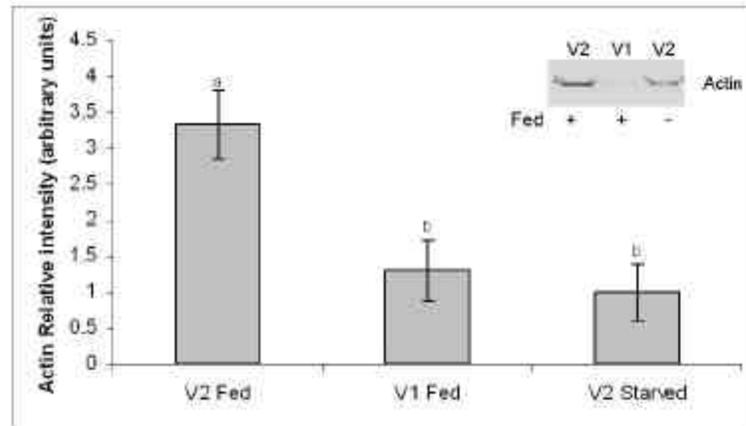
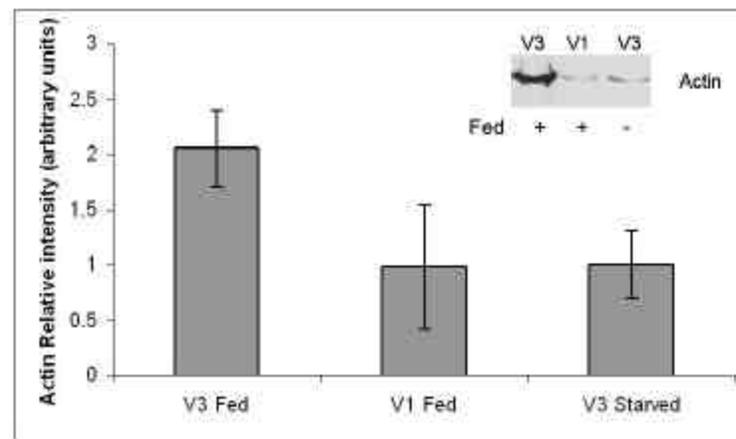
### Changes in growth due to starvation from minimal viable weight

Minimal viable weight is the minimal mass required to successfully complete metamorphosis. In *M. sexta* this is a mass of 3g which generally occurs by the second day of the fifth instar (V2) (Nijhout, 1975). When the larva attains a mass of 6g on the third day of the stage (V3), it no longer needs to feed in order to successfully complete metamorphosis. Additionally, the prothoracic glands grow significantly between V2 and V3 (Chapter 1, Figure 3). It was hypothesized that the glands would be most sensitive to extreme changes in nutritional state surrounding the period when prothoracic gland growth accelerates. To test this hypothesis, larvae weighing approximately 2.5g (V1) were starved for 30 hours. The resulting starved larvae weighed 3.5g on average, while normally fed age-matched counterparts weighed between 3.5g and 6g. Weight-matched fed larvae tended to be large V1 larvae. It was expected that there would be a decrease in actin abundance in starved prothoracic glands relative to age-matched fed glands, while weight-matched glands would have similar actin abundance. As seen in Figure 9A, actin abundance in starved glands was significantly reduced relative to age-matched (V2) (Dunn's Multiple Comparisons test,  $p < 0.05$ ) and nearly identical to weight-matched (V1) larvae (Dunn's Multiple Comparisons test,  $p > 0.05$ ). This indicated that the prothoracic glands were no longer growing if the larvae have been starved for 30 hours.

In order to ensure that nutrient deprivation occurred for enough time to impact both growth and transcript levels of insulin receptor and 4EBP, additional larvae were starved for 54 hours. When larvae were starved longer, they were still able to gain only

one gram in weight to achieve an average weight of 3.5g while their age-matched fed counterparts weighed anywhere from 6.5g to 8.5g. Again to control for differences due to weight alone, prothoracic glands from weight-matched larvae were used to assess a change in growth compared to starved larvae. Weight-matched larvae were comparable to large day one larvae (V1). Again, it was expected that prothoracic gland growth as assessed by actin abundance would be decreased in starved larvae.

As seen in Figure 9B, the trend in actin protein abundance in starved prothoracic glands relative to age-matched V3 controls and weight-matched V1 controls was not quite significant (Kruskal-Wallis nonparametric ANOVA,  $KW=4.308$ ,  $p=0.0584$ ). This was likely due to the small sample size and variability of actin abundance, particularly within V1 larvae. It is probable that the prothoracic glands of larvae ceased to grow once they were starved as V1 larvae.

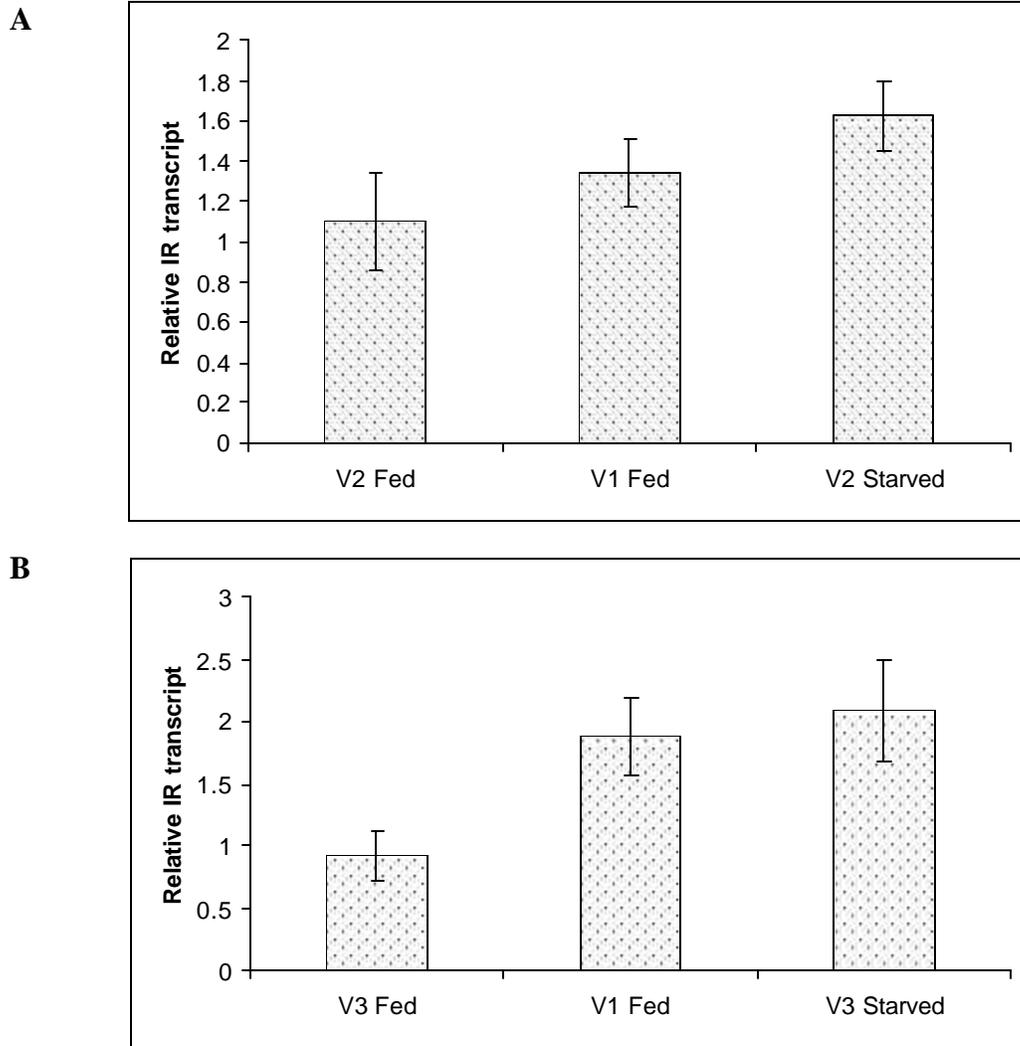
**A****B**

**Figure 9.** Effect of starvation on actin abundance in prothoracic glands of larvae starved from minimal viable weight. **A.** Larvae weighing approximately 2.5g were starved for 30 hours. Quantification of Western blots analyzed using ImageJ (Abramoff et al., 2004). Bars represent means from 4 independent experiments  $\pm$  SE. The trend in actin abundance was significant (Kruskal-Wallis nonparametric ANOVA, KW=7.538,  $p=0.0040$ ). Actin abundance from starved larvae was significantly different from V2 as designated by “b” (Dunn’s Multiple Comparison’s,  $p<0.05$ ), but not significantly different to weight matched V1 larvae as designated by “a” (Dunn’s Multiple Comparison’s,  $p>0.05$ ). Inset: Representative Western blot. Each lane represents a pair of glands from a single larva. The actin band corresponds to an expected size of 45 kD. **B.** Larvae were starved for 54 hours. Quantification of Western blots analyzed using ImageJ (Abramoff et al., 2004). Bars represent means from 4 independent experiments  $\pm$  SE. The trend in actin protein content was not quite significant (Kruskal-Wallis nonparametric ANOVA, KW=4.308,  $p=0.0574$ ). Inset: Representative Western blot. Each lane represents a pair of glands from a single larva.

### **Changes in insulin receptor transcript due to starvation from minimal viable weight**

As a measure of insulin sensitivity, 4EBP and insulin receptor transcript were quantified using real-time PCR. It was expected that transcript levels of both insulin receptor and 4EBP would increase due to starvation as is seen in flies (Tettweiler et al., 2005; Puig and Tjian, 2006). Figure 10A reveals that there was only a slight increase in insulin receptor transcript between larvae that have been starved for 30 hours and their age-matched fed counterparts (V2) (one-way ANOVA,  $F=1.757$ ,  $p=0.2063$ ).

To see if a longer span of time caused an increase in insulin receptor transcript in prothoracic glands of starved larvae, larvae were starved from 2.5g for an additional 24 hours. It was expected that larvae deprived of nutrients for 54 hours would have a large increase in both insulin receptor and 4EBP transcript relative to both weight-matched fed (V1) and age-matched fed (V3) controls. As seen in Figure 10B, even though the larvae were starved for 54 hours, the trend in insulin receptor transcript was still not quite significant (one-way ANOVA,  $F=3.628$ ,  $p=0.0502$ ). It appeared that the insulin receptor of prothoracic glands of larvae starved from minimal viable weight are not as sensitive to nutritional deprivation as initially thought.



