EXPRESSION OF HIF-1 ALPHA AND HIF-1 BETA IN INSECTS THROUGHOUT JUVENILE DEVELOPMENT

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

Insects grow exponentially from hatching to adult. This growth is punctuated by periodic molts during which the exoskeleton, including the large airways of the respiratory system, is enlarged and replaced. Major tracheae increase in size after molting, fixing gas exchange capacity within an instar. Therefore, I hypothesize that larvae may become hypoxic at the end of each instar.

One regulator of responses to hypoxia is the oxygen-sensing protein complex hypoxia-inducible factor 1 (HIF-1). In hypoxia, HIF-1α and HIF1β dimerize to form the HIF-1 complex, a transcription factor that controls expression of hypoxia-responsive genes. To test my hypothesis, I measured gene expression of HIF-1α and HIF-1β across various stages of larval development in the tobacco hornworm, *Manduca sexta*. As predicted, levels of HIF-1α and HIF-1β increase within an instar and decrease after molting. Understanding normal development of insect respiratory systems is important, because insects are crop pests and critical pollinators.

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CHAPTER ONE: INTRODUCTION

For normal growth and development, most animals must maintain an adequate level of oxygen for aerobic production of adenosine triphosphate (ATP). Maintaining oxygen homeostasis is critical for survival in all animals. At the cellular level there are mechanisms in place to compensate for a decrease in oxygen and to maintain energy production. A key player in maintaining energy by oxygen regulation is hypoxia inducible factor 1 (HIF-1). HIF-1 responds to changes in oxygen availability and provides cells with instructions for gene transcription to maintain oxygen homeostasis.

Hypoxia is described as a decrease in the concentration of available oxygen. Hypoxia may occur at high altitude, during diving or drowning, in habitats with restricted airflow, such as underground burrows or cavities. Aquatic animals that live in certain waters (e.g., high altitude lakes or those with algal blooms) may also experience hypoxia. Hypoxia can also occur just at the tissue level when oxygen delivery is decreased, either through a decrease in supply (e.g., myocardial infarction) or an increase in demand that outstrips supply (e.g., heavy exercise). In many organisms, decreases in tissue oxygen levels activate HIF-1, which leads to the activation of hypoxia-related genes that aid in increasing oxygen delivery and, thus, aerobic ATP production.

In this chapter, I review the oxygen-signaling pathway of the nuclear transcription factor, HIF-1, and its subunits, HIF-1α and HIF-1β. In addition, I describe the role of HIF-1α and HIF-1 in oxygen sensing. Finally, I will describe one situation in which HIF-1 may be important in insect development. Because many physiological processes in insects and mammals are similar, understanding how HIF-1 functions in insects may give us insight into its function in mammals. Understanding its role in insect development is particularly important, because juvenile insects

are common pest species on crops. In addition, insects are notably hypoxia tolerant and are able to thrive in harsh conditions, and understanding how HIF-1 functions in a hypoxia-tolerant animal could provide inspiration for treatment of hypoxia-related injuries and illnesses in humans.

HIF-1 Overview

Insects that experience hypoxic conditions have mechanisms to cope with decreased oxygen. In low oxygen, insects may have altered morphological traits, such as increased tracheal branching (Centanin et al., 2008) and diameter (Loudon, 1989) or changes in behavior, such as increased abdominal pumping and ventilation frequency (Greenlee & Harrison, 1998b, 2004b). Therefore, researchers knew that there must be some signal that initiates those changes, so scientists searched for a putative oxygen sensor. One such molecule was identified in the early 1990's, HIF-1 (Semenza, Nejfelt, Chi, & Antonarakis, 1991).

HIF-1 is an important transcription factor in the response to hypoxia. In all animals that experience hypoxia, HIF-1 is responsible for initiating the transcription of hypoxia-related genes (i.e., genes that contain a hypoxia response element, HRE). As such, HIF-1 controls many vital physiological pathways, such as angiogenesis, erythropoiesis, glucose and iron transport, glycolysis, tricarboxylic acid cycle, cell proliferation, and apoptosis. (Ke & Costa, 2006; Prabhakar & Semenza, 2012). Many of the gene products result in increased oxygen delivery, including, but not limited to vascular endothelial growth factor (VEGF; Forsythe et al., 1996; Iyer et al., 1998), inducible nitric oxide synthase (NOS; Palmer & Johns, 1998), transferrin (Rolfs, Kvietikova, Gassmann, & Wenger, 1997), and glucose transporter-1 (GLUT-1; Ebert, Firth, & Ratcliffe, 1995; Iyer et al., 1998). The discovery of the HRE (5'-RCGTG-3') in the erythropoietin gene (EPO) uncovered more knowledge of the HIF-1 pathway (Goldberg,

Dunning, & Bunn, 1988; Semenza et al., 1991). Following that discovery, the binding of HIF-1 to the HRE was shown to occur under hypoxic conditions (Semenza et al., 1991), firmly establishing HIF-1 as an oxygen sensor.

Evolutionarily, the genes encoding for HIF-1α and HIF-1β have been conserved across a range of taxa (Loenarz et al., 2011; Nikinmaa & Rees, 2005). A search of the NCBI database revealed 888 taxa, ranging from chordates to viruses, with conserved HIF-1α gene sequences (http://www.ncbi.nlm.gov/nuccore; April 18, 2016). Due to HIF-1's highly conserved sequence and its role in the response to hypoxia, the interest in studying HIF-1 has grown over the years.

HIF-1 Structure and Function

HIF-1 is composed of two protein subunits, HIF-1α and HIF-1β. Analysis of the cDNA sequences of HIF-1α reveals a nucleotide sequence of approximately 826 base pairs (B. H. Jiang, Zheng, Leung, Roe, & Semenza, 1997). HIF-1β contains 789 base pairs and has been termed the aryl hydrocarbon receptor nuclear translocator (ARNT) protein (Wang, Jiang, Rue, & Semenza, 1995). HIF-1β is a ubiquitiously expressed protein that is capable of dimerizing with other proteins due to its bHLH PAS domain (Wang et al., 1995), whereas HIF-1α is only known to bind with HIF1- β and the defining subunit of the HIF-1 complex (Figure 1;Huang, Arany, Livingston, & Bunn, 1996).

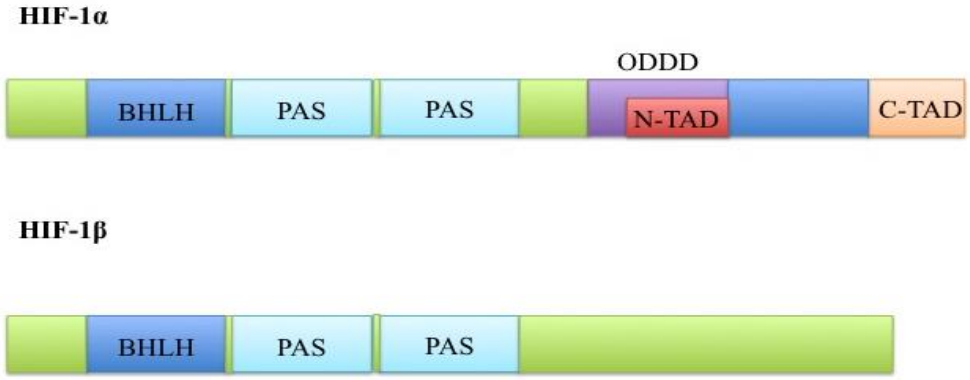

Figure 1. The structure of the HIF-1α and HIF-1β genes. These genes contain a basic helix-loop-helix (bHLH) motif and Per-ARNT-Sim (PAS) domains. The bHLH and PAS domains aid in dimerization and the binding of the subunits to DNA. The carboxy-terminal transactivation domains, N-TAD and C-TAD, serve as regulatory and transactivation regions. The oxygen-dependent degradation domain, ODDD, is the site where HIF-1 is tagged for degradation by proline hydroxlase. Re-drawn from Semenza (1999).

HIF-1α and HIF-1β Gene Regulation

Hypoxia-related gene expression is controlled by the HIF-1α gene, which encodes for the HIF-1α protein subunit. Many things control the gene expression of HIF-1α. The HIF-1α gene has been shown to increase when there is an increase in reactive oxygen species (ROS) in a dose dependent manner. Specifically thrombin induces the NFκB pathway that increases HIF-1α expression by increasing ROS (Bonello et al., 2007). Further support of ROS's role in HIF-1α gene regulation was found by Huang et al. (1996) they showed that in hypoxia and in normoxia ROS activate HIF-1α. However, Kim, Cho, Chun, Park, and Kim (2002) showed that, in rats, there was no change in HIF-1α mRNA expression in hypoxia, but they did see a increase in protein expression. Other findings show that HIF-1 mRNA increases in the presence of lipopolysaccharides (LPS; Frede, Stockmann, Freitag, & Fandrey, 2006). More researchers have shown that angiotensin II (ANG II) increases HIF-1 mRNA expression, but there was no change in hypoxia (Page, Robitaille, Pouyssegur, & Richard, 2002). Fukuda et al. (2002) showed that insulin-like growth factor (IGF) doesn't induce mRNA expression but it does increase protein

expression. Other regulators of HIF-1α include the PI3K and NFκB pathways, which have been shown to increase HIF1- α mRNA expression upon activation and decrease HIF-1α mRNA expression upon inhibition (BelAiba et al., 2007). Together, these data indicate that regulation of HIF-1 at the transcription level may be more complex than previously thought.

HIF-1 Protein Regulation

In normoxic rodents, HIF-1α proteins are continuously expressed, ubiquitinated, and degraded (Figure 1). HIF-1α contains an oxygen-dependent degradation domain (ODDD) (Figure 2; Huang, Gu, Schau, & Bunn, 1998). If oxygen levels are adequate, the HIF-1α subunit is flagged for proteasomal degradation. In normoxia, HIF-1α proteins are bound by the von Hippel-Lindau protein (VHL) and prolyl-hydroxlases, which recruit ubiquitin protein ligases that mediate enzymatic degradation of HIF-1α by a proteasome (Kamura et al., 2000; Maxwell et al., 1999). As shown by Kallio et al. (1999), degradation of HIF-1α increases under normoxic conditions and significantly decreases in hypoxic conditions, further supporting the hypothesis that HIF-1α protein levels are increased by hypoxia. Interestingly, HIF-1α protein may also be stabilized in normoxia by insulin, insulin-like growth factor, transforming growth factor, interlukin-1, epidermal growth factor, and platelet-derived growth factor (Feldser et al., 1999; Gorlach et al., 2001; Haddad & Land, 2001; Hellwig-Burgel, Rutkowski, Metzen, Fandrey, & Jelkmann, 1999; Richard, Berra, & Pouyssegur, 2000; Stiehl, Jelkmann, Wenger, & Hellwig-Burgel, 2002; Zelzer et al., 1998), suggesting that the role of HIF-1a is more complex than previously thought.

HIF-1β is a common protein that mediates more than just HIF-1 activity. In normoxia, the HIF-1β subunit is continuously expressed, similar to the HIF-1α subunit. However, unlike HIF-1α, HIF-1β is not degraded and is available for binding with proteins. As an ARNT protein,

HIF-1β is capable of heterodimerizing with other proteins such as aryl hydrocarbon receptor (AHR) (Reyes, Reiszporszasz, & Hankinson, 1992) and single-minded (SIM) (Sogawa et al., 1995). However, in hypoxic conditions, the HIF-1β subunit moves into the nucleus.

Once HIF-1α and HIF-1β translocate to the nucleus, they dimerize to form the HIF-1 complex and bind to the HRE on target genes (B. H. Jiang, Rue, Wang, Roe, & Semenza, 1996). Then, RNA polymerase binds to the HIF-1 complex and the HRE, and transcription of hypoxia-relatedgenes occurs (Lando, Peet, Whelan, Gorman, & Whitelaw, 2002; Woo, Jeong, Park, & Kwon, 2004), increasing oxygen delivery to the oxygen-deprived areas (Lee, Bae, Jeong, Kim, & Kim, 2004; Semenza, 2002, 2003).