**Figure 10.** Abundance of insulin receptor transcript in prothoracic glands of larvae starved from minimal viable weight. **A.** Larvae weighing approximately 2.5g were starved for 30 hours. RNA was collected from the prothoracic glands of 4-17 larvae and pooled for individual samples of starved ( $n=6$ ), age-matched fed larvae ( $n=5$ ) and weight-matched fed larvae ( $n=7$ ). 100 ng of RNA from each sample was reverse transcribed with gene specific primers. cDNA was analyzed using quantitative real time PCR and calculated using the Pfaffl method where the mean level of the V2 fed transcript was set to 1 and samples were normalized to the *Manduca* ribosomal protein *rpL17A* (Pfaffl, 2001; Rewitz et al., 2006). Bars are means  $\pm$  SE. The trend in the transcript levels was not significant (one way ANOVA,  $F=1.757$ ,  $p=0.2063$ ). **B.** Larvae were starved for 54 hours. RNA was collected from the prothoracic glands of 10-16 larvae and pooled for individual samples of starved ( $n=6$ ), age-matched fed larvae ( $n=6$ ) and weight-matched fed larvae ( $n=7$ ). The mean level of V3 fed transcript was set to 1. The transcript levels of insulin receptor was not quite significant (one way ANOVA,  $F=3.628$ ,  $p=0.0502$ ).

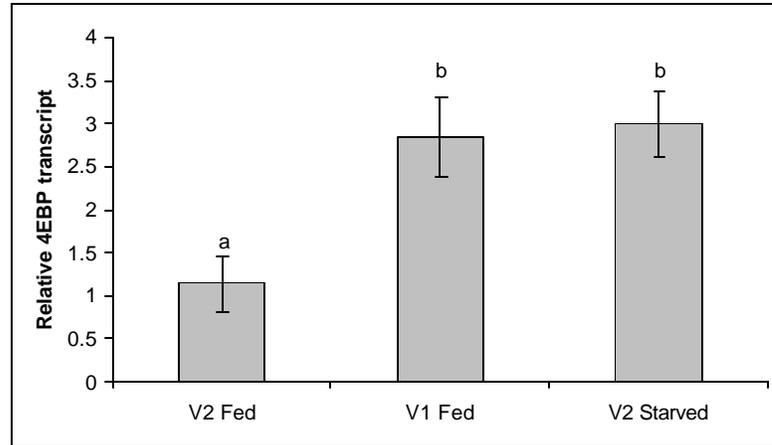
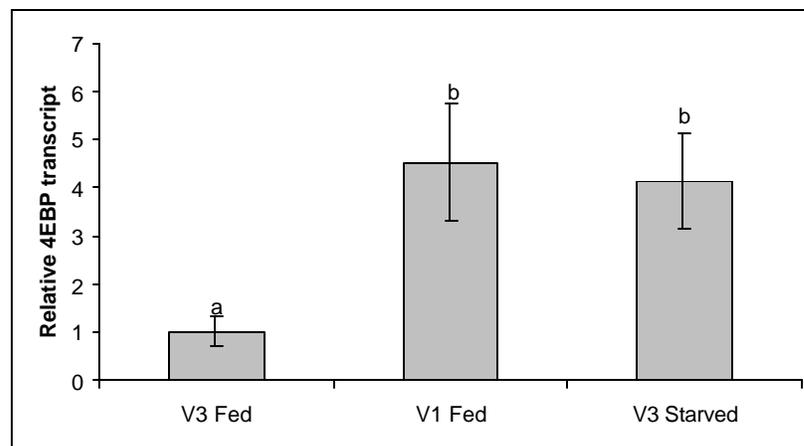
## **Changes in 4EBP transcript and protein due to starvation from minimal viable weight**

Transcript levels of 4EBP were also assessed in prothoracic glands of larvae that were starved from minimal viable weight for 30 hours. Even though there was no significant change in insulin receptor transcript, 4EBP transcript proved to be more sensitive to starvation at this time. 4EBP transcript levels showed a significant increase in starved larvae relative to their age-matched (V2) controls (Tukey-Kramer Multiple Comparisons test,  $p < 0.05$ ), while there was no change in 4EBP transcript between starved and weight-matched (V1) fed larvae (Tukey-Kramer Multiple Comparisons test,  $p > 0.05$ ) (Figure 11A).

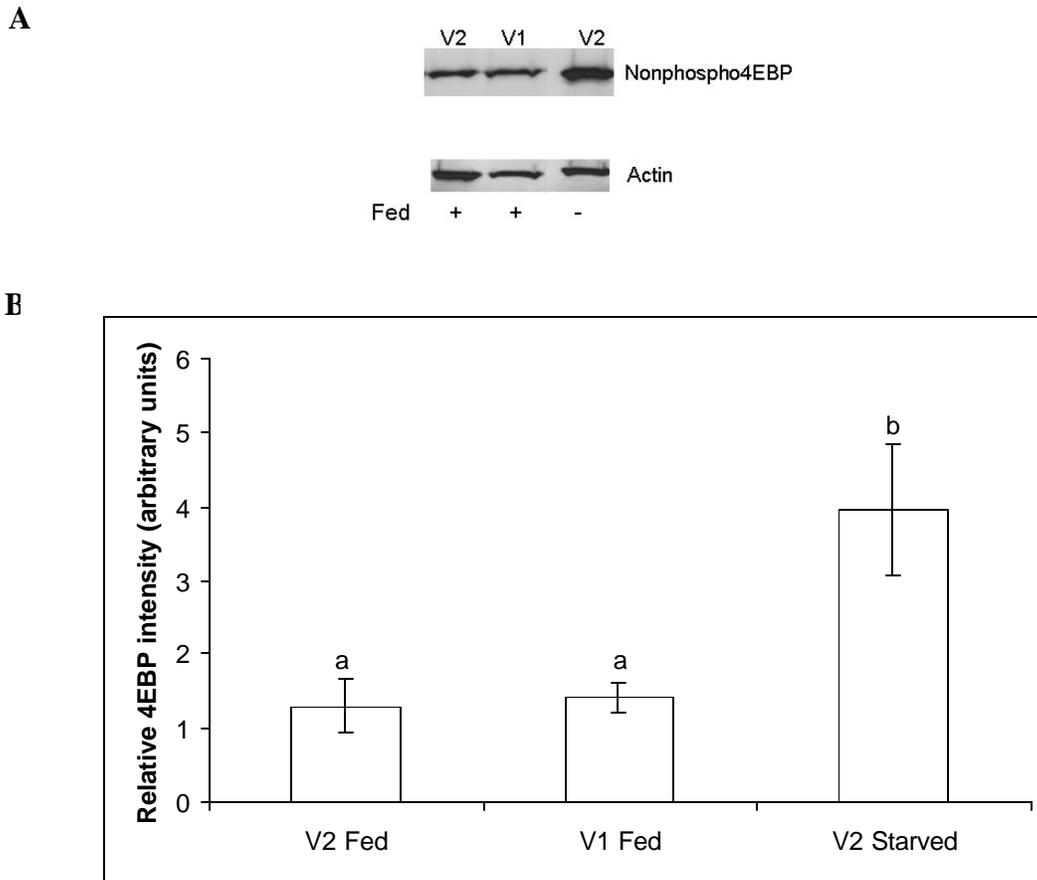
When larvae were starved for 54 hours a similar change was seen (Figure 11B). A 54-hour starvation resulted in a significant difference in 4EBP transcript in prothoracic glands of V3 larvae in comparison to fed V3 controls (Tukey-Kramer Multiple Comparisons test,  $p < 0.05$ ). There was no significant difference between 4EBP transcript in prothoracic glands from starved V3 larvae in comparison to fed V1 (weight matched) controls (Tukey-Kramer Multiple Comparisons test,  $p > 0.05$ ). It appears that 4EBP transcript levels were more sensitive to changes in nutritional state than insulin receptor transcript when the larvae were starved from minimal viable weight.

Figure 12 shows non-phospho4EBP protein levels from glands of larvae starved from 2.5g for 30 hours. There was a significant increase of non-phospho4EBP in prothoracic glands when the larvae were starved relative to their age matched fed counterparts (Tukey-Kramer Multiple Comparisons test,  $p < 0.05$ ). This was similar to

4EBP transcript levels. However, unlike 4EBP transcript levels, there was a significant difference in non-phospho4EBP of starved larvae relative to weight matched controls (Tukey-Kramer Multiple Comparisons test,  $p < 0.05$ ).

**A****B**

**Figure 11.** Abundance of initiation factor 4E binding protein (4EBP) transcript in prothoracic glands of larvae starved from minimal viable weight. **A.** Larvae weighing approximately 2.5g were starved for 30 hours. RNA was collected from the prothoracic glands of 4-17 larvae and pooled for individual samples of starved ( $n=6$ ), age-matched fed larvae ( $n=5$ ) and weight-matched fed larvae ( $n=7$ ). cDNA was analyzed using quantitative real time PCR and calculated using the Pfaffl method where the mean level of the V2 fed transcript was set to 1 and samples were normalized to the *Manduca* ribosomal protein *rpL17A* (Pfaffl, 2001; Rewitz et al., 2006). Bars are means  $\pm$  SE. The trend in transcript levels was significant (one way ANOVA,  $F=5.424$ ,  $p=0.0169$ ). Transcript in starved larvae was significantly different from V2 fed larvae as designated by “a” (Tukey-Kramer Multiple Comparisons Test,  $p<0.05$ ), but not significantly different from V1 fed larvae as designated by “b” (Tukey-Kramer Multiple Comparisons Test,  $p>0.05$ ). **B.** Larvae were starved for 54 hours. RNA was collected from the prothoracic glands of 10-16 larvae and pooled for individual samples of starved ( $n=5$ ), age-matched fed larvae ( $n=6$ ) and weight-matched fed larvae ( $n=6$ ). The mean level of the V3 fed transcript was set to 1. The trend in transcript levels was significant (one way ANOVA,  $F=5.953$ ,  $p=0.0135$ ). There was a significant difference in transcript between fed V3 and starved V3 as designated by “a” (Tukey-Kramer Multiple Comparisons Test,  $p<0.05$ ) but not between fed V1 and starved V3 as designated by “b” (Tukey-Kramer Multiple Comparisons test,  $p>0.05$ )