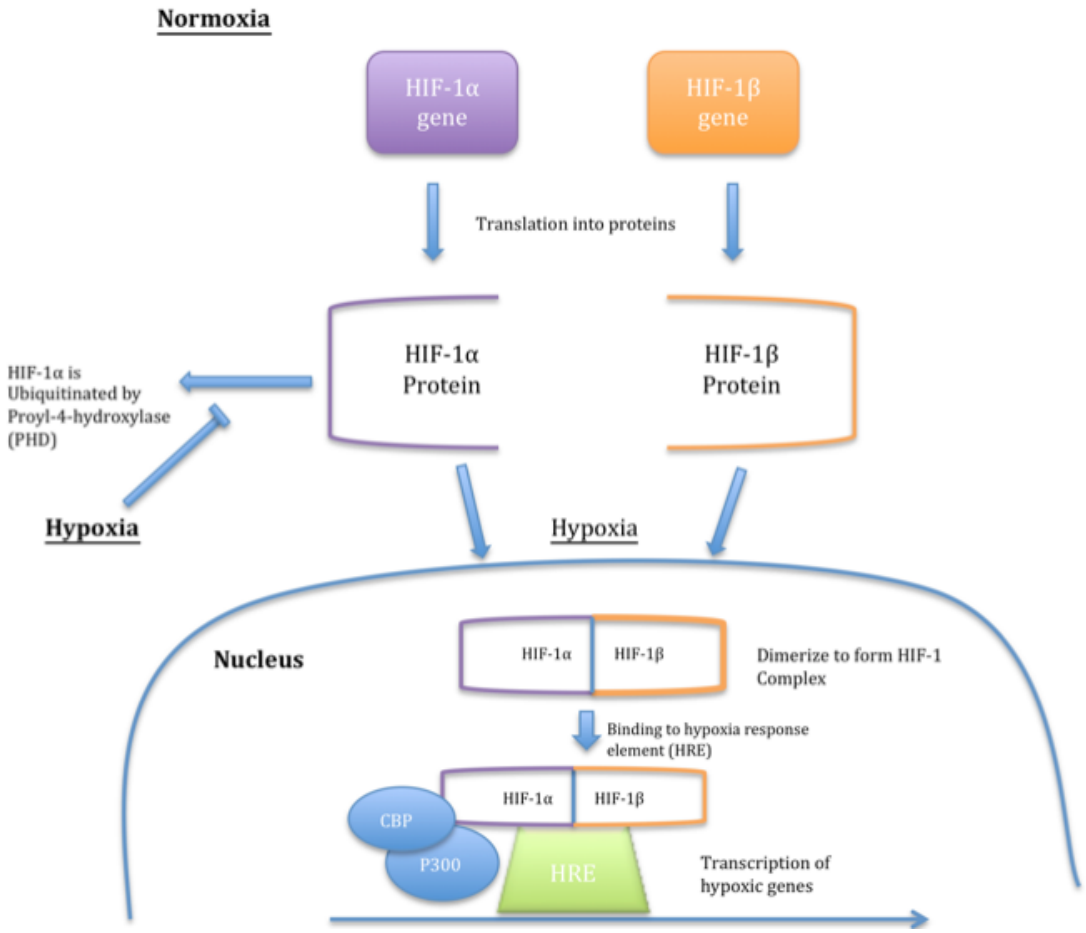

Figure 2. The process by which the hypoxia inducible factor 1 complex forms. First, HIF-1α and HIF-1β mRNA are transcribed and translated in normoxia. Both are continuously expressed in rodents, but HIF-1α is ubiquitinated and degraded in the presence of oxygen. When hypoxia occurs, HIF-1α is stabilized and can dimerize with HIF-1β, forming the HIF-1 complex. HIF-1α and HIF-1β translocate to the nucleus, where they dimerize to form the HIF-1 complex. Refer to text for more detail.

HIF-1 as a Transcription Factor: Regulation of Target Genes

Each HIF-1 subunit has special domains that are required for DNA binding. The N-termini of HIF-1α and HIF-1β proteins contain basic helix-loop-helix (bHLH) domains, which are responsible for DNA binding, and hence their ability to act as a transcription factor (Wang et al., 1995; Wang & Semenza, 1995). Both HIF-1α and HIF-1β also contain Per-ARNT-Sim homology (PAS) domains that are responsible for their heterodimerization (Hoffman et al.,

1991). The C-terminus of the HIF-1α subunit is composed of two transactivation domains (TADs), which regulate HIF-1 target genes. The TADs are known as N-TAD and C-TAD (B. H. Jiang et al., 1997; Pugh, Orourke, Nagao, Gleadle, & Ratcliffe, 1997). Two co-activators, CREB binding protein (CBP) and p300 interact with C-TAD and are both needed for HIF-1-mediated gene transcription to occur (Lando et al., 2002). N-TAD also stimulates HIF-1 transcription (Ke & Costa, 2006).

HIF-1 regulates gene transcription of many different proteins that contribute to increases in ATP production and oxygen delivery. One such protein is GLUT-1. GLUT-1 mRNA contains a HIF-1 binding site similar to that of EPO and other hypoxia-related genes and its expression is upregulated in hypoxia (Ebert et al., 1995). Enzymes, such as aldolase-A (ALD-A), enolase-1, lactate dehydrogenase-A (LDH-A), phosphofructokinase-L (PFK-L), and pyruvate kinase-M (PKM) have also been shown to increase as a result of transcriptional up-regulation of glycolytic genes in hypoxia. Upregulation of ALD-A, LDH-A, PFK-L, and PKM was shown by using agents such as cobalt chloride and desferroxamine that mimic hypoxia and upregulate EPO mRNA and HIF-1 activity (Firth et al., 1995; Semenza et al., 1994). Conservation of the HIF-1 binding site in the glycolytic genes leads to the conclusion that they are all upregulated by the same hypoxia signaling pathway that activates EPO transcription, HIF-1 (Semenza, Roth, Fang, & Wang, 1994).

HIF-1 in Invertebrates

Although HIF-1 is well studied in vertebrate systems (Acker & Acker, 2004; Gassmann & Wenger, 1997; Lando, Gorman, Whitelaw, & Peet, 2003; Schofield & Ratcliffe, 2004; Semenza, 2013; Semenza et al., 1994; Tam et al., 2009), little is known about HIF-1 in invertebrate systems. HIF-1 has been sequenced and characterized in few invertebrates,

including roundworms (*Caenorhabditis elegans*; H. Q. Jiang, Guo, & Powell-Coffman, 2001; Powell-Coffman, Bradfield, & Wood, 1998), fruit flies (*D. melanogaster*; Ma & Haddad, 1999; Nambu, Chen, Hu, & Crews, 1996; Ohshiro & Saigo, 1997; Sonnenfeld et al., 1997), and water fleas (Daphina magna; T. A. Gorr, Cahn, Yamagata, & Bunn, 2004). In *D. melanogaster*, HIF-1 function is similar to that of its mammalian homolog (Gorr et al. 2006). As in mammals, *D. melanogaster* HIF-1 levels peak in hypoxic tissues (~3-4% oxygen) (Lavista-Llanos et al., 2002). *D. melanogaster* HIF-1 is highly homologous to mammalian HIF-1; therefore, it has been widely used in a variety of research studies.

HIF-1 in Insects

Much of our understanding about the molecular regulation of insect HIF-1 comes from studies done on the tracheal respiratory system of *D. melanogaster*. The tracheal system develops during embryogenesis under the control of the *trachealess* gene (Isaac & Andrew, 1996; Wilk, Weizman, & Shilo, 1996), starting as a monolayer of cells wrapped in a tubular structure, then forming an interconnected network of tubes (Metzger & Krasnow, 1999). HIF-1α is pivotal to the development of normal tracheae, the large tubes of the respiratory network. As HIF-1α accumulates in the tracheolar cells, the blind-ended tubular cells that deliver oxygen to tissues from the tracheae, tracheal branching is induced , thereby increasing oxygen supply to newly developing tissues (Centanin et al., 2008). This occurs due to the high affinity of HIF-1 to the tracheolar cells expressing genes with HRE, giving the cells a higher sensitivity to hypoxia than any other cell (Centanin et al., 2008). Growth of the tracheal system is also controlled by HIF-1-activation of the *breathless* (*btl*) gene (Centanin et al., 2008), which encodes the fibroblast growth factor (FGF) receptor (Klambt, Glazer, & Shilo, 1992). The FGF receptor is activated by

the *branchless* (*bnl*) ligand (Sutherland, Samakovlis, & Krasnow, 1996) leading to increased cell motility and tracheal branching.

HIF-1 and Molting

One unresolved issue involving both HIF-1 and the insect tracheal system is the relationship between oxygen demand and molting. The process of molting in insects is also known as ecdysis or shedding of the exoskeleton, and it allows for growth of sclerotized or hardened structures (Chapman 1998). In insects, molting occurs periodically throughout the juvenile stages, and the duration between molts is called an instar. Before molting can occur, the old exoskeleton separates from the new epidermal layer, in a process called apolysis. Then, epidermal cells continue to enlarge, and molting fluid is secreted. Molting fluid contains proteases and chitinases that break down the old exoskeleton. Finally the insect abdomen undergoes contractions which helps to extricate the insect from its old exoskeleton (Chapman, 1998). Because an insect's respiratory system is an invagination of its exoskeleton, the tracheal system is also shed during a molt and increases in size only after molting. Thus, growth of the respiratory system may be limited until after molting, resulting in a possible imbalance between oxygen supply and demand.

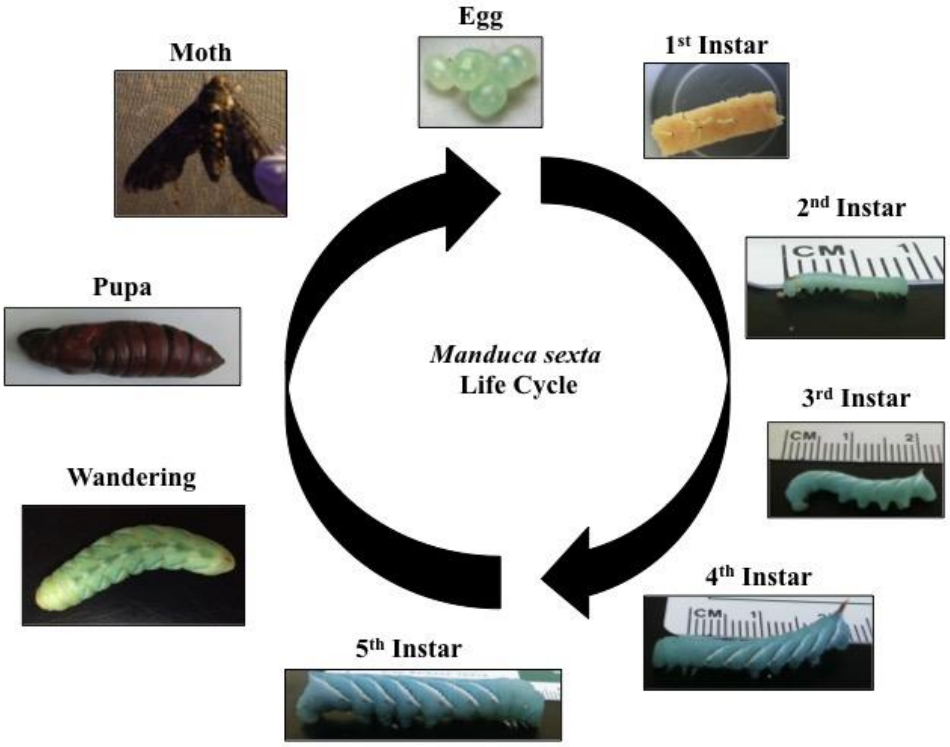

Figure 3. Life cycle of Manduca sexta, the tobacco hornworm. Body mass increases continuously from hatching to wandering. Between each of the instars, larvae molt. Wandering caterpillars molt before undergoing metamorphosis, and pupae molt prior to emerging as adult moths.

There are two potential times during juvenile growth when HIF-1 might be involved. First, within an instar there is an increase in oxygen demand (Greenlee and Harrison, 2005 and Greenlee and Harrison 2004), but the tracheal system does not increase in size (Callier & Nijhout, 2011), possibly resulting in hypoxia (Figure 2). As insects develop, they can more than double their body mass within an instar (Greenlee & Harrison, 2004a, 2005; Kirkton et al., 2012). Because absolute metabolic rate increases as body size increases, there is an increase in oxygen demand. If the tracheal system does not increase proportionately with body size, oxygen supply may not increase at the same rate as does oxygen demand. Indeed, in grasshoppers, *Schistocerca americana*, oxygen delivery does not match demand as they progress through an intermolt period, as evidenced by their decreased tolerance to hypoxia (Greenlee & Harrison,

2004b) and decreased air sac volumes (Clarke, 1957; Kirkton et al., 2012; Lease, Wolf, & Harrison, 2006). Caterpillars also had decreased hypoxia tolerance at the end of an instar compared to the beginning of the instar as metabolic rates increased (Greenlee & Harrison, 2005), suggesting that tracheal oxygen supply does not match demand. Callier and Nijhout (2011) showed that the tracheal system of *M. sexta* is fixed in volume within an instar, limiting oxygen delivery until they molt and increase the size of the tracheal system.