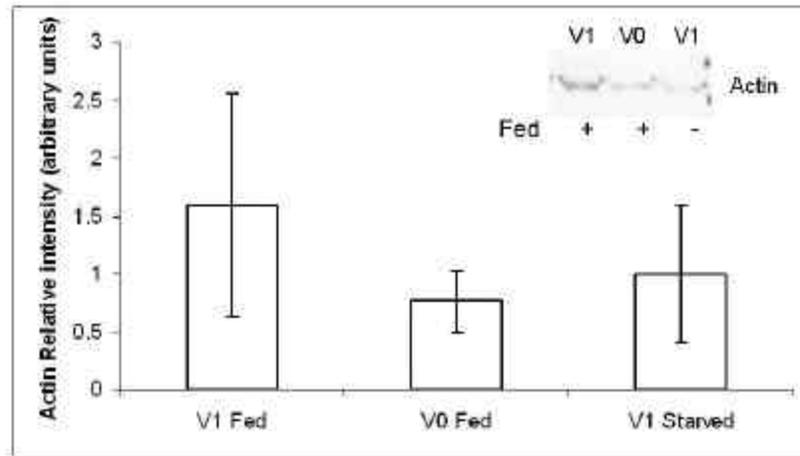
**Figure 12.** Effect of starvation on 4EBP protein abundance in prothoracic glands of *Manduca sexta* starved from minimal viable weight. **A.** Representative Western blot. Larvae that weighed approximately 2.5g were starved for 30 hours. Each lane represents varying numbers of glands to approximate equal loading of total protein. The non-phospho4EBP band corresponds to an expected size of 20 kD and the actin band corresponds to the expected size of 45 kD. **B.** Quantification of Western blots analyzed using ImageJ (Abramoff et al., 2004). Bars represent means from 8 independent experiments  $\pm$  SE. There was a significant trend in the transcript level (one way ANOVA,  $F=5.927$ ,  $p=0.0091$ ). Difference in 4EBP from starved larvae was significant to both age matched V2 (Tukey-Kramer Multiple Comparisons test,  $p<0.05$ ) and weight matched V1 (Tukey-Kramer Multiple Comparisons test,  $p<0.05$ ) larvae as designated by “b”, but there was no difference between V2 fed and V1 fed 4EBP protein as designated by “a” (Tukey-Kramer Multiple Comparisons test,  $p>0.05$ ).

## **Changes in insulin receptor and 4EBP transcript due to starvation upon entry into the fifth instar**

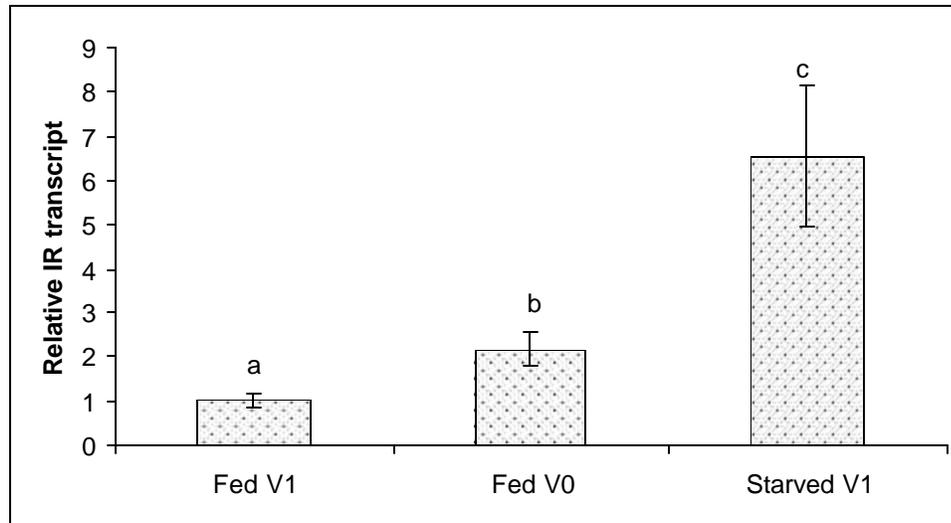
Although starvation caused the prothoracic glands to stop growing and significantly increased 4EBP, there was no significant up-regulation of the insulin receptor. This may have been due to the ability of the larvae to access stored carbohydrates. In order to more fully deplete nutrient reserves, larvae were starved upon entry into the fifth instar. It was expected that there would be a significant increase in both insulin receptor and 4EBP transcript when larvae were starved at this time. Prior to ecdysis, the head capsule of the larva slips forward in preparation for the molt. Once this happens the molt will occur within 12 to 24 hours. Larvae were separated once their head capsule slipped forward and starved for 30 hours after they molted.

Relatively little growth occurs in the prothoracic glands for the first 30 hours after the larvae molt into the fifth instar (Chapter 1, Figure 3). This was reflected in the small amount of weight gain between newly molted larvae (V0) that weigh about 1.5g, and larvae in the first day of the fifth instar (V1) that weigh between 2g to 3g. When larvae were starved for 30 hours upon entry into the fifth stage, they weighed about 1.5g to 2g. This was similar to the weight of age-matched controls fed ad libitum. Thus, it was not surprising that the trend in actin abundance in prothoracic glands of starved V1, fed V1 and weight matched V0 was not significant (Kruskal-Wallis nonparametric ANOVA, KW=0.5000, p=0.4552) (Figure 13). This was likely to be due to the fact that at this time there was only a small amount of growth that occurs in the glands normally, so although the starved glands have stopped growing, it can not be detected by measuring relative actin levels.

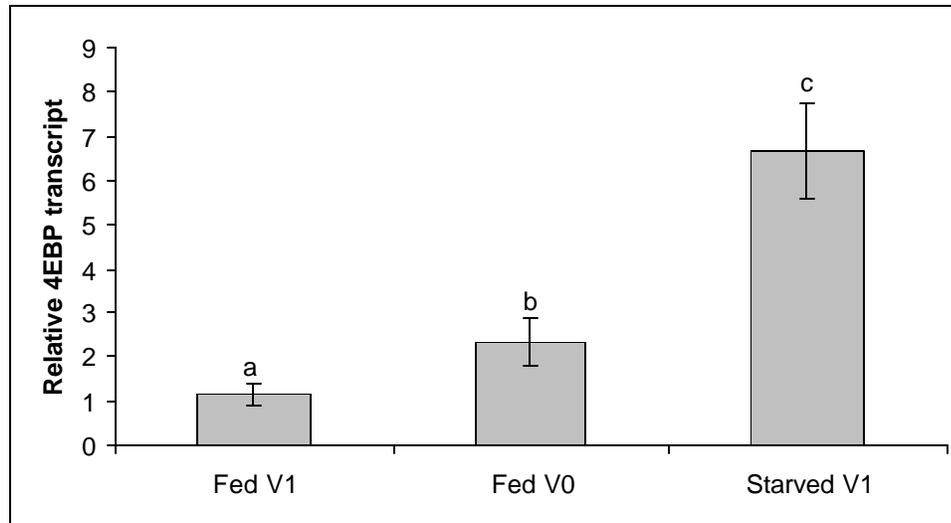
As seen in Figure 14, after the 30 hour starvation there was a significant increase in insulin receptor transcript in glands from starved larvae and age-matched (V1) fed larvae (Tukey-Kramer Multiple Comparisons test,  $p < 0.001$ ). There was also a significant transcript increase between glands from starved and weight-matched (V0) fed larvae (Tukey-Kramer Multiple Comparisons test,  $p < 0.01$ ) (Figure 14). This same trend was observed for the 4EBP transcript (Figure 15). Again there was a significant increase in 4EBP transcript from starved V1 glands in comparison to both V1 fed (Tukey-Kramer Multiple Comparisons test,  $p < 0.001$ ) and weight matched V0 fed larvae (Tukey-Kramer Multiple Comparisons test,  $p < 0.001$ ). However, in 4EBP protein levels the trend was not quite significant even though there appeared to be an increase in non-phospho4EBP in starved glands (Kruskal-Wallis nonparametric ANOVA,  $KW = 4.025$ ,  $p = 0.0713$ ) (Figure 16). The trend in non-phospho4EBP was similar to the trend of 4EBP transcript levels. The increase in insulin receptor and 4EBP transcript levels was noteworthy because it occurs at a time when both of these transcripts are normally most abundant (Chapter 1, Figures 4 and 5).



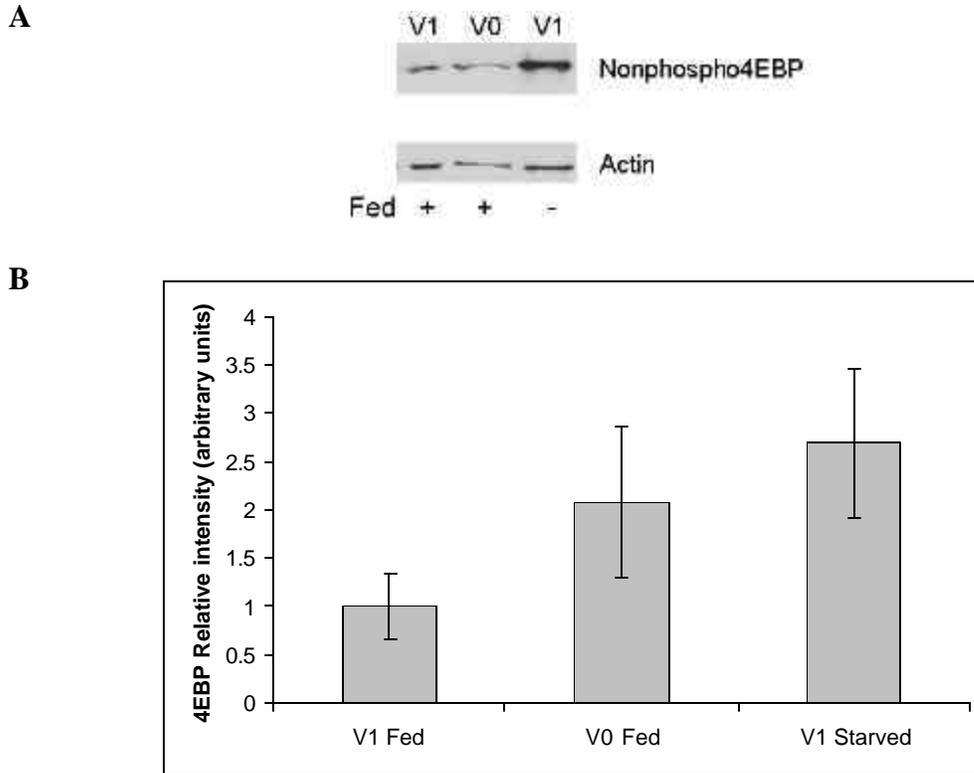
**Figure 13.** *Effect of starvation on actin abundance in prothoracic glands of Manduca sexta larvae starved upon entry into the fifth instar.* Quantification of Western blots. Larvae were starved for 30 hours. Blots were analyzed using ImageJ (Abramoff et al., 2004). Bars represent means of 4 independent experiments  $\pm$  SE. The trend in actin protein was not significant (Kruskal-Wallis nonparametric ANOVA, KW=0.5000, p=0.4552). Inset: Representative Western blot. Each lane represents a pair of glands from the same animal. The actin band corresponds to the expected size of 45 kD.



**Figure 14.** Abundance of insulin receptor transcript in prothoracic glands of *Manduca sexta* larvae starved upon entry into the fifth instar. Larvae were starved for 30 hours. RNA was collected from the prothoracic glands of 10-30 larvae and pooled for individual samples of starved ( $n=5$ ), age-matched fed larvae ( $n=7$ ) and weight-matched fed larvae ( $n=7$ ). 100 ng of RNA from each sample was reverse transcribed with gene specific primers. cDNA was analyzed using quantitative real time PCR and calculated using the Pfaffl method where the mean level of the V1 fed transcript was set to 1 and samples were normalized to the *Manduca* ribosomal protein *rpL17A* (Pfaffl, 2001; Rewitz et al., 2006). Bars are means  $\pm$  SE. The trend in transcript levels in insulin receptor was significant (one way ANOVA,  $F=13.475$ ,  $p=0.004$ ). The transcript levels in starved larvae significantly increased in comparison to V1 fed larvae as designated by “a” and “b” (Tukey-Kramer Multiple Comparisons test,  $p<0.001$ ). There was also a significant increase of starved larvae relative to weight-matched larvae (Tukey-Kramer Multiple Comparisons test,  $p<0.01$ ) as designated by “c”.



**Figure 15.** Abundance of initiation factor 4E binding protein (4EBP) transcript in prothoracic glands of *Manduca sexta* larvae starved upon entry into the fifth instar. Larvae were starved for 30 hours. RNA was collected from the prothoracic glands of 10-30 larvae and pooled for individual samples of starved ( $n=5$ ), age-matched fed larvae ( $n=7$ ) and weight-matched fed larvae ( $n=7$ ). 100 ng of RNA from each sample was reverse transcribed with gene specific primers. cDNA was analyzed using quantitative real time PCR and calculated using the Pfaffl method where the mean level of the V1 fed transcript was set to 1 and samples were normalized to the *Manduca* ribosomal protein *rpL17A* (Pfaffl, 2001; Rewitz et al., 2006). Bars are means  $\pm$  SE. The trend in 4EBP transcript levels was significant (one way ANOVA,  $F=20.404$ ,  $p<0.0001$ ). The transcript levels in starved larvae were significantly increased in comparison to V1 fed larvae as designated by “a” and “b” (Tukey-Kramer Multiple Comparisons test,  $p<0.001$ ). Also there was a significant increase in transcript in starved larvae relative to weight-matched V0 larvae as designated by “c” (Tukey-Kramer Multiple Comparisons test,  $p<0.001$ ).



**Figure 16.** Initiation factor 4E binding protein (4EBP) protein abundance in prothoracic glands of *Manduca sexta* larvae starved upon entry into the fifth instar. **A.** Larvae were starved for 30 hours. Representative Western blot. Each lane represents varying numbers of glands to approximate equal loading of total protein. The non-phospho4EBP band corresponds to the expected size of 20 kD and the actin band corresponds to the expected size of 45 kD. **B.** Quantification of Western blots analyzed using ImageJ (Abramoff et al., 2004). Bars represent means from 4 (V0 fed, V1 starved) or 5 (V1 fed) independent experiments  $\pm$  SE. The trend in 4EBP protein levels was not significant (Kruskal-Wallis nonparametric ANOVA, KW=4.025, p=0.0713).

## DISCUSSION

The attainment of critical weight coincides with a series of well defined endocrine changes that leads to the commitment of the larva to undergo metamorphosis. Even though much is known about the endocrine changes that occur in conjunction with critical weight, it is unclear what signal or signals are involved in the determination of critical weight in Lepidoptera. Insulin signaling is a likely candidate to signal critical weight. In order to validate this hypothesis, this study demonstrated the nutritional sensitivity of the prothoracic glands through changes in insulin receptor and 4EBP transcript abundance during developmentally significant time points in the fifth instar of the tobacco hornworm, *Manduca sexta*.

Initially it was believed that the time the glands would be most sensitive to nutrient deprivation would coincide with the period that the glands are undergoing rapid growth. The majority of prothoracic gland growth occurs between the second day and fourth days of the fifth instar (Sedlak et al., 1983; Smith and Pasquarello, 1989; Hanton et al., 1993; Smith, 1995). On the second day, the larvae attain a minimal viable weight of 3g, i.e. the minimal weight that the larva can survive metamorphosis (Nijhout, 1975). By the fourth day, normally growing larvae attain the critical weight of 6g, a weight at which no more growth was necessary for metamorphosis to occur without delay. It was expected that the prothoracic glands of larvae starved from V1 (2.5g) through the period of maximal growth (V3), would demonstrate significant increases in both insulin receptor and 4EBP transcripts.

When larvae were starved at 2.5g for 30 hours the prothoracic glands stopped growing, as indicated by a decrease in actin abundance. Larvae starved for 54 hours showed a similar trend. However, when larvae were starved for 30 hours there was no appreciable increase in transcript levels of insulin receptor in prothoracic glands. Larvae starved for an extra day (54 hours) prior to attaining minimal viable weight (2.5g) also did not have significant increase in insulin receptor transcript.

In contrast to insulin receptor, upon starvation 4EBP transcript was rapidly up-regulated. When larvae were starved at 2.5g, transcript levels of 4EBP did significantly increase relative to the age-matched fed controls. This was true when larvae were starved for either 30 or 54 hours. As a negative regulator of growth, it made sense that the transcript of 4EBP increased after a 30 hour starvation even though insulin receptor transcript did not. This indicates, however, that transcription of the insulin receptor may not be as sensitive to changes in nutritional status at minimal viable weight as was initially thought. Prior to starvation these larvae were feeding for 24 hours, and this may have allowed them to accrue nutritional stores. It is possible that the nutritional stores differentially maintained circulating carbohydrates and amino acids. If carbohydrate levels were maintained while circulating amino acids decreased, it could explain why 4EBP transcript increased while insulin receptor transcript did not. Unlike insulin receptor, 4EBP transcript is regulated through TOR which is able to sense circulating amino acid levels (Avruch et al., 2009). There is some evidence from *Drosophila* prothoracic gland that suggests that the TOR pathway and the insulin pathway act independently of one another while both work to modulate growth period of the larvae (Layalle et al., 2008). In order to obtain an insulin-sensitive response in prothoracic

glands, larvae were starved upon entry to the fifth instar, when nutritional reserves would be lowest.

Upon entry into the fifth instar, larvae have attained “threshold size”, i.e. have entered the final instar. However, these larvae have not yet attained minimal viable weight. It was expected that larvae starved for 30 hours post-ecdysis would have an increase in insulin receptor and 4EBP transcript in the prothoracic glands. It was expected that there would not be a significant change in growth due to a 30 hour starvation because little growth occurs in the glands at this time.

Both insulin receptor transcript and 4EBP transcript levels increased significantly in glands from larvae starved upon entry into the fifth instar, relative to age-matched fed counterparts. This result indicates that the insulin receptor transcript was sensitive to changes in nutritional state of the larva. The trend in 4EBP protein abundance was similar to the trend seen in transcript abundance. As expected, there was no detectable change in actin abundance between starved and fed prothoracic glands in these larvae.

There was also an increase of both insulin receptor and 4EBP transcripts in V1 starved larvae relative to fed larvae that had the same weight (V0). This indicates that there is no correlation between the weight of the larva and insulin receptor or 4EBP transcript abundance. As demonstrated by Figure 10 and 11, there was no change in transcript levels of 4EBP between prothoracic glands of V2 starved larvae and fed larvae of the same weight (V1). However, if there were a correlation, then the trend in transcript would also have been the same in the larvae starved upon entry into the last larval stage, and this was not the case.

Taken together this data established that the prothoracic glands are most nutritionally sensitive upon entry to the fifth instar. This is reinforced by the fact that the relative increase in insulin receptor and 4EBP transcripts is large when starved upon entry into the fifth instar, even though during normal development there are relatively high levels of insulin receptor and 4EBP transcript (Chapter 1, Figures 4&6). The changes in insulin receptor and 4EBP transcript demonstrated here indicate that the prothoracic glands are able to indirectly sense extrinsic (nutritional) cues. These findings support the hypothesis that insulin signaling may be integral in signaling critical weight of the larva. The glands may be translating those nutritional cues ultimately into the physiological response of ecdysone secretion. These results raise the question if changes in insulin signaling affect the ability of the glands to secrete ecdysone.

## **CHAPTER THREE**

Starvation-elicited changes in ecdysone secretion by prothoracic glands of fifth instar  
larvae

## INTRODUCTION

In the previous chapter it was established that prothoracic glands in fifth instar *M. sexta* stop growing in response to nutrient deprivation. In addition, transcript levels of both insulin receptor and 4EBP in the glands increase in response to starvation. To understand the relevance of growth on metamorphosis, it is essential to determine the impact of glandular growth on the ability of the prothoracic glands to secrete ecdysone. Insulin/IGF signaling has been implicated in both the growth and function of vertebrate and invertebrate steroidogenic tissues. One well-studied example is mammalian ovarian function, which has been linked to insulin, IGF1 and IGF2. Specifically, there is a great deal of evidence that both insulin and IGF1 have a stimulatory effect on estradiol production by mammalian granulosa cells (Hsueh et al., 1984; Giudice, 1992; Spicer and Echternkamp, 1995). The exact mechanism remains unclear, but it is due in part to the ability of insulin-like hormones to enhance the action of gonadotropins on ovarian follicular steroidogenesis (Adashi et al., 1990; Monget et al., 2002; Spicer et al., 2002). More recently, IGF2 has also been shown to stimulate estradiol and progesterone secretion by granulosa cells (Spicer and Aad, 2007). There is also evidence of insulin/IGFs influencing steroidogenesis in other mammalian glands such as the adrenals. For example in humans, IGFs play a pivotal role in adrenal development during puberty. IGFs enhance the ability of the gland to function normally even though there does not appear to be a direct link between IGFs and adrenal steroidogenesis (Guercio et al., 2003).