Interestingly, in the 5th instar, both tracheal mass and tracheal volume increased with body size in *M. sexta* larvae (Helm & Davidowitz, 2013). However, tracheal mass did not increase as much as the mass of the larvae. The tracheal mass makes up ~9% of the total body mass and decreases by ~ 0.6% with each additional gram of growth in the larvae, suggesting that oxygen supply may still not be adequate to keep up with increases in total body mass and, thus, oxygen demand (Helm & Davidowitz, 2013). Together, these findings support the hypothesis that insects are increasingly oxygen-limited as they grow within an instar (Callier & Nijhout, 2011; Greenlee & Harrison, 2005). If oxygen limitation is severe, insects may become hypoxic as they reach the end of an instar, which could trigger HIF-1 signaling.

The second situation, in which HIF-1 may be involved in insect growth, occurs at the end of the instar. When apolysis is occurring and the old cuticle and molting fluid are present, the tracheae and spiracles may become blocked. Tracheae and spiracle blockage could cause short-term hypoxia, eventually resulting in the molting of the larvae. In either situation, insects should become hypoxic near the end of an instar.

Future Directions

Because of their unique physiology, potential to naturally become hypoxic during their life cycle, and homologous genes, insects can provide exciting new directions in physiological research studies involving HIF-1 and its role in development. Further exploring the effects of hypoxia and the regulation of HIF-1 may help us understand the physiological adaptions to changes in oxygen. More research needs to be done on the role of HIF-1 in relationship to development. If we can bridge the gap between the relationship of HIF-1 and development it may further help us understand the underlying mechanisms of how an insect develops when they are oxygen limited.

CHAPTER TWO: INTRODUCTION

Insects exhibit two types of growth, continuous and discontinuous. First, continuous growth occurs in soft structures and is evidenced by measures of body mass, which increase exponentially from hatching to the penultimate instar. Discontinuous growth occurs in structures that are formed from the hardened exoskeleton and only increase in size after molting. The juvenile stage is punctuated with periodic molts during which the exoskeleton, including the large airways of the respiratory system, are enlarged and replaced (Chapman, 1998). Because the major tracheae only increase in size after a molt, researchers hypothesize that gas exchange capacity may be fixed within an instar (Callier & Nijhout, 2011; Clarke, 1957; Greenlee & Harrison, 2004a, 2005).

A consequence of the fixed respiratory capacity is that it may not keep up with increases in oxygen demand (Callier & Nijhout, 2011; Clarke, 1957; Greenlee & Harrison, 2004a, 2005). In grasshoppers, *Schistocerca americana*, oxygen delivery does not match demand as they progress through an intermolt period, and grasshoppers exhibit decreased tolerance to hypoxia (Greenlee & Harrison, 2004b), decreased jump performance (Kirkton et al., 2012), and decreased tracheal system volumes (Clarke, 1957; Kirkton et al., 2012; Lease et al., 2006). Caterpillars also may exhibit signs of mismatches between oxygen supply and demand, as they exhibit decreased hypoxia tolerance at the end of an instar compared to the beginning of the instar (Greenlee & Harrison, 2005). Callier and Nijhout (2011) showed that the tracheal system of *M. sexta* is fixed in volume within the third and fourth instars, limiting oxygen delivery until after caterpillars molt when the size of the tracheal system increases. Interestingly, in the 5^{th} instar, both tracheal mass and tracheal volume increased with body size in *M. sexta* larvae (Callier & Nijhout, 2011; Helm & Davidowitz, 2013). However, tracheal mass did not increase as much as the mass of the

larvae, suggesting that oxygen supply may be inadequate to keep up with increases in total body mass and, thus, oxygen demand (Helm & Davidowitz, 2013). Indeed, as insects develop, they can more than double their body mass within an instar (Greenlee & Harrison, 2004a, 2005; Kirkton et al., 2012), which could result in an increase in oxygen demand so high that it may exceed the oxygen delivery capacity of the tracheal system near the end of an instar. Together, these findings support the hypothesis that insects are increasingly oxygen-limited as they grow within an instar (Callier & Nijhout, 2011; Greenlee & Harrison, 2005).

If oxygen limitation within an instar is severe enough (i.e., increases in oxygen demand strongly outpace oxygen supply), insects may even become hypoxic as they reach the end of an instar, in turn triggering the hypoxia-inducible-factor (HIF-1) signaling pathway. Some researchers have hypothesized that being hypoxic at the end of an instar may even be a key signal for insects to molt to the next instar, since only after molting will the tracheal system increase in size (Callier & Nijhout, 2011; Greenlee & Harrison, 2005). Alternatively, insects may become hypoxic at the end of an instar as a consequence of the molting process itself. After apolysis and before ecdysis, the tracheal system may be blocked with molting fluid and old tracheal tissue, possibly leading to short-term hypoxia. In either case, there may be a mis-match between oxygen supply and demand or blocked tracheal system. In this paper, I will test the hypothesis that growing larvae become hypoxic at the end of each larval instar.

To determine if insects are hypoxic at the end of an instar, I need a measurable indicator of hypoxia. Hypoxia-inducible factor 1 (HIF-1) is that indicator. HIF-1 is an important transcription factor and a key regulator of cellular responses to hypoxia (Semenza et al., 1991). HIF-1 is composed of two protein subunits, HIF-1α and HIF-1β, both of which can be measured by quantitative PCR. HIF-1α is continuously expressed, ubiquitinated, and degraded in

normoxia (Thomas A. Gorr, Gassmann, & Wappner, 2006). HIF-1β is also continually expressed in normoxia, but not broken down (Huang et al., 1998; Pugh et al., 1997). In hypoxic conditions, HIF-1α dimerizes with HIF-1β, forming the HIF-1 complex (Wang & Semenza, 1995). HIF-1 binds to the HIF response element (HRE) on the promoter region of hypoxia-responsive genes and induces gene expression (Semenza et al., 1991). Levels of HIF-1 protein has been shown to increase during hypoxia in many animals including roundworms (Caenorhabditis elegans; H. Q. Jiang et al., 2001; Powell-Coffman et al., 1998), fruit flies (D. melanogaster; Ma & Haddad, 1999; Nambu et al., 1996; Ohshiro & Saigo, 1997; Sonnenfeld et al., 1997), water fleas (Daphina magna; T. A. Gorr, Cahn, et al., 2004), low-land butterflies (*Melitaea cinxia*; Marden et al., 2012), and cowpea bruchids (*Callobruchus maclatus*; Ahn, Zhou, Dowd, Chapkin, & Zhu-Salzman, 2013). HIF-1 is responsible for triggering many vital physiological pathways that maintain oxygen homeostasis, such as angiogenesis, erythropoiesis, glucose and iron transport, glycolysis, tricarboxylic acid cycle, cell proliferation, and apoptosis (Ke & Costa, 2006; Prabhakar & Semenza, 2012). To test the hypothesis that insects are hypoxic at the end of an instar, I measured HIF-1α and HIF-1β gene and protein expression in two model insect species, *M. sexta* and *D. melanogaster*, at the beginning and the end of each instar.

Materials and Methods

Manduca sexta Methods for Animal Care

We obtained *M. sexta* larvae from Carolina Biological Supply (Burlington, NC, USA) as eggs. For all experiments, larvae were reared in a 25°C incubator with a photoperiod of 16 hours light:8 hours dark as we have previously done (Vishnuvardhan et al., 2013). Each larva was housed in a clear plastic 9 oz cup (ULINE; Pleasant Prairie, Wisconsin, USA) and fed a wheat germ diet (modified from; Ojeda-Avila, Woods, & Raguso, 2003). Food was replaced every 2 days. Through daily observation, the age of *M. sexta* larvae within an instar was determined by the presence or absence of a head capsule and exuvia. A head capsule is the sclerotized exoskeleton of the head. As the animal develops and grows, this structure becomes too small and appears to slip forward on the anterior of the caterpillar prior to molting. This indicates the end of an instar and impending molt. Once the head capsule and exoskeleton is shed, animals were considered to be at the beginning of a new instar. Larvae used for collecting growth data were weighed on an analytical balance (Mettler Toledo; Columbus, OH, USA) and head width was measured with digital calipers (Fisher Scientific; Waltham, MA, USA).

Manduca sexta HIF-1 3rd Instar Hypoxia Control Experiment

To validate that HIF-1α is increased in hypoxia in *M. sexta*, animals were exposed to hypoxia for varying amounts of time. To initiate hypoxia, caterpillars were submerged in water for 0, 10 or 30 minutes and 1, 4, or 8 h. Ten caterpillars were used for each time point and were covered with plastic to ensure submersion for the entire time period. . Once the caterpillars were taken from the water, the gut was dissected out and they were immediately frozen on dry ice for either RNA or protein isolation.

Manduca sexta Sample Collection for Development Study

Ten caterpillars were collected from the colony at the beginning and end of each instar. Age of caterpillars was determined as described above. Because insects varied in size throughout their lives, samples were collected differently for some ages (Table 1.0). The smallest insects were pooled to obtain enough tissue for each assay. The mid-range size insects were large enough that individual larvae had enough tissue for protein and RNA but were too small to dissect out tracheal tissue. In the largest insects, tracheal tissue could be isolated. To determine whether sample collection procedure affected mRNA or protein expression, I measured mRNA and protein expression using both sample collection procedures. For the largest insects, the gut was removed, and the 5^{th} instar samples were cut longitudinally in half to achieve an appropriate sample size. Samples were prepared for either protein or RNA extraction.

Drosophila melanogaster Methods for Animal Care

Adult flies were set up in bottles 5 days (about 120 hours) before collection of early third instar larvae. Flies were reared at Arizona State University (Tempe, AZ) on standard fly food medium (cornmeal/corn syrup). To obtain flies of the correct age, fly food was placed under a dissecting microscope to identify early third instar larvae. Larvae were collected based on timing, size, and physical marks (size and coloration). Early third instar larvae were either frozen immediately or transferred to a Petri dish containing standard fly food media. Petri dishes with up to 60 larvae were placed in one of three different oxygen-regulated chambers in the Roxy system (Sable Systems, Las Vegas, Nevada). The oxygen levels were maintained at hypoxia (10%), normoxia (21%), or hyperoxia (30%). Larvae were maintained at these oxygen levels for either 24 or 48 hours, at which point they were collected as late 3^{rd} instar larvae and immediately frozen at -20°C and shipped to NDSU on dry ice.

RNA Isolation

Each caterpillar sample (individual or pooled) was placed in 500 µl of Trizol per 500 mg of sample (TRI Reagent; Fisher Scientific; Waltham, MA, USA) and placed on dry ice. Dissection took a maximum of 1 min per sample. *Drosophila* melanogaster samples were stored at -80°C until they were used for RNA isolation.

Table 1

Description of sample collection across instars for RNA and protein isolation

Species	Developmental stage	Sample collection
Manduca sexta	1st instar	10 larvae per tube with 10 tubes totaling 200 larvae
	2nd instar	individual whole larvae
	3rd instar	individual whole larvae
	4th instar	Exp 1: tracheal tissue dissected out from individual larvae Exp 2: individual whole body samples halved.
	5th instar	Exp 1: tracheal tissue dissected out from individual larvae Exp 2: individual whole body samples halved.
Drosophila melanogaster	3rd instar	whole body polled number

RNA was extracted as specified by the manufacturer's protocol (TRI Reagent; Fisher Scientific; Waltham, MA, USA). RNA pellets were re-dissolved in 30-100 µl RNase-free deionized water. Each of the samples was diluted 1:30 and quantified using 1 µl of sample on a

Nanodrop1000 spectrophotometer (Thermo Scientific; West Palm Beach, FL, USA). RNA stock samples were stored at -80°C until further use.

Complementary DNA Synthesis

Complementary DNA was made from mRNA using an RT-cDNA synthesis kit following the manufacturer's protocol (Quanta Bioscience, Gaithersburg, MD, USA). To check for genomic DNA contamination, the negative control reactions were run with no reverse transcriptase added. Complementary DNA was made using real-time PCR on controls, standards, and samples in total volumes of 10 µl, including 2 µl of template and 8 µl of master mix (5.0 µl RT master mix, 0.5 µl of each gene-specific primer, and 2.5 µl of RNase-free deionized water).