In invertebrates, there is abundant evidence demonstrating that insulin can have a direct impact on the ability of prothoracic glands to secrete ecdysone. In *D. melanogaster*, changes in PI3K signaling in prothoracic glands have been shown to alter the ability of the glands to secrete ecdysone, leading to a change in the overall body size of the animal (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005). In particular, mutations that decreased PI3K signaling specifically in the prothoracic gland resulted in a reduction in transcription of two steroidogenic biosynthesis pathway genes (Colombani et al., 2005). Conversely, when mutations increased PI3K signaling there was an increase in the transcript of two steroidogenic genes (Colombani et al., 2005). Although ecdysone secretion could not be directly measured, it appeared that insulin signaling in the prothoracic glands was able to directly stimulate ecdysone secretion, which led to the cessation of feeding and the determination of final body size (Colombani et al., 2005). It is important to note that mutations that caused the up-regulation of other growth enhancing proteins, dMyc and cyclin D/cdk4, were able to increase the size of the prothoracic gland, but did not impact final body size (Colombani et al., 2005).

There is additional evidence that supports the postulate that prothoracic glands are able to assess final body size in *D. melanogaster*. For example, the over-expression of PTEN, a phosphatase in the insulin pathway that is responsible for suppressing growth, in the prothoracic glands resulted in smaller prothoracic glands and larger adults (Mirth et al., 2005). The over-expression of PTEN only impacted overall growth of the larva when expressed in prothoracic glands and corpora allata, not when it was expressed in corpora allata alone, indicating that the prothoracic glands were responsible for body size assessment (Mirth et al., 2005). Ecdysone secretion in these larvae was significantly

reduced (Mirth et al., 2005). Similarly, expression of a dominant-negative (dn) PI3K in prothoracic glands of flies reduced the transcriptional activation of two ecdysone signaling targets, E74A and E74B (Caldwell et al., 2005). However, when PI3K was over-expressed, the resulting glands were larger and the adults were smaller, but no significant increase in ecdysone secretion was detected (Mirth et al., 2005). Taken together this data demonstrates that in flies there is a direct impact of insulin signaling on prothoracic gland function that results in the determination of final body size.

Like flies, insulin signaling has been found to directly impact ecdysone secretion in Lepidoptera. In the silkworm, *B. mori*, insulin has been shown to directly stimulate ecdysone secretion late in the last larval stage. The majority of the growth in the prothoracic glands of *B. mori* starts on the sixth day of the instar and continues until the larvae pupate. Growth of the glands was found to coincide with an increase in bombyxin/insulin titers, which rises after the larvae have ceased feeding (Saegusa et al., 1992). It appears that insulin is important for both growth in the glands and ecdysone secretion. Glands of larvae from the first four days of the last larval stage showed no increase in ecdysone secretion *in vitro* when incubated with insulin for 8 hours (Gu et al., 2009). However, glands from days four through pupariation secreted significantly more ecdysone when insulin-treated (Gu et al., 2009). Additionally, there was a significant increase in ecdysone secretion *in vivo* when day 6 larvae from the last larval stage were injected with insulin (Gu et al., 2009).

In previous chapters, evidence was presented that insulin signaling may regulate the growth of *M. sexta* prothoracic glands. The prothoracic glands stop growing in response to starvation concurrent with changes in insulin receptor and 4EBP transcripts.

It was hypothesized that the reduction in nutrients that prevents the growth of fifth instar prothoracic glands would also impact the ability of the glands to produce ecdysone. A complicating factor is that ecdysone secretion can be inhibited by JH. In normally feeding larvae, JH levels drop by the second day of the fifth instar (Wolfgang and Riddiford, 1986; Baker et al., 1987). This is permissive of the release of PTTH from the brain and allows the prothoracic glands to respond to PTTH (Nijhout and Williams, 1974b; Bollenbacher et al., 1981). It has been shown that when larvae are starved, levels of JH remain high until feeding was resumed (Cymborowski et al., 1982; MacWhinnie et al., 2005). However, there is some evidence that insulin can override the inhibitory effects of JH. For example, in fifth instar wing discs, the presence of insulin causes the expression of the molecular marker of pupal commitment, Broad transcription factor, despite the presence of JH (Koyama et al., 2008). Therefore, it was expected that the addition of insulin to starved larvae would increase the amount of ecdysone, despite the high titers of JH. Finally, although we were not anticipating a late effect, given that bombyxin/insulin titers are elevated and cause late growth in *B. mori* there may be an unanticipated effect of insulin on ecdysone later in the fifth stage.

## MATERIALS AND METHODS

### *Animals*

Larvae were reared on artificial diet as described in earlier chapters. Larvae used in the present study wandered on day 5 of the fifth instar. All starved larvae were fed a non-nutritive 3% agar block for moisture (Jones et al., 1981). Larvae starved prior to ecdysis into the fifth instar were identified at head capsule slippage (24-36 hours pre-ecdysis) and put on 3% agar diet. Larvae were monitored to ensure ecdysis occurred the next day otherwise they were eliminated from the study. Glands used for Western blots were removed under lepidopteran saline, rinsed quickly in Grace's medium, placed in 2x SDS sample buffer and boiled for 3 minutes at 90°C.

### *Western Blot analysis*

Samples were run on gels and transferred as previously described. Primary antibodies included anti-non-phospho4EBP and anti-actin (all antibodies were obtained from Cell Signaling). Blots were prepped and developed as previously described. All blots were analyzed using ImageJ (Abramoff et al., 2004). Samples were normalized to actin and compared to a reference sample.

### *Ecdysone radioimmunoassay*

Larval prothoracic glands were dissected into lepidopteran saline and maintained in Grace's medium for periods of less than 1 hour prior to experimentation. Brain extract was prepared by rapid homogenization of *M. sexta* brains from newly ecdysed pupae in a

small volume of cold Grace's medium (2  $\mu$ l medium/brain), followed by boiling for 3 minutes and centrifugation to remove cellular debris. Brain extract was then filtered using Amicon Ultra filters (Millipore) to remove molecules that were greater than 10 KDa in size, including insulin. Individual glands (fed V2, V5, V6) or pairs of glands (fed V0, fed V1, starved V1, starved V2) were placed in Grace's insect culture medium (Invitrogen) or droplets containing brain extract for designated times. Experiments were terminated by placing glands directly into 2X sample buffer (for western blots) and boiling for 3 minutes, while medium was removed and stored at -20 °C for ecdysone RIAs. Ecdysone RIAs were conducted as previously described (Warren et al., 1984). The ecdysone antibody was produced in rabbits against an ecdysone-22-succinyl thyroglobulin synthesized by Dr. D.H.S. Horn (C.S.I.R.O., Canberra, Australia). [<sup>3</sup>H] Ecdysone (60 mCi/mmol) was obtained from GE Healthcare.

#### *Statistical analysis*

Ecdysone RIA data was subjected to a one way ANOVA test for statistical significance, with a Student-Keuls-Newman Multiple Comparisons post hoc test applied to compare specific experimental differences. For Western blot analysis, the one-way ANOVA was used to determine statistical significance. A Tukey-Kramer Multiple Comparisons post hoc test was applied to compare treatments. In all cases,  $p < 0.05$  was used to determine significance. All statistical analysis was performed using InStat v.3.0 (GraphPad Software, 2008).

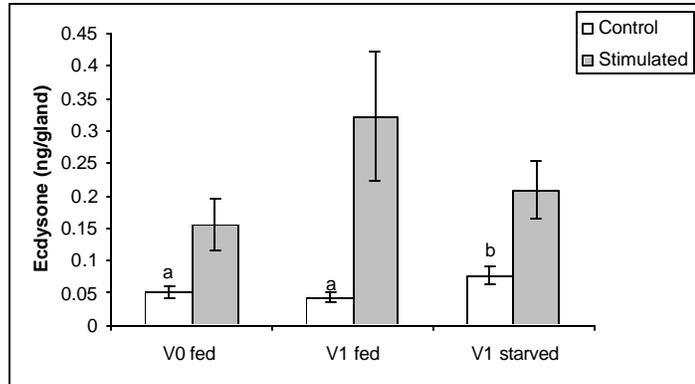
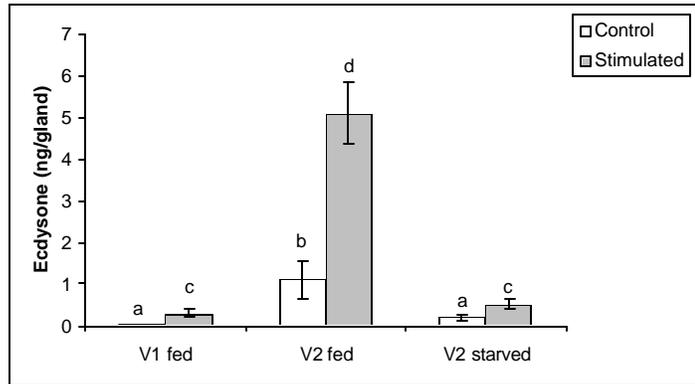
## RESULTS

### **Changes in ecdysone secretion due to starvation upon entry into the fifth instar**

It was shown that when the fifth instar larvae are starved, the prothoracic glands stop growing. It was expected that concurrent with the inhibition of growth, the glands would decrease their ability to secrete ecdysone. The head capsule slips forward at the end of the fourth instar, indicating that the larva will molt in the next 12 to 24 hours. These larvae were identified and starved for 24 hours post ecdysis into the fifth instar. Normally at this time in development the glands do not have high secretory capacity (Lee et al., 1995) and little growth is occurring (Chapter 1, Figure 3). However, this was when nutrient deprivation had the largest impact on insulin receptor and 4EBP transcript (Chapter 2, Figures 10 & 11), therefore it was predicted that there would be a reduction in ecdysone secretion. Figure 17A shows the ability of starved prothoracic glands to secrete ecdysone both basally and when the glands were stimulated with PTTH-containing brain extract. Starvation impacted basal ecdysone secretion, but not in the way expected. As seen in Figure 17A, basal levels of secretion were significantly higher in the starved glands relative to age-matched V1 fed controls (Student-Newman-Keuls test,  $p < 0.05$ ). This result may simply be due to a small sample size and a high variance between samples. Further experimentation is required to confirm this finding. There was no difference in basal secretion in glands from starved larvae in comparison to weight matched V1 fed larvae (Student-Newman-Keuls test,  $p > 0.05$ ). The ability of the glands to secrete ecdysone in response to stimulation was not significantly different in starved glands, weight-matched or age-matched glands (one-way ANOVA,  $F = 1.254$ ,  $p = 0.3105$ ).