Quantitative Polymerase Chain Reaction

HIF-1α and HIF-1β mRNA levels were determined using quantitative real-time polymerase chain reaction (qPCR) using a Stratagene Mix 3000p detection system (Stratagene; La Jolla, CA, USA) and Perfecta SYBR Super mix (Quanta Biosciences; Gaithersburg, MD, USA). Primers for HIF-1α and HIF-1β (Integrated DNA Technologies; Skokie, IL, USA) were synthesized and validated using PCR (Table 2,3,4,5). Quantitative PCR reactions for both HIF-1α and HIF-1β were carried out in 10 µl volumes. Eight µl of master mix (5 µl SYBR mix, 0.5 µl each of forward and reverse primers, and 2.5 µl nuclease-free water per reaction) were added to each well followed by 2 µl of sample. Wells were mixed by gentle pipetting 10 times each. Samples, standards, and controls were all run in duplicate. Plates were capped with PCR strip caps and centrifuged for 5 min. Thermocycler parameters were as follows: 95°C for 10 min, 45 cycles of 95°C for 30 seconds, and 58°C for 1 minute (Bergan, Kittilson, & Sheridan, 2013). The data were normalized with the RpS3 housekeeping gene. From that correction, copy number was calculated and used for statistical analysis.

Table 2

M. sexta qPCR primers

Oligonucleotide Name	Forward sequence (5'-3')	Reverse sequence (5'-3')	Expected product size
HIF-1α	GTGATGCGTCTAGCTATATCC	ACAAATGGCTGAACTCGAAC	279 bp
HIF-1β	CATGAAAGCTTTGAGAGGTA	GGTCGTATAAACATGACGAA	221 bp
rpS3 (Housekeeping Gene)	CCGCATCCGCGAGTTG	CGGACTGTTCCGGGATGTT	59 bp
Actin (Housekeeping Gene)	ACGAGGCCCAGAGCAAGAG	GGTGTGGTGCCAGATCTTCTC	101 bp

Table 3

M. sexta standard curve clone sequences

Oligonucleotide Name	Sequence 5'-3'
HIF-1α	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
HIF-1β	CCGCTTACACGCTACGTGAAATTTGTATAGTTTGTTATTTTTAATCATAACATAAACTCATTCNTAAATAGTATTTC

Table 4

D. melanogaster qPCR primers

Oligonucleotide Name	Forward sequence (5'-3')	Reverse sequence (5'-3')	Expected product size
DMS (HIF-1α)	CGAGTTTAGCGGCAACCAGT	TGTGCGGGGTCCTACTTTCA	785 bp
DMT (HIF-1β)	TCGGGCAGGGTGATCTATGT	TCGGGATGGATGTGCTCATAC	945 bp
DMH (PHD)	GGCACCAATTGGTTTAC	GGATGTCCCTTGAGTCTCATTT	747 bp
DMActin (Housekeeping Gene)	GGACTCGTACGTGGGTGATGA	TCTCCATATCGTCCCAGTTGGT	88 bp

Table 5

D. melanogaster standard curve sequences

Oligonucleotide Name	Forward sequence (5'-3')	Reverse sequence (5'-3')
DMS (HIF-1α)	ACGCGATGATGACGATTCCG	TGTGCGGGGTCCTACTTTCA
DMT (HIF-1β)	GCGCATAAGACCAGCCGAAT	TCATATCGTTGGCTGCCGTG
DMH (PHD)	GAGCGCCGCTATGAGGATCT	CAGTGACCGCGTTTGTCCAC

Protein Isolation

Each sample was homogenized using an electric tissue homogenizer (IKA; Wilmington, NC, USA) in 100-500µl of cell lysis buffer (Cell Signaling; Danvers, MA, USA). Then, samples were centrifuged at 12,000 x g at 4°C for 10 min. The supernatant was then transferred to a fresh microfuge tube. Protein concentrations were determined using Bio-Rad protein assay as per

manufacturer's protocol (Bio-Rad Laboratories; Hercules, CA, USA). Briefly, 10 µl of sample or standard and 200 µl of reagent were pipetted into the wells of a 96-well plate, and 200 µl of protein assay was added to each well. All samples and BSA standards (bovine serum albumin) were run in duplicate. The wells were carefully mixed on a shaker for 30 seconds and then incubated for 5 min at room temperature. Absorbance was read at 595 nm on a micro plate reader (Bio-Rad Laboratories; Hercules, CA, USA).

Western Blotting

First, proteins were separated by molecular weight using electrophoresis. Fifty µg of sample were loaded into the wells of Bio-Rad 10% pre-cast TGX gels according to the manufacturer's protocol (Bio-Rad Laboratories; Hercules, CA, USA). Gels were electrophoresed for 30 minutes at 200V in 1% sodium dodecyl sulfate (SDS) running buffer. Separated proteins were transferred to nitrocellulose membranes using transfer buffer following the manufacturer's protocol (TransBlot Turbo Kit; Bio-Rad; Hercules, CA, USA). Membranes were blocked in 40 ml of 5% dry milk (HyVee; Des Moines, IA, USA) in tris-buffered saline with 0.1% Tween 20 (TBS-T) for 1 hour on a rocking shaker. Then the membrane was washed 3 times for 5 min with TBS-T. The membrane was then probed with the primary antibody (rabbit-anti- *Drosophila* HIF-1α; a gift from Drs. Jim Marden and Pablo Wappner) at a 1:1,000 dilution in (40 ul of anti-HIF-1 α in 40 ml of 5% bovine serum albumin) at 4°C overnight on a shaker. The next day the membrane was washed 3 times for 5 min with TBS-T. The membrane was then incubated with goat anti-rabbit IgG secondary antibody HRP conjugate (Thermo Scientific; Rockford, IL, USA) at a 1:5,000 dilution (8 µl of secondary antibody in 40 ml of 5% TBS-T and 2 g of dry milk), for 1 hour on a rocker. After 1 hour the secondary antibody was poured off and frozen for later use. The membrane was washed 3 times 10 min with 1X TBS-T. Antibodies were visualized using

the West-Femto kit following manufacturer's protocol (ThermoFisher; Grand Island, NY, USA) using a 30 min exposure time on a chemi-imager (Alpha-Innotech; Miami, FL, USA) for.

Statistics

Statistical analyses were performed using IBM SPSS Version 22. Two-factor analysis of variance (ANOVA) was used to identify differences in body mass and head width among groups, with instar and stage as the factors. Non-parametric Kruskal-Wallace tests were used to identify differences in gene and protein expression among instar and stages. Tamhane post-hoc tests were used to identify differences between specific stages and instars. Means +/- S.E.M. are shown throughout.

Results

Manduca sexta Body Size

Body mass increases continuously both across and within each instar (Fig.4, stage x instar interaction, $F_{3,73} = 6.13$, $p < 0.002$) . In contrast, head width increases only after each molt, remaining constant within each instar (Fig. 4, effect of instar, $F_{4, 67} = 318.7$, $p < 0.001$, effect of stage, $F_{1,67} = 1.2$, $p = 0.28$).

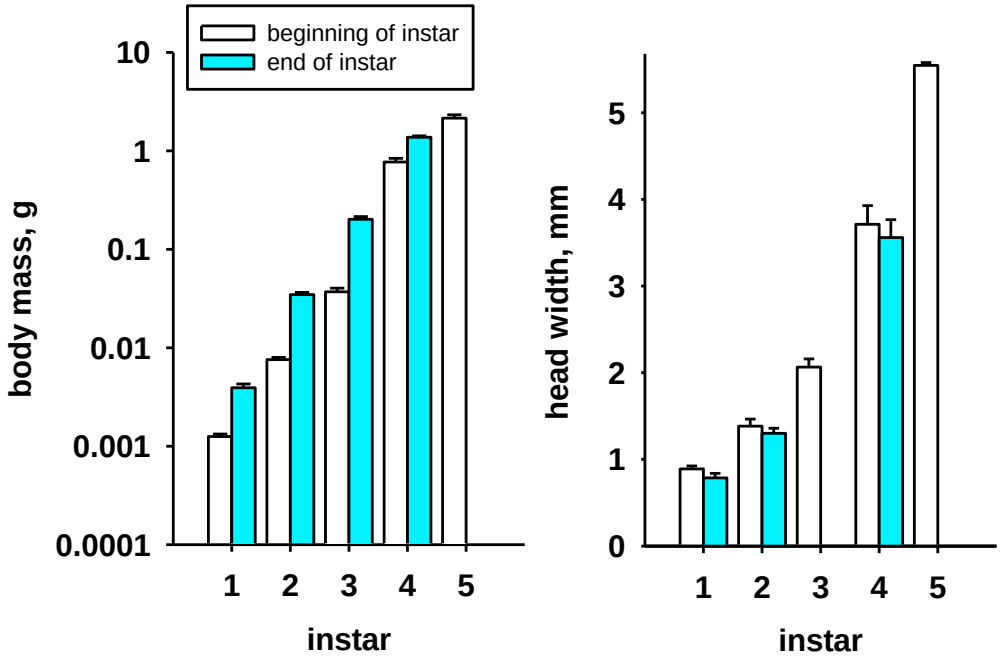

Figure 4. Body mass (left) continuously increases within and across all instars. Head width (right) increases across instars, but not within an instar.

Manduca sexta Hypoxia Control Experiment

Submersion in water to simulate hypoxia resulted in increased expression of HIF-1α mRNA, but not protein. Third instar larvae with head-capsules at the end of their instar were submerged in water from 10 minutes to 8 hours. Expression of mRNA varied significantly with time ($F_{5,24}$ =3.72, p <0.02). However, pair-wise post-hoc tests did not detect significant differences between specific groups. From time 0 to 4 and 8 hours, mRNA expression approximately doubled (t0 = 3376 ± 353, t4 = 6167 ± 1088, t8 = 5359 ± 631) (Fig. 5). There was no variation in HIF-1α protein expression with submersion (Appendix A, Fig. 6, X^2 = 4.41, p = 0.492).

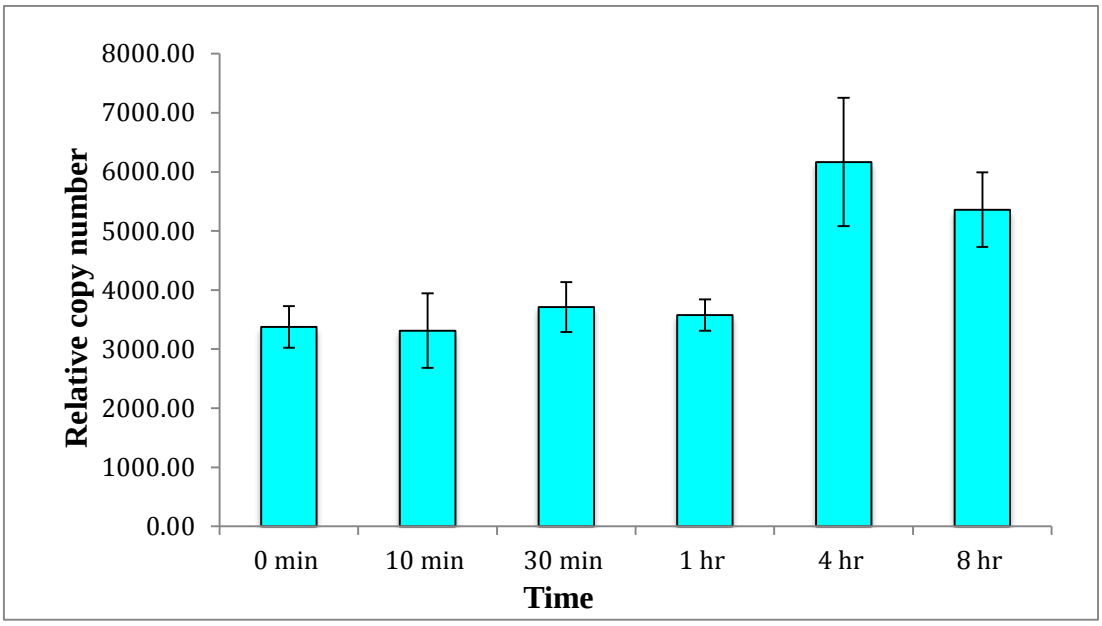

Figure 5. HIF-1α mRNA expression in 3th instar end of the instar (headcapsuled) *M.sexta* that were submerged in water from 0 minutes to 8 hours. There was no significant change in HIF-1α mRNA expression after being submerged in water. However there was an effect of time