Due to the fact that during normal development the glands were able to secrete relatively little hormone on the first day of the instar, the larvae were starved for a second day. This allowed for comparison between glands that were capable of higher levels of ecdysone secretion. As demonstrated in Figure 17B, the trend in basal secretion was significant in the expected direction (one way ANOVA,  $F=4.430$ ,  $p=0.0225$ ). Specifically, basal secretion from glands of larvae that were starved for 48 hours showed a significant decrease in secretion relative to age-matched fed larvae (Student-Newman-Keuls Multiple Comparisons test,  $p<0.05$ ). As expected, the basal secretion in V2 starved larvae was similar to the basal secretion of V1 weight-matched fed larvae (Student-Newman-Keuls Multiple Comparisons test,  $p>0.05$ ) as basal secretion in V1 larvae was normally low.

The trend in stimulated secretion following two days' starvation was significant as well (one way ANOVA,  $F=33.579$ ,  $p<0.0001$ ). When glands were stimulated there was a massive decrease in the ability of the V2 starved glands to secrete ecdysone relative to the V2 age-matched fed controls (Student-Newman-Keuls Multiple Comparisons test,  $p<0.001$ ). Upon stimulation, the starved glands did only moderately better than their weight-matched fed counterparts, which again was expected (Student-Newman-Keuls Multiple Comparisons test,  $p>0.05$ ).

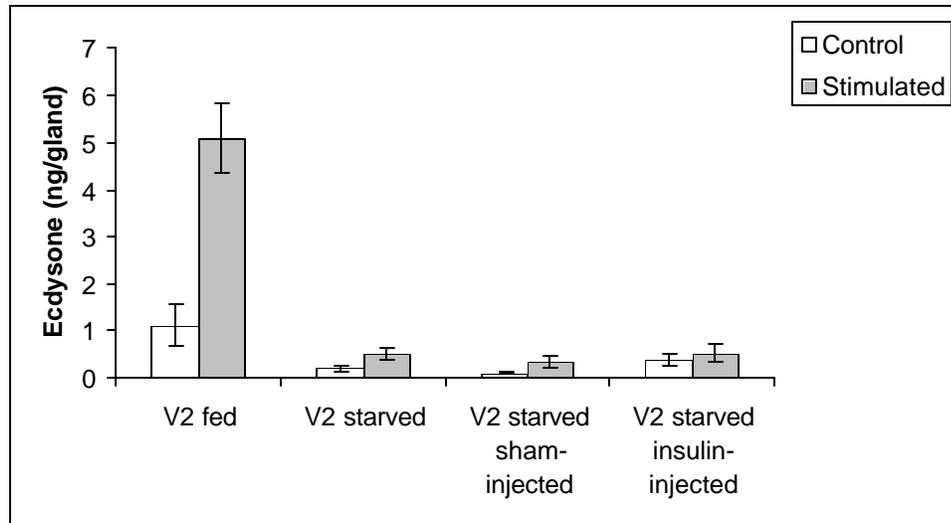
**A****B**

**Figure 17.** Effects of starvation on *in vitro* ecdysone secretion in *Manduca sexta* larvae.

**A.** Larvae were starved for 24 hours upon entry into the fifth instar. Glands were removed and incubated for 2 hours in Grace's medium with PTH-containing brain extract (stimulated) or medium alone (control). Following incubation, secreted ecdysteroids were assayed using an ecdysone RIA. Starved larvae ( $n=7$ ) were compared to weight matched (V0,  $n=5$ ) and age matched (V1,  $n=8$ ) control larvae. Each bar represents the mean  $\pm$  SE. There was a significant trend in the basal secretion of the glands due to starvation (one-way ANOVA,  $F=3.733$ ,  $p=0.0453$ ). Also there was a significant increase between the basal secretion of the starved larvae and age matched V1 fed controls as designated by "b" (Student-Newman-Keuls test,  $p<0.05$ ), but not between starved larvae and weight matched V0 controls as designated by "a" (Student-Newman-Keuls test,  $p>0.05$ ). There was no significant difference in the secretion upon stimulation (one-way ANOVA,  $F=1.254$ ,  $p=0.3105$ ). **B.** Larvae were starved for 48 hours after entry into the fifth instar. Secretion from glands of starved larvae ( $n=9$ ) was compared to weight matched (V1,  $n=8$ ) and age matched (V2,  $n=10$ ) control larvae. Each bar represents the mean  $\pm$  SE. The trend in basal secretion due to starvation was significant (one way ANOVA,  $F=4.108$ ,  $p=0.0292$ ). There was a significant decrease in basal secretion of starved larvae in comparison to fed V2 larvae (Student-Newman-Keuls Multiple Comparisons test,  $p<0.05$ ) as designated by "b", but not in comparison to weight matched V1 larvae (Student-Newman-Keuls Multiple,  $p>0.05$ ) as designated by "a". The trend in stimulated secretion was significant as well (one way ANOVA,  $F=31.331$ ,  $p<0.0001$ ). There was a significant decrease in secretion of stimulated glands from starved larvae in comparison to glands from age matched V2 fed larvae (Student-Newman-Keuls test,  $p<0.001$ ) as designated by "d".

### **Effect of insulin on ecdysone secretion in larvae starved for 48 hours**

In order to determine if there was a direct effect of insulin on ecdysone secretion, newly molted fifth instar larvae were injected with insulin and given a nonnutritive 3% agar block for 48 hours. After 48 hours, their prothoracic glands were removed and ecdysone titers were assessed *in vitro*. To ensure that injection itself did not alter ecdysone secretion, newly molted fifth instar larvae were sham-injected and starved for 48 hours. Finally, other larvae were simply starved. Fed larvae were included as controls to demonstrate normal ecdysone secretion. It was predicted that insulin would increase the ability of prothoracic glands from starved larvae to secrete ecdysone. Figure 18 demonstrates that glands removed from insulin-injected starved larvae showed no significant increase in basal ecdysone secretion relative to starved or sham-injected starved larvae (Kruskal-Wallis nonparametric ANOVA,  $KW=4.519$ ,  $p=0.1044$ ). Additionally, there was no significant difference in stimulated ecdysone secretion when the starved larvae were treated with insulin (Kruskal-Wallis nonparametric ANOVA,  $KW=1.026$ ,  $p=0.5986$ ). This indicated that insulin alone in starved larvae does not rescue ecdysone secretion.

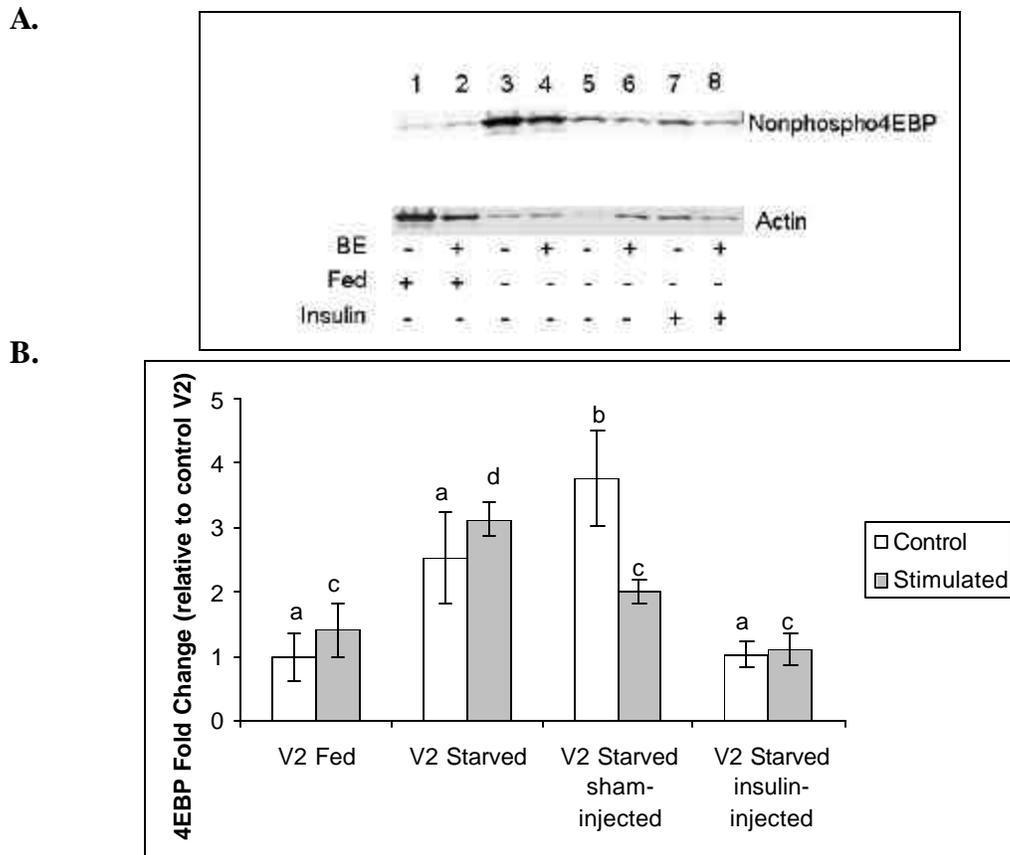


**Figure 18.** Effects of insulin injection on ecdysone secretion in *Manduca sexta* larvae starved for 48 hours. Fed larvae ( $n=10$ ) were fed *ad libitum*. Starved larvae were fed nonnutritive 3% agar. Treated larvae were injected with sterile distilled water or 50  $\mu$ g insulin post head capsule slippage and removed from normal diet for 48 hours. Glands were removed and incubated for 2 hours in Grace's medium with PTTH-containing brain extract (stimulated) or medium alone (control). Following incubation, secreted ecdysone was assayed using an ecdysone RIA. Fed larvae were not included in statistical analysis. Starved larvae ( $n=9$ ) were compared to sham-injected ( $n=9$ ) and insulin-injected ( $n=10$ ) larvae. Each bar represents the mean  $\pm$  SE. There was no significant difference between the basal secretion of any of the starved glands (Kruskal-Wallis nonparametric ANOVA,  $KW=4.519$ ,  $p=0.1044$ ). There was no significant difference between the stimulated secretion of any of the starved glands (Kruskal-Wallis nonparametric ANOVA,  $KW=1.026$ ,  $p=0.5986$ ).