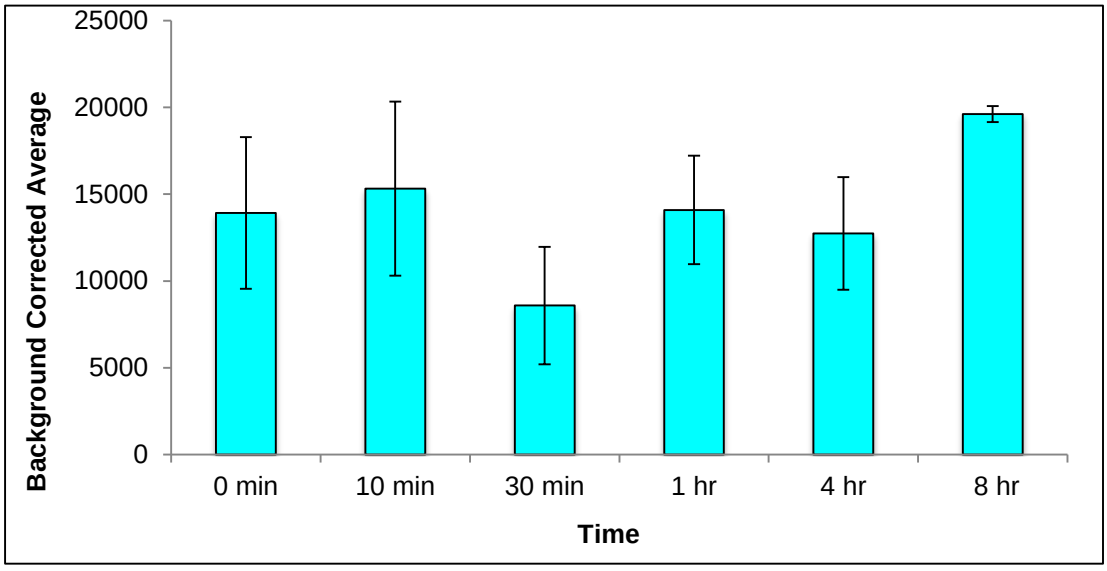

Figure 6: HIF-1α protein expression in 3th instar *M.sexta* caterpillars at the end of the instar (with head-capsules) that were submerged in water from 0 minutes to 8 hours. There was no effect of submersion on protein expression of HIF-1 α.

Within-Instar HIF-1 mRNA Expression

HIF-1α expression varied by stage of the instar differently depending on the instar of the animal (instar x stage interaction; $F_{9, 95} = 22.84$, $p < 0.001$). For example, in the first instar, there was no difference from freshly molted or beginning of the instar larvae (FM) to headcapsuled or end of the instar larvae (HC), while in the 3rd instar; HIF -1α expression was higher at the end compared to the beginning of the instar (Fig. 7; 2nd instar p = 0.000; 3rd instar p = 0.038.). Post-hoc tests identified significant differences within an instar for only the 2nd and 3rd instars for HIF-1α gene expression. HIF-1β gene expression also varied by stage differently depending on the instar of the animal (instar x stage; $F_{9,92} = 10.716$). As with HIF-1 α, in the first instar, there was no difference from FM to HC animals, while in the 2nd and 3rd instars, HIF -1β expression was higher at the end compared to the beginning of the instar (HIF-1β: 2nd instar, p = 0.007; 3rd instar, p = 0.004).

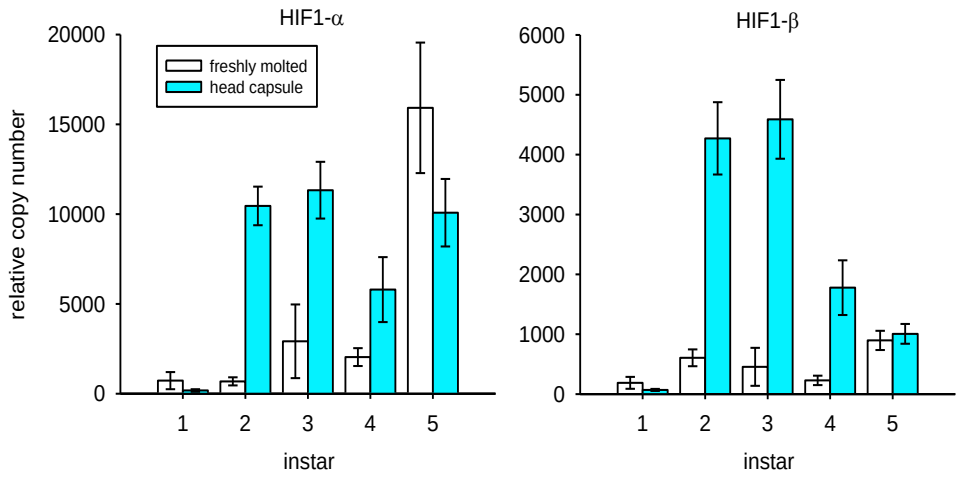

Figure 7. HIF-1 α (left) and HIF-1 β (right) mRNA expression across and within instars. *indicates significantly higher expression at the end of the instar.

HIF-1 Protein Expression

We detected HIF-1α protein (120 kDa) at the end of the 1st, 2nd, 3rd, 4th and 5th instars (indicated by HC in Appendix B). Densitometric measurements indicated significant differences in expression among the instar-stage groups (Fig. 8) (Kruskal-Wallis test, $X^2 = 27.74$, df = 10, $p < 0.01$). To identify differences within an instar, we compared 95% confidence intervals between the beginning and end of each instar (Table 6). Confidence intervals were overlapping in most of the within instar comparisons, indicating that there was no difference in protein expression from beginning to end of the instar. The fifth instar differed from beginning to end of the instar with expression more than 4 times higher at the beginning of the instar compared to the end of the instar (Table 6).

Table 6

Ninety-five percent confidence intervals between the beginning and end of each instar

Instar	Stage	Mean	Lower bound	Upper bound
1st	Beginning of instar	660.86	459.82	861.9
	End of instar	404.6	85.54	723.67
2nd	Beginning of instar	123.4	63.64	183.05
	End of instar	324.9	-117.21	766.93
3rd	Beginning of instar	0.368	-0.80	1.53
	End of instar	4060.5	-3924.44	12,045.46
4th	Beginning of instar	7065.9	-5391.30	19530.12
	End of instar	20,901.2	15129.40	26673.02
*5th	Beginning of instar	30,311.3	26801.76	33820.88
	End of instar	7554.7	-10213.74	25323.18

**Means it is significant*

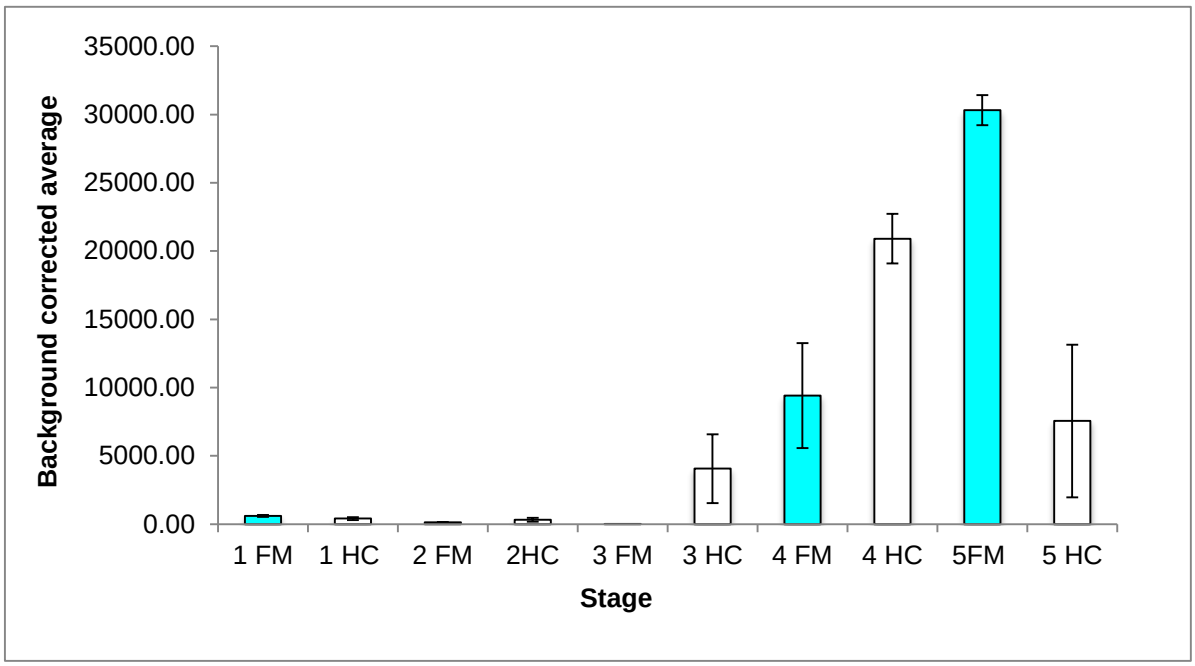

Figure 8. Densitometry values from Western blots showing HIF-1α protein expression within and across each instar. FM indicates the beginning of each instar and HC indicates the end of each instar.

Drosophila melanogaster Gene Expression

Expression of HIF-1 α varied differently over time, depending on the percent oxygen (Fig. 9, oxygen x hour, $F_{22,92} = 2.7$, $p < 0.002$). At the beginning of the third instar, HIF-1 α levels were similar, but by the end of the third instar flies in 40% oxygen expressed three times less HIF-1α. HIF-1β expression level did not vary with oxygen treatment, but differed as flies developed within the third instar (Fig. 10, effect of time, $F_{12,92} = 2.71$, $p < 0.006$). Expression of prolyl hydroxylase varied differently over time, depending on the percent oxygen (Fig. 11, oxygen x hour, $F_{22,92} = 2.12$, $p < 0.02$). By 25 – 36 hours, flies in 10% oxygen had 5-fold higher expression of prolyl hydroxylase compared to the other two groups.

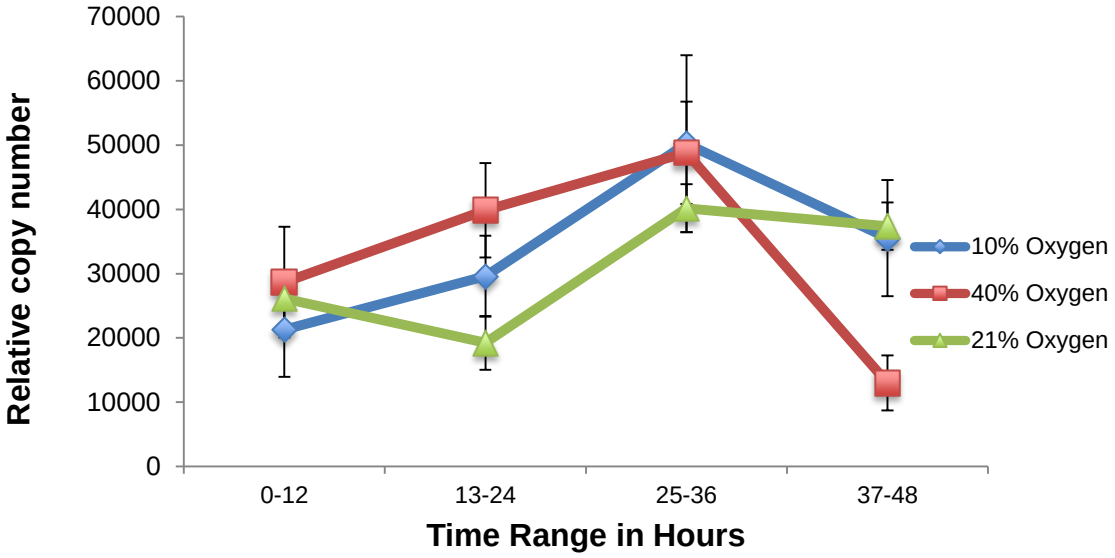

Figure 9. HIF-1α mRNA expression from 0-48 hours in the third instar. Hypoxia (10% oxygen, blue diamond), normoxia (21% oxygen, green triangle) and hyperoxia (40% oxygen, red square).

Drosophila melanogaster HIF-1α Protein Expression

HIF-1α protein was detected by western blots (Fig 12; Appendix C). Densitometric measurements showed a significant relationship with time from the beginning of the 3rd instar to the end of the 3rd instar indicated by the 48 hour time point. However there was not a significant relationship between the different time points.

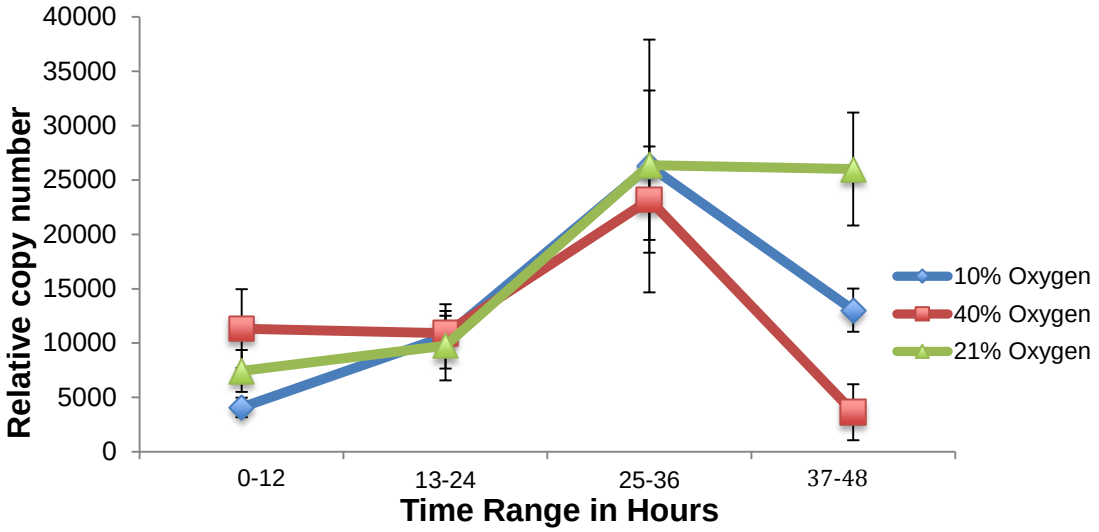

Figure 10. HIF-1β mRNA expression from 0-48 hours in the 3rd instar. Hypoxia (10% oxygen, blue diamond), normoxia (21% oxygen, green triangle) and hyperoxia (40% oxygen, red square)

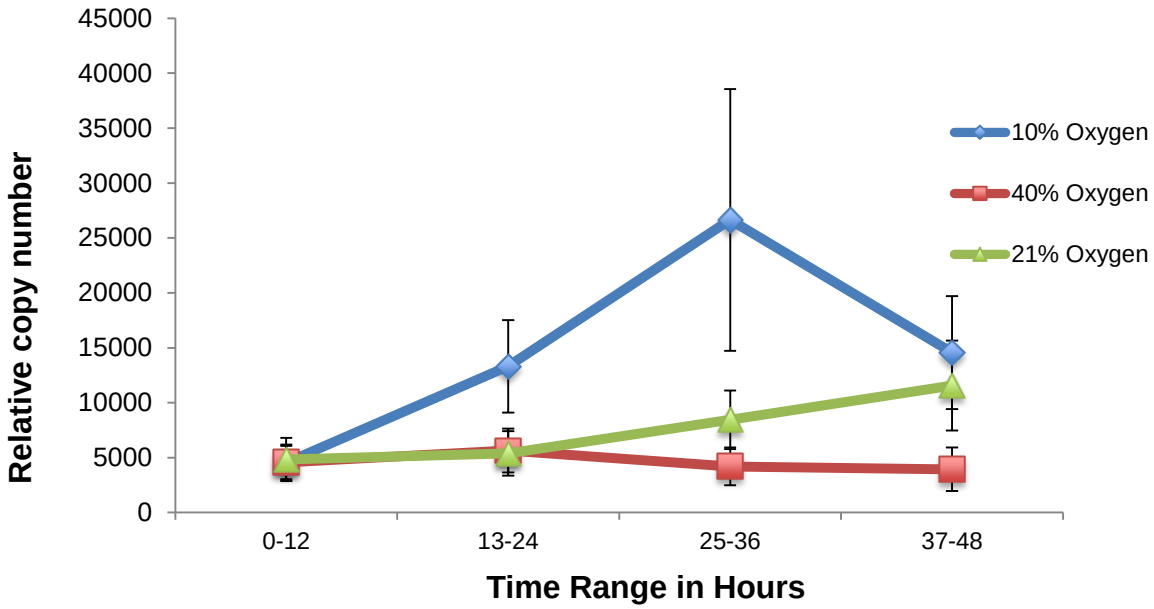

Figure 11. PHD mRNA expression from 0- 48 hours in the 3rd instar. Hypoxia (10% oxygen, blue diamond), normoxia (21% oxygen, green triangle) and hyperoxia (40% oxygen, red square).

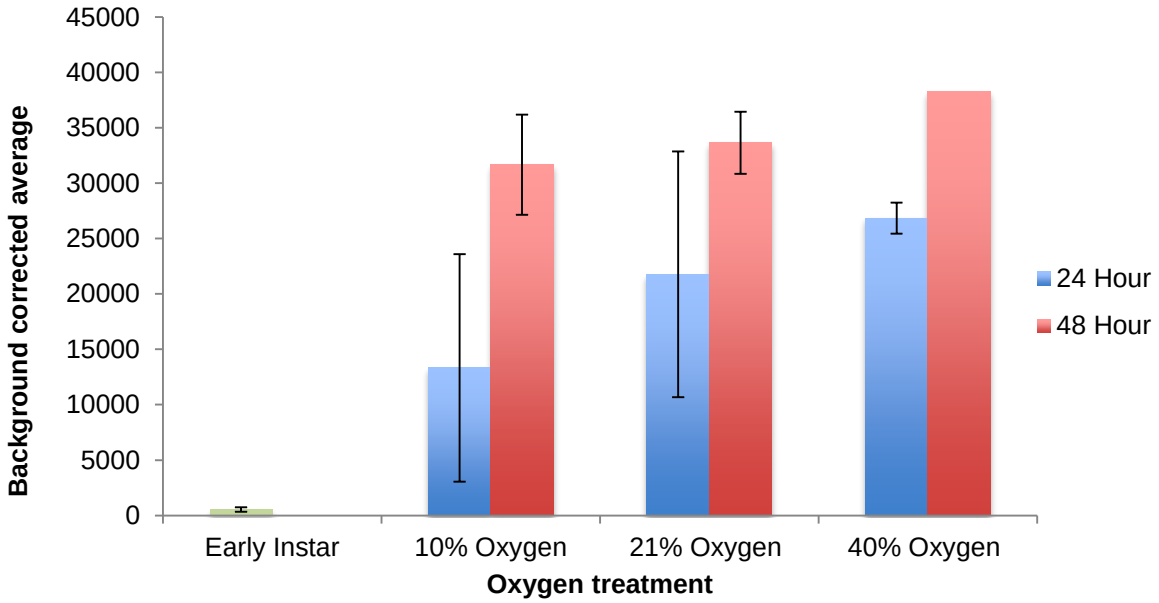

Figure 12. Densitometry of HIF-1α protein expression within 3rd instar larvae of *D. melanogaster*

Discussion

With this research, I have shown that body mass more than doubles within an instar, while sclerotized structures, such as the head, remain a constant size, supporting the hypothesis that insects may be constrained within an instar until they can molt. I also showed for the first time that insects may become hypoxic near the end of an instar, with HIF-1 α and HIF-1 β gene expression increasing at the end of the 2nd and 3rd instars in *M. sexta*. These data are correlated with that of *D. melanogaster*, in which HIF-1α increases at the end of the third instar. These data are exciting, because they suggest that insects are becoming hypoxic at the end of some instars. In addition, these data show that developmentally, HIF-1 may play an important role in molting. These data also give us insight into how insects are able to tolerate extremely low-oxygen conditions.

HIF-1α and HIF-1β gene expression in the 2nd and 3rd instars of *M. sexta* was significantly increased at the end of the instars. This suggests that caterpillars in those instars, but

not the 1st, 4th or 5th are becoming hypoxic. These findings are partially in alignment with previous work in *M. sexta* showing that critical PO_2 was higher at the end of the 3rd and 5th instars (Greenlee & Harrison, 2005), and that tracheal volume is fixed within the 3rd and 4th instars (Callier & Nijhout, 2011). However, in this study, animals at the end of the 5th instar did not have increased HIF-1α, suggesting that caterpillars at those instars are not hypoxic (Figs. 7 and 8). Because qPCR and Western blotting were repeated with whole animal samples of caterpillars from the 4th and 5th instars (Appendix D) and showed similar patterns as those from tracheae only, this suggests that this is a biologically relevant result and not just a result of different sampling methods,. One possible reason for this discrepancy between the critical PO2 study and this one is that, in this study, the 5th instar animals were younger by 5 days than those used for critical PO_2 study (Greenlee & Harrison, 2005). Another possibility is that 5th instar caterpillars increase tracheal volume enough to prevent hypoxia, a hypothesis supported by the work of Helm and Davidowitz (2013) who showed that tracheal mass and volume increase in the 5th instar. First instar larvae maybe small enough and have low enough oxygen demand that they do not experience hypoxia.

If caterpillars in the 2nd and 3rd instars are hypoxic at the end of these instars, this suggests that the tracheal system may not be able to support the increase in body size and thus oxygen demand, in turn causing them to be hypoxic. This makes physiological sense, because HIF-1α aids in activation of tracheal branching to oxygen-deprived tissues (Jarecki, Johnson, & Krasnow, 1999). These data are intriguing because some instars appear to get hypoxic and others do not, suggesting that there may be other physiological mechanisms in place to cope with decreases in oxygen availability, such as increased convective ventilation (Greenlee & Harrison, 1998a; Kestler, 1991).

HIF-1α mRNA and protein expression increased when caterpillars were placed in hypoxic conditions, providing further support that the increase in HIF-1α at the end of the instar indicates that animals are hypoxic (Figs. 5 and 6). Although, we tested this in 3rd instar caterpillars at the end of the instar, it indicates that expression can be increased above what is normally expressed at that time. This finding is interesting, for several reasons. First, HIF-1α is typically thought to be regulated at the protein level (Semenza, 1999), so increased HIF-1α gene expression is not expected and most researchers typically measure protein expression or expression of downstream target genes (BelAiba et al., 2007; Fukuda et al., 2002; Page et al., 2002). The literature suggests that HIF-1α mRNA expression does not increase in response to hypoxia (Fukuda et al., 2002; Kim et al., 2002; Page et al., 2002). One reason for this may be that HIF-1α mRNA does not have a HRE binding site. This is evidenced by the numerous other genes with HRE that show upregulation in hypoxia or in response to overexpression of HIF-1α, , such as glucose transporter-1 (GLUT-1; Ebert et al., 1995), vascular endothelial growth factor (VEGF; Forsythe et al., 1996), and erythropoietin (EPO; Semenza et al., 1994).

HIF-1β expression was also not expected to change in hypoxia, since it is continuously expressed and not typically stimulated in hypoxia (Huang et al., 1998; Pugh et al., 1997). However, in our study, we found increases that matched expression of HIF-1α. It is possible that the increase in HIF-1α results in upregulation of HIF-1β to increase the amount of that subunit available for binding to create the heterodimeric HIF-1, although HIF-1β does not have a HRE, so the mechanism for this is unclear. Alternatively, HIF-1β is involved in many pathways involving growth and developments, so it is possible that the increase in HIF-1β is unrelated to hypoxia.

HIF-1α protein expression patterns across instars were similar compared to the gene expression patterns. We compared Western blots using 4th and 5th instar whole body and with isolated tracheal samples. Both patterns are similar, indicating that the differences we see between the 2nd and 3rd instars and the 4th and 5th instars are not due to sampling techniques. Western blots were analyzed for HIF-1α expression using a band that appears at 120 kDa. However, there has been much controversy in the literature about the expected molecular weight of HIF-1α. Wang et al. (1995) found HIF-1α at 120 kDa. Marden et al. (2012) believes it to be 87 kDa in the lowland butterfly, and T. A. Gorr, Tomita, Wappner, and Bunn (2004)identified it at 180 kDa. To resolve this discrepancy, I calculated expected HIF-1α sizes from translated nucleotide sequences published on NCBI and in the literature (for references see Table 7). Based on these data and our lab's partial sequence of HIF-1α, I determined that the HIF-1α band should be approximately 120 kDa. In addition to this information, I focused on the band at 120 kDa, because it was consistently expressed across all instars and increased at the end of some instars and in hypoxia. In addition to the 120 kDa band, a consistent band is expressed near 75 kDa in all of the western blots, which may correspond to HIF-1β. Because I used a polyclonal antibody, and HIF-1α and HIF-1β when aligned showed 3.4% identity in sequences (Fig. 13). Unfortunately, because the antibody was donated from a lab that did not create the antibody, we do not know what peptide sequence was used to generate the antibody. If our antibody is picking up HIF-1β protein, this would be consistent with the expression pattern of a band at 75 kDa which is, commonly associated with HIF-1β (Clark et al., 2007; Hoskins et al., 2007; Wang & Semenza, 1995; Zimin, Smith, Sutton, & Yorke, 2008).