In order to confirm that the insulin that was injected into the larvae could affect the prothoracic glands *in vivo*, Western blots were run and 4EBP protein content was quantified. It was expected that active insulin would reduce the cellular content of non-phospho4EBP, which normally increases due to starvation (Puig and Tjian, 2006). The results are shown in Figure 19. Starvation, as expected, increased glandular levels of non-phospho4EBP (compare, for example, starved and fed V2, subsequently stimulated with PTTH-containing brain extract). By contrast, injection of insulin reduced non-phospho4EBP to levels indistinguishable from V2 fed controls. Further, there was a significant decrease in non-phospho4EBP between insulin-injected and sham-injected starved larvae (Tukey-Kramer Multiple Comparisons test,  $p < 0.01$ ). This demonstrated that the reduction in 4EBP was not due to injection alone. It is unclear why there appears to be a decrease of non-phospho4EBP levels in prothoracic glands from sham-injected larvae that were treated with brain extract. If the brain extract alone were acting to lower non-phospho4EBP levels, then it would be expected that this result would be seen in all the treatments.

JH levels have been shown to remain high when larvae are starved (Cymborowski et al., 1982; MacWhinnie et al., 2005). Additionally, the presence of JH at this time is inhibitory to both the release of PTTH and secretory capability of the glands (Ciancio et al., 1986). Therefore, an effort was made to remove JH from starved larvae so that the effect of insulin in the absence of JH could be assessed. To do this, larvae were head-ligated just after ecdysis into the fifth instar, thus effectively removing the source of the JH while mimicking starvation. Head-ligated larvae were injected with insulin, sham injected, or left alone, in order to determine the effect of insulin on

ecdysone secretion in the absence of JH. Unfortunately in all cases ecdysone titers were not measurable. This was likely due to the top portion of the prothoracic glands being damaged by the ligation, resulting in drastic reductions in ecdysone secretion.



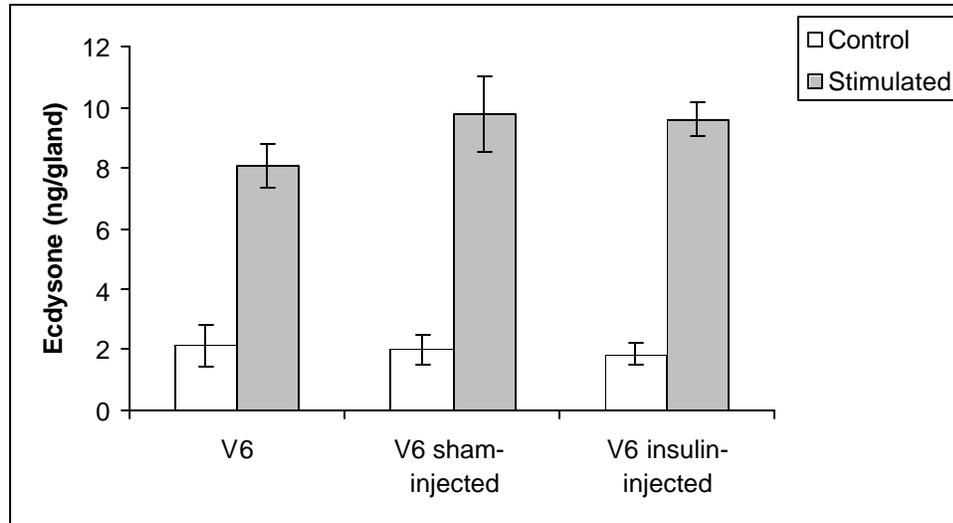
**Figure 19.** Effects of insulin injection on 4EBP in starved *Manduca sexta* larvae. **A.** Representative Western blot. Fed larvae were fed *ad libitum*. Starved larvae were fed nonnutritive 3% agar. Treated larvae were injected with sterile distilled water or 50 ug insulin post head capsule slippage and removed from normal diet for 48 hours. Glands were then removed and incubated for 2 hours in PTTH-containing brain extract or medium alone. The samples were subsequently run on gels and transferred. The non-phospho4EBP band corresponds to the expected size of 20 kD, and the actin band corresponds to the expected size of 45 kD. **B.** Quantification of non-phospho4EBP Western blots analyzed using ImageJ (Abramoff et al., 2004). V2 fed larvae ( $n=6$ ) were compared to starved V2 ( $n=5$ ), water injected ( $n=5$ ) and insulin injected ( $n=5$ ) larvae. Each bar represents the mean  $\pm$  SE. The trend in non-phospho4EBP protein in control prothoracic glands was significant (one-way ANOVA,  $F=7.787$ ,  $p=0.0017$ ). There was no significant difference between V2 fed larvae and insulin-injected larvae as designated by “a” (Tukey-Kramer Multiple Comparisons test,  $p>0.05$ ). There was a significant difference in protein abundance in V2 fed larvae in comparison to V2 starved larvae injected with water (Tukey-Kramer Multiple Comparisons test,  $p<0.01$ ) as designated by “b”. A similar trend was seen in prothoracic glands that were brain extract-treated (one-way ANOVA,  $F=7.787$ ,  $p=0.0017$ ). In these matched-pairs, there was a significant increase in 4EBP abundance in prothoracic glands of starved V2 in comparison to fed V2 (Tukey-Kramer Multiple Comparisons test,  $p<0.01$ ) as designated by “d”. There was also a significant decrease in non-phospho4EBP in prothoracic glands from larvae that were insulin treated relative to larvae that were not treated (Tukey-Kramer Multiple Comparisons test,  $p<0.01$ ).

### **Effect of insulin on ecdysone secretion in post-wandering larvae**

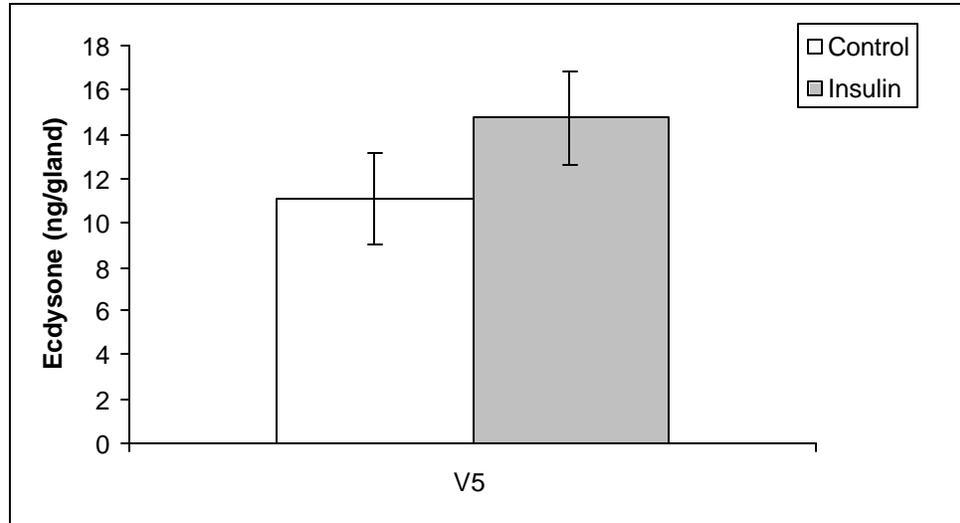
On the fifth day of the last larval stage, *M. sexta* larvae exhibit wandering behavior due to a “commitment” pulse of ecdysone (Introduction, Figure 1). At this point the larvae cease feeding. In *B. mori*, this is when the majority of growth occurs in the prothoracic glands concurrent with an increase in bombyxin/insulin titers (Saegusa et al., 1992). Also, it has been shown that insulin causes a significant increase in ecdysone secretion in larvae late in the last instar (Gu et al., 2009). Although the growth pattern of the glands differs between *M. sexta* and *B. mori*, it was of interest to determine if the effect of insulin on ecdysone secretion later in the fifth instar seen in *B. mori* was the same in *M. sexta*. To this end, larvae were injected with insulin, or sham-injected, on the day of wandering. The next day glands were removed in pairs and incubated for 1.5 hours in PTTH-containing brain extract or medium alone. The medium was then assayed for ecdysone content. As seen in Figure 24 insulin injection had no effect on subsequent secretory activity. There was no difference in the basal secretion in glands removed from treated larvae or control larvae (Kruskal-Wallis nonparametric ANOVA, KW=0.1050, p=0.9489). Additionally, when the glands were stimulated with PTTH, there was no change in ecdysone secretion (Kruskal-Wallis nonparametric ANOVA, KW=3.185, p=0.2034). This indicated that insulin had no direct effect on ecdysone secretion in post-wandering larvae.

In order to further confirm the idea that insulin did not directly impact ecdysone secretion and refute the notion that the result was due to the inability of insulin to act *in vivo*, glands were removed from larvae at the time of wandering and incubated in insulin for eight hours. As demonstrated in Figure 25, there was no significant difference in the

basal secretion of ecdysone from glands incubated in insulin as compared to matched controls (paired  $t$ -test,  $p < 0.1775$ ). This further confirmed that insulin has no direct effect on ecdysone secretion in post-wandering larvae.



**Figure 20.** Effects of insulin injection on ecdysone secretion in wandering *Manduca sexta* larvae. Larvae were injected with sterile distilled water or 50  $\mu$ g of insulin on the day they displayed wandering behaviors. The following day glands were removed and incubated for 1.5 hours in PTTH-containing brain extract (stimulated) or medium alone (control). Following incubation, secreted ecdysone was assayed using an ecdysone RIA. Each bar represents the mean  $\pm$  SE of 8 individual glands. There was no significant difference in basal secretion (Kruskal-Wallis nonparametric ANOVA,  $KW=0.1050$ ,  $p=0.9489$ ). There was also no significant difference in secretion due to stimulation (Kruskal-Wallis nonparametric ANOVA,  $KW=3.185$ ,  $p=0.2034$ ).