Figure 13. Partial sequence alignment. HIF-1α and HIF-1β have 3.4 % sequence identity with 77 identical sites out of a total of 2, 246 residues.

Data obtained from the 3rd instar *D. melanogaster* larvae provide further support that insects become hypoxic at the end of their instar. HIF-1α mRNA and protein expression increase from the beginning to the end of 3rd instar. Since the data points are the same for HIF-1α mRNA expression in 10% and 21% oxygen this may suggests that *D. melanogaster* larvae are hypoxic at the end of the instar. Alternatively, maybe animals reared in hypoxia are smaller in body mass therefore do not need to produce as much HIF-1α as a larvae reared in 21% oxygen (Klok, Kaiser, Lighton, & Harrison, 2010).

Expression patterns of HIF-1β mRNA in *D. melanogaster* supported the hypothesis that HIF-1β is an oxygen independent molecule (Huang et al., 1998; Pugh et al., 1997). Our data showed that HIF-1β gene expression did not vary between the treatment groups, but it did vary over time as the flies aged (Fig. 10). The variation we see from the beginning to the end of the instars could be due to the increase in body size that may occur during the third instar and possible changes in metabolic demands of the flies.

PHD is an oxygen sensor that hydroxylates HIF-1α while in normoxia and under hypoxia PHD activity is reduced (Bruick & McKnight, 2001; Epstein et al., 2001; Kallio et al., 1998). Therefore, I predicted that it would increase as insects become hypoxic at the end of the instar because I predicted that HIF-1α would increase and and therefore there would be more HIF-1α to break down. However, PHD mRNA levels decreased from beginning to end of the third instar, even though we saw increases in HIF-1α. Interestingly, in previous research, expression of variant B of PHD, which is what we measured, is increased in hypoxia (Acevedo, Centanin, Dekanty, & Wappner, 2010). Together, these data support the hypothesis that insects are normoxic at the beginning of an instar and hypoxic at the end of an instar.

Results from *D. melanogaster* also supported the results from *M. sexta* in that the protein expression of HIF-1α was more affected by hypoxia. HIF-1α protein expression increased at the end of the instar in 10% oxygen compared to that of the early instars and those end of the instars that were raised in 21% oxygen. However, contradictory to our prediction and the literature there appeared to be an even bigger increase of HIF-1α protein expression in hyperoxia. One possibility is that reactive oxygen species increased levels of HIF-1α, as ROS have been shown to increase HIF (Bonello et al., 2007; Huang et al., 1996; Kim et al., 2002).

Table 7

Published band sizes for HIF-1α

Organism	NCBI Protein Identification number	Amino acid size (a.a.)	Protein weight (kDa)	Author
Drosophila melanogaster	AAC47303.1	1505	165.74	Nambu et al. (1996)
D. melanogaster	AAV37017.1	776	85.2	Stapleton submitted
D. melanogaster	NP_001138129.1	426	47.61	Hoskins et al. (2007)
D. ananassae	XP_014767115.1	1601	177.21	Clark et al. (2007)
D. erecta	XP_015009087.1	1495	164.41	Zimin et al. (2008)
D. sechellia	XP_002037318.1	213	140.94	Clark et al. (2007)
D. simulans	XP_002105468.1	1493	164.15	Clark et al. (2007)
D. yakuba	XP_002099350.1	1517	167.21	Clark et al. (2007)
Manduca sexta	---	1450	116.71	Greenlee unpublished

Insects will provide exciting new directions in future studies investigating HIF-1 and its relationship with development. Further exploring the effects of hypoxia and the regulation of HIF-1 may help us understand the maintenance of physiological adaptions to changes in oxygen. More research needs to be done on the role of HIF-1 in relationship to development, such as distinguishing exact time points when *M. sexta* are hypoxic, making larvae hypoxic manually,

and HIF-1's role in molting. If we can distinguish the exact time *M. sexta* are hypoxic we may be able to understand HIF-1 role in development more clearly. It would also be a big step in indicating HIF-1's role in molting if we could inhibit or stabilize molting of *M. sexta* by manipulating HIF-1. If we can bridge the gap between the relationship of HIF-1 and development, it may further help us understand the underlying mechanisms of how an insect develops when they are oxygen limited.

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APPENDIX A. *MANDUCA SEXTA* DROWNING EXPERIMENT

Western blots using anti-HIF-1α for 3rd instar at end of each instar drown in water for 0 min, 10 min, 30 min, 1 hr, 4 hr, and 8 hr. the order is as follows (A) 0 min (B) 10 min (C) 30 min (D) 1 hr (E)4 hr and 8 hr.

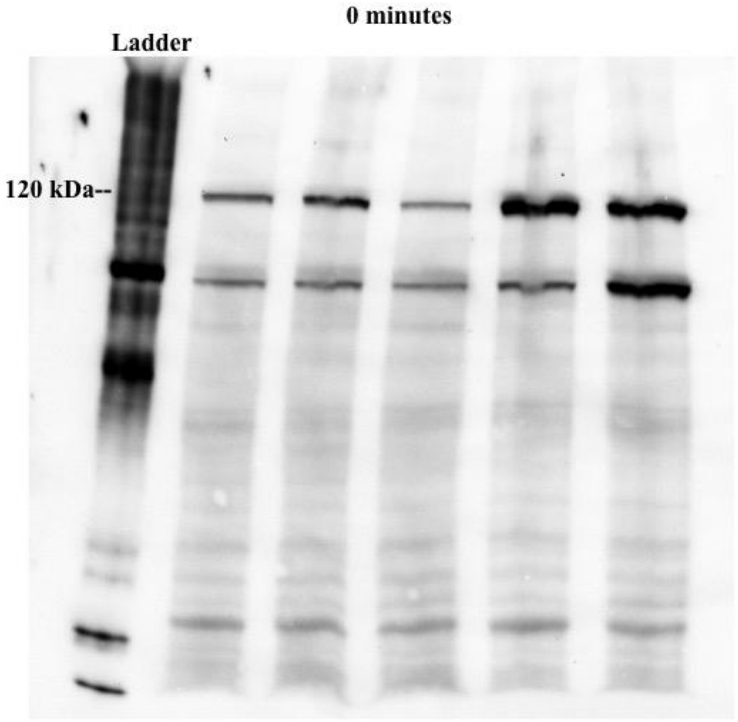

Figure A1. Protein expression for *M. sexta* 3rd instar control.

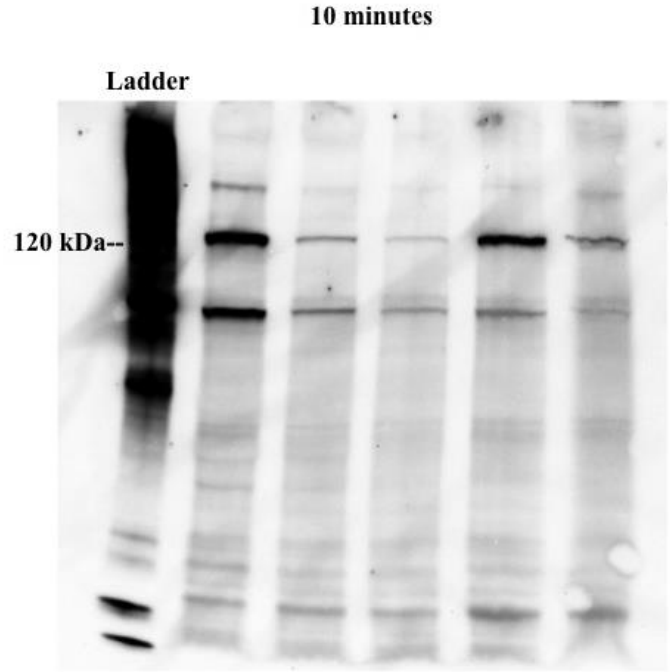

Figure A2. Protein expression of *M. sexta* 3rd instar larve submerged in water for 10 minutes.

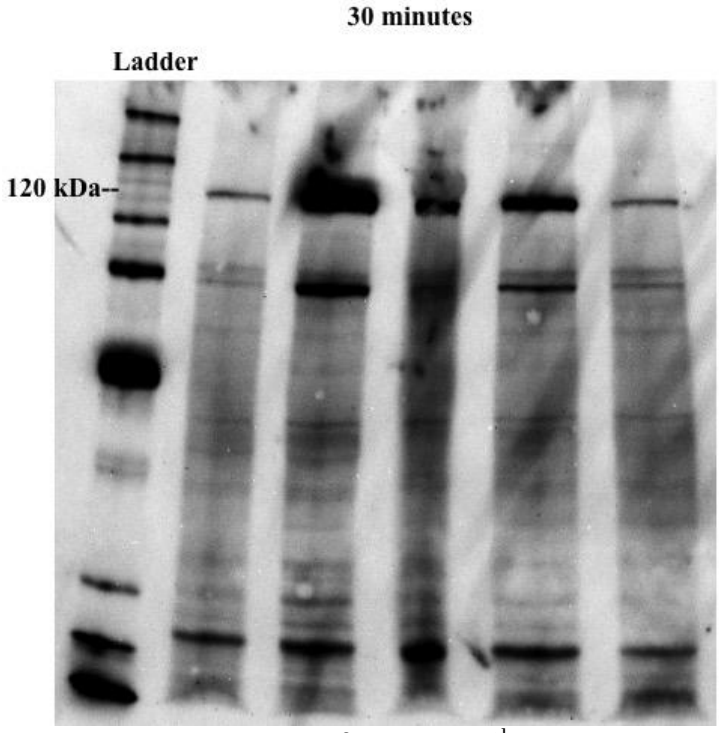

Figure A3. Protein expression of *M. sexta* 3rd instar larve submerged in water for 30 minutes.

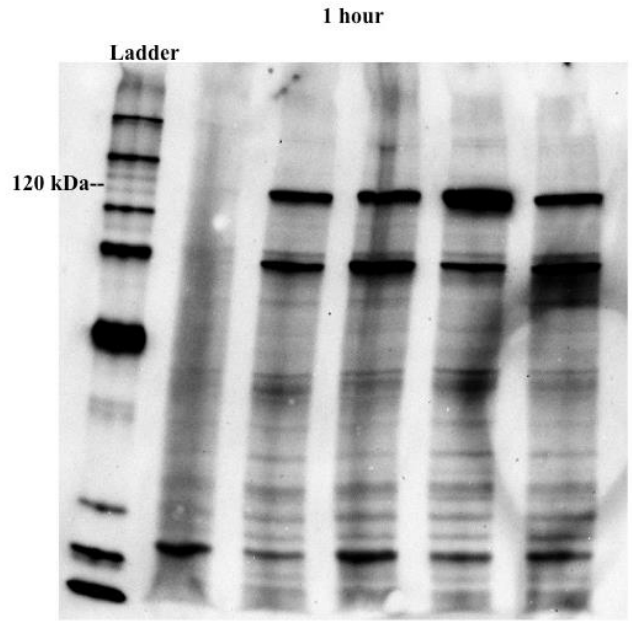

Figure A4. M. sexta 3rd instar submerged in water for 1 hour protein expression.

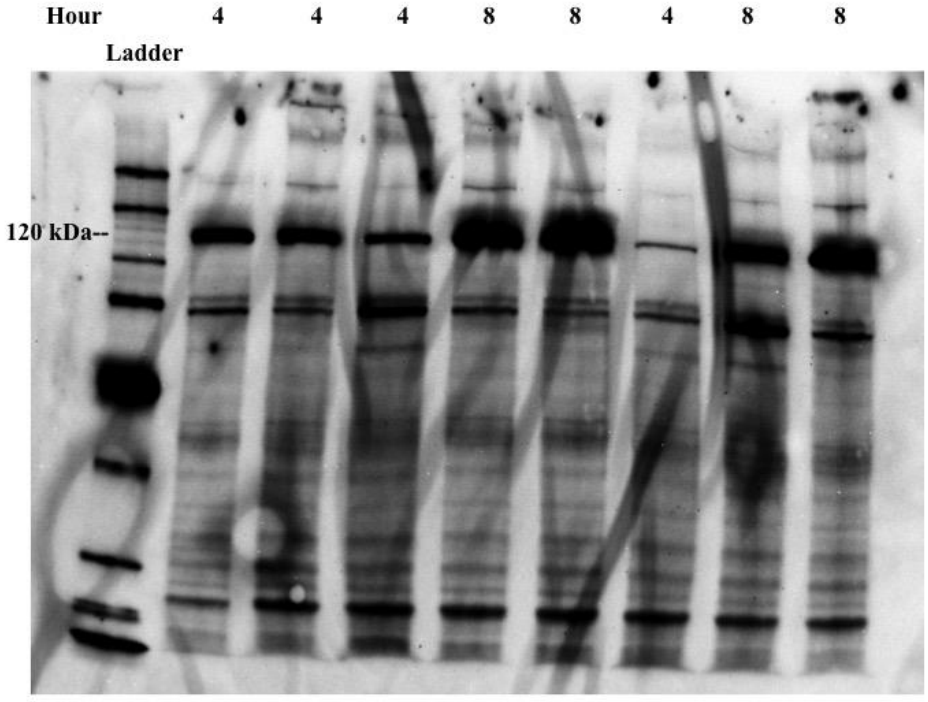

Figure A5. M. sexta 3rd instar submerged in water for 4 and 8 hours protein expression.