**Figure 21.** Effects of 8 hour insulin incubation on ecdysone secretion from prothoracic glands of wandering *Manduca sexta* larvae. Glands from wandering larvae were removed and incubated for 8 hours in Grace's medium with 9.3  $\mu$ M insulin (stimulated) or medium alone (control). Following incubation, secreted ecdysteroids were assayed using an ecdysone RIA. Each bar represents the mean  $\pm$  SE of 9 individual glands. Insulin had no significant effect on ecdysone secretion (paired t-test,  $p < 0.1775$ ).

## DISCUSSION

It was hypothesized that in *M. sexta*, prothoracic glands were responsible for the assessment of critical weight due to insulin-directed growth. Concurrent with critical weight is a decline of JH that is permissive of the release of PTTH that results in ecdysone synthesis and release. Therefore, if insulin was regulating the determination of critical weight it was expected that there would be an impact of insulin on ecdysone secretion. It has been shown that in response to nutrient deprivation there was an increase in insulin receptor transcript, particularly upon entry to the fifth instar. It was expected that a decrease in ecdysone secretion would coincide with nutrient deprivation, and that the effect would be reversed when the larvae were treated with insulin.

In *M. sexta* the growth of the prothoracic glands between days one and four of the fifth instar is correlated with the ability of the glands to secrete ecdysone (Sedlak et al., 1983; Smith and Pasquarello, 1989; Hanton et al., 1993; Lee et al., 1995). As shown in Chapter 2, glandular growth is inhibited by starvation, accompanied by a large increase in insulin receptor and 4EBP transcript due to nutrient deprivation. Therefore, it was of interest to determine if there would be a concurrent reduction in ecdysone secretion due to starvation. When larvae were starved upon entry into the fifth instar for 24 hours there was an unexpected increase in basal ecdysone secretion by the prothoracic glands of starved larvae in comparison to age-matched fed larvae. Secretion during the first 24 hours was very low, in both starved and fed glands; hence further experiments are needed to confirm a real difference.

In order to better determine the effect of nutrient deprivation on ecdysone secretion when the prothoracic glands are normally able to produce more ecdysone (V2-

V4), larvae were starved for an additional day. When the larvae were starved for two days, there was a significant decrease in the ability of the glands to secrete ecdysone relative to fed controls. This indicated that nutritional deprivation impacted the steroidogenic activity of the prothoracic glands.

The effects of starvation on ecdysone secretion were not reversed by insulin injection. This was despite a clear effect of injected insulin on glandular levels of 4EBP. It is possible that, despite this efficacy, insufficient levels of insulin were achieved to fully restore ecdysone secretory capacity. However, under a treatment of a single injection of insulin, insulin does not appear to directly stimulate the secretion of ecdysone.

A complicating factor was that circulating JH levels remain high in starved larvae, when they would normally decrease (Cymborowski et al., 1982; Lee and Horodyski, 2006; Tauchman et al., 2007). It is likely that JH acts as an inhibitory signal in starved prothoracic glands as it does in developing prothoracic glands. Unfortunately, the effect of insulin on ecdysone secretion in the absence of JH could not be quantified. Recent experiments on *M. sexta* wing discs indicate that insulin is capable of initiating commitment to pupate despite the presence of JH (Koyama et al., 2008). However, as shown in the present study, insulin does not appear to be capable of overriding JH to stimulate the prothoracic glands to secrete ecdysone.

In the silkworm, *Bombyx mori*, there is a direct link between ecdysone secretion and insulin signaling (Gu et al., 2009). The difference in this species and *M. sexta* may be explained by the difference in growth pattern of the prothoracic glands. Specifically, prothoracic gland growth in *B. mori* occurs concurrently with the commitment peak of

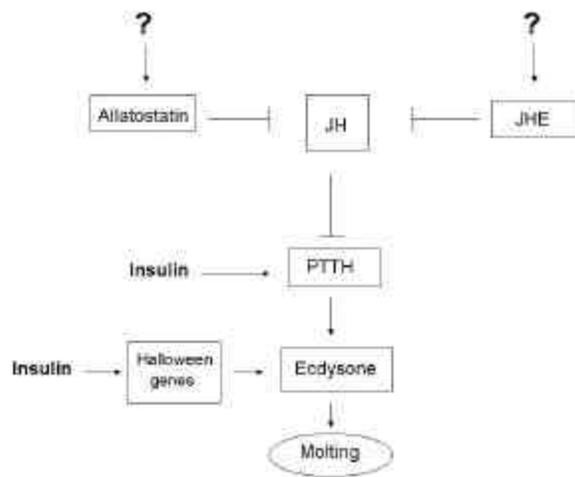
ecdysone when the larvae have ceased feeding (Mizoguchi et al., 2001). In contrast, the majority of the growth in the glands of *M. sexta* occurs prior to the commitment peak, while the larvae are still feeding. Therefore in *B. mori*, insulin is essential for growth and ecdysone secretion at a time when the two processes occur concurrently, as opposed to discrete processes as seen in *M. sexta*.

In *D. melanogaster* although there seems to be a direct link between insulin and ecdysone secretion, the means by which insulin stimulates ecdysone secretion is largely unknown. One complicating factor is that it is difficult to obtain direct measurements of ecdysone secretion in *D. melanogaster* due to their small size. Synthesis of ecdysone and secretion of ecdysone are closely linked as the glands themselves do not actually store hormone (Nijhout, 1994). Therefore, one way to measure ecdysone secretion in flies due to changes in insulin signaling is the quantification of ecdysone biosynthetic pathway members, particularly cytochrome P450 genes known as the Halloween genes (Colombani et al., 2005). In flies, two of the Halloween genes have been shown to increase in response to mutations that up-regulate PI3K signaling in prothoracic glands (Colombani et al., 2005). In contrast, using direct measurements of ecdysone secretion, no increase is detected in PI3K enlarged glands (Mirth et al., 2005).

In *M. sexta*, insulin alone was not able to directly stimulate ecdysone. Instead it seems that other hormones may be playing a larger role than initially thought. For example, JH may be ultimately driving the metamorphic molt. It has been shown that JH is an important regulator of ecdysone secretion, as in its presence the glands were inhibited (Nijhout and Williams, 1974b; Ciancio et al., 1986). The elimination of JH by surgical removal of the corpora allata (allatectomy), the tissues that synthesize and

secrete JH, gives a clearer picture of the role of JH. For example, it has been shown that allatectomy in fourth instar *M. sexta* larvae caused pupation (Kiguchi and Riddiford, 1978). This implies that the removal of JH alone is able to drive the metamorphic molt. Further proof of the importance of JH is that similar studies have suggested a decline in JH activates the prothoracic glands in *B. mori* (Sakurai et al., 1989). Therefore, in order to understand the switch that initiates the metamorphic molt, it is critical to determine the regulation of JH decline.

The decline of JH in the beginning of the fifth instar is known to be under the control of two hormones, allatostatin and juvenile hormone esterase (JHE) (Figure 22). Allatostatin works by reducing the synthesis of JH from the corpora allata. Once there is a decline in JH titers, there is an increase of JHE from the fat body (Baker et al., 1987). JHE works by catabolizing remaining JH in the hemolymph (Hammock and Roe, 1985). Once JH levels decline, PTTH release is enhanced, as is glandular response to PTTH (Nijhout and Williams, 1974b; Ciancio et al., 1986). Understanding the regulation of both allatostatin and JHE could provide insight into the primary regulation of ecdysone secretion (Figure 22).



**Figure 22.** Proposed model for insulin involvement in initiation of the metamorphic molt. Abbreviations: JH= juvenile hormone and JHE= juvenile hormone esterase. Insulin may be working to enhance PTTH secretion once JH levels have declined due to regulation by allatostatin and JHE. Additionally, insulin may increase ecdysone secretion by up-regulating the transcription of the Halloween genes (enzymes involved in steroidogenesis).

Even though insulin alone is not driving this process, the role of insulin may still be integral to key elements in the initiation of metamorphosis (Figure 22). For example, insulin may have a role in stimulating the biosynthesis of enzymes involved in steroidogenesis, such as the Halloween genes. Transcripts of three of these genes have been characterized in *M. sexta* and have been shown to increase concomitantly with ecdysone titers (Rewitz et al., 2006). More recently one of these genes, Spook (Spo), was identified as a possible rate-limiting enzyme in the ecdysone biosynthetic pathway (Rewitz et al., 2009). As there was no direct effect of insulin on total ecdysone secretion in *M. sexta*, it is likely that the transcription and translation of Spo is directly regulated by PTTH alone, while it is possible that insulin may impact the synthesis of downstream members of the Halloween genes in preparation of ecdysone secretion. It would be

interesting to quantify transcripts of these genes in response to insulin stimulation and nutrient deprivation. If the transcripts are up-regulated by insulin, this would allow for the secretion of ecdysone from the prothoracic glands to occur both rapidly and in large quantities upon PTTH stimulation.

A second possibility, it is that insulin may be enhancing the release of PTTH from the brain. A high amount of insulin in the hemolymph would then be able to ensure maximal stimulation of the glands by PTTH. Alternatively, insulin may be up-regulating the PTTH receptor on the prothoracic glands, allowing the glands to be more responsive to PTTH. Just recently the sequence of the PTTH receptor in *D. melanogaster* was deduced (Rewitz et al., in press). This sequence could be used to determine the sequence of the PTTH receptor in *M. sexta*. Once the sequence is obtained it could be used to quantify PTTH receptor transcript in prothoracic glands, first developmentally, then in response to both nutrient deprivation and insulin stimulation. This would demonstrate that insulin is responsible for the sensitivity of the glands to PTTH.

Although insulin was not sufficient to replace nutrients causing a direct stimulation in ecdysone secretion in *Manduca sexta* prothoracic glands, it is likely to be involved in processes integral to metamorphosis that have yet to be fully understood. Vertebrate models indicate that although insulin/IGFs are important in the regulation of steroidogenic tissues, they do not directly stimulate secretion of hormones. Instead, they work farther upstream to regulate factors capable of directly stimulating steroidogenesis. Further experimentation is necessary to determine the exact role of insulin, in *M. sexta*, in the regulation of prothoracic glands function. This knowledge will determine if *M. sexta*

prothoracic glands function in a way analogous to the vertebrate model, as the present study suggests, or in a similar manner to the prothoracic glands of *D. melanogaster*.

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