APPENDIX B. *MANDUCA SEXTA* WESTERN BLOTS

Western blots using anti-HIF-1α for 1st-5th instar at the beginning (FM) and end of each instar (HC). (A) 1st instar larve, (B) 2nd instar larve, (C) 3rd instar larvae, (D) 4th instar larvae, (E) 5th instar larve.

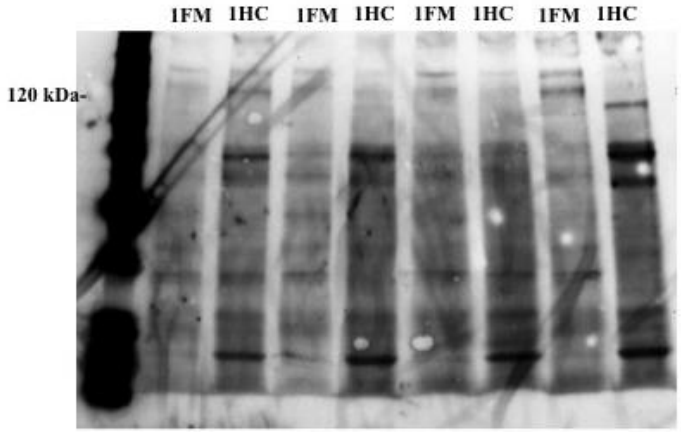

Figure B1. M. sexta 1st instar beginning and end of the instar protein expression.

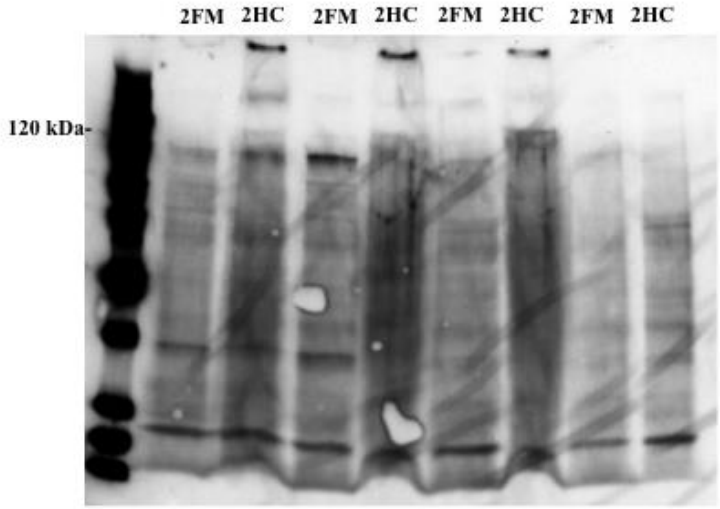

Figure B2. M. sexta 2nd instar beginning and end of the instar protein expression.

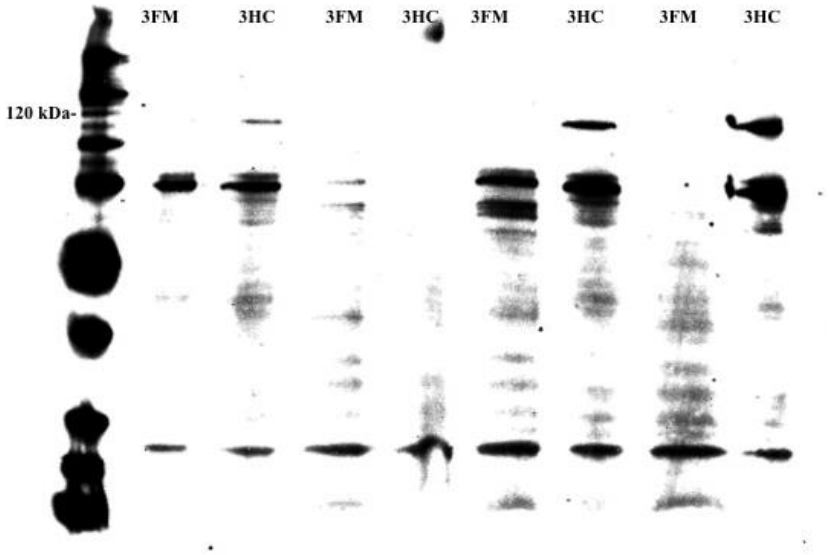

Figure B3. M. sexta 3rd instar beginning and end of the instar protein expression.

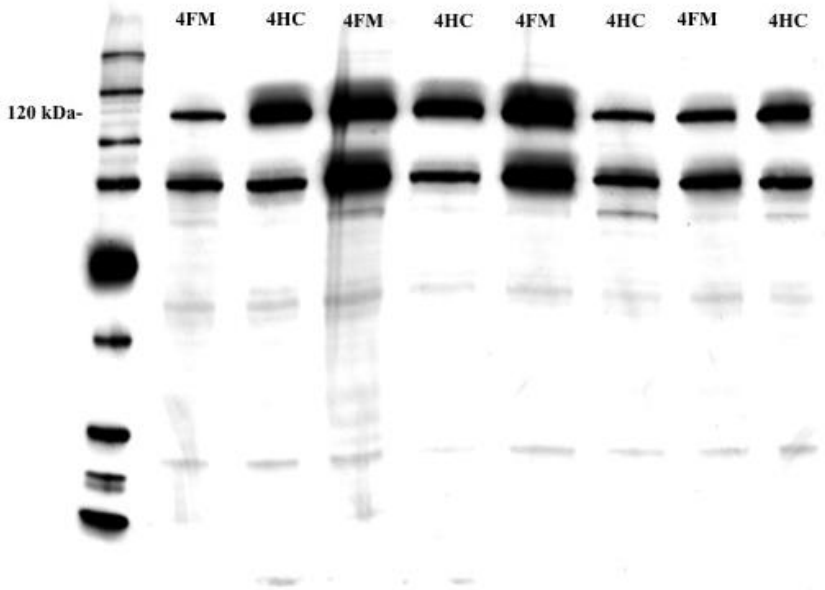

Figure B4. M. sexta 4th instar beginning and end of the instar protein expression.

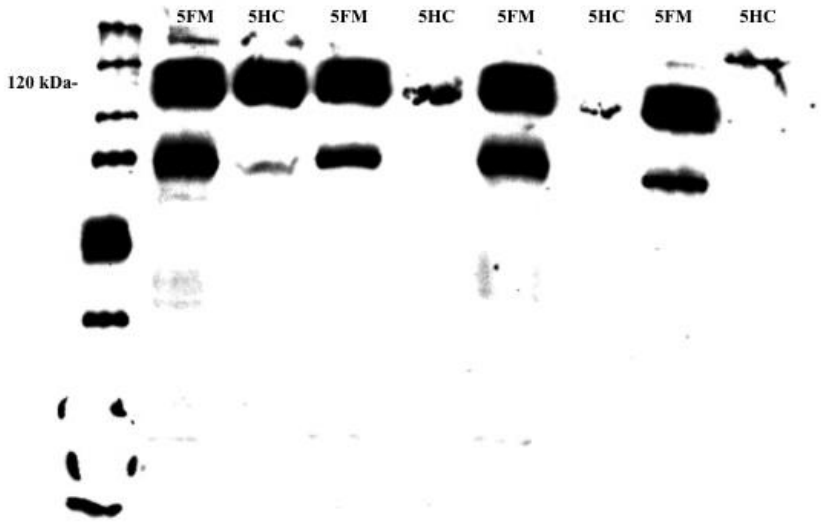

Figure B5. M. sexta 5th instar beginning and end of the instar protein expression.

APPENDIX C. *D. MELANOGASTER* WESTERN BLOT

Western blot of HIF-1α protein expression in *Drosophila melanogaster* at 0, 24, and 48 hours in the third instar. Flies were reared at 21, 10, or 40% oxygen. HIF-1α was detected at 120 kDa.

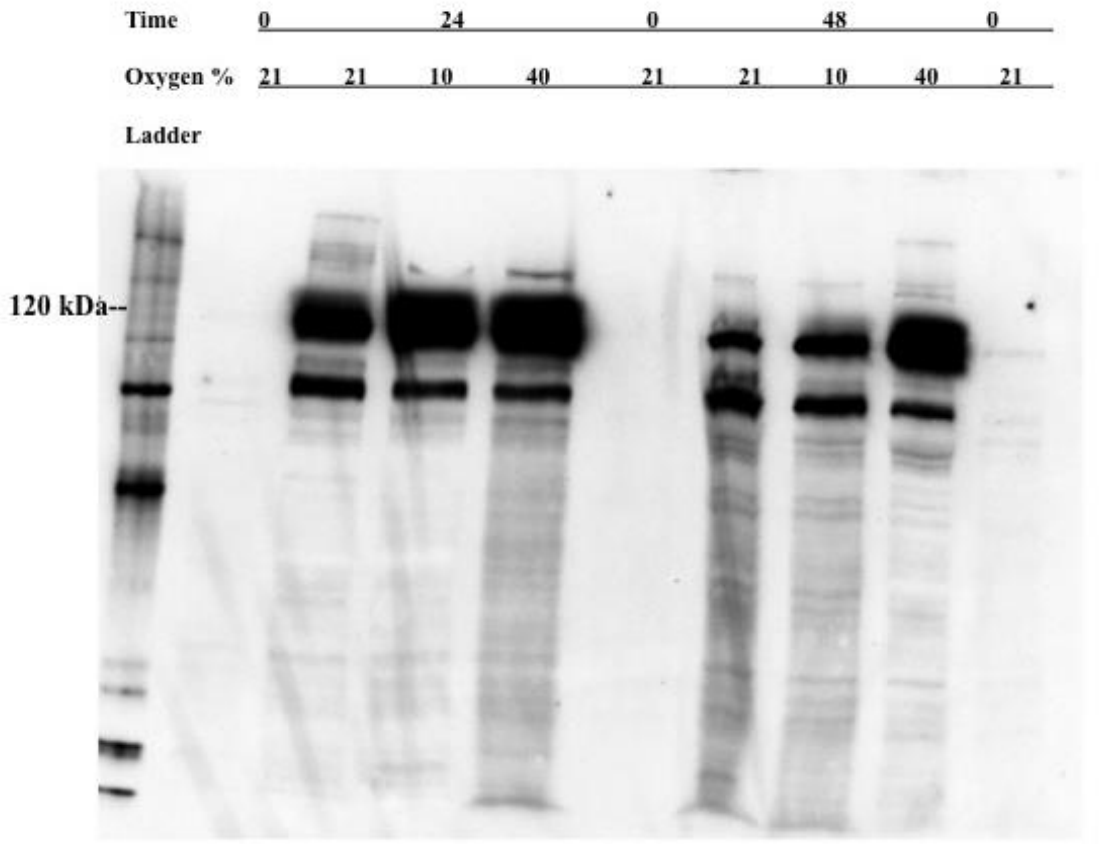

Figure C1. Protein expression of 3rd instar *D. melanogaster* reared in 10,20,40% oxygen for 0-48 hours.

APPENDIX D. WHOLE BODY EXPERIMENTS

To determine whether the differences that we saw in RNA and protein expression were due to different sample collection methods (i.e., tracheae only versus whole body), whole body samples were collected from 4th and 5th instar caterpillars, and western blots were run. Expression of HIF-1α (Fig.6) was the same as that observed from tracheal samples (Fig. 5). (A) Densitometry values from Western blots (B) showing HIF-1α protein expression within and across the 4th and 5th instars using proteins extracted from whole body samples rather than tracheae only. FM indicates the beginning of each instar and HC indicates the end of each instar. (B) HIF-1α mRNA expression within and across the 4th and 5th instars using proteins extracted from whole body samples rather than tracheae only. FM indicates the beginning of each instar and HC indicates the end of each instar.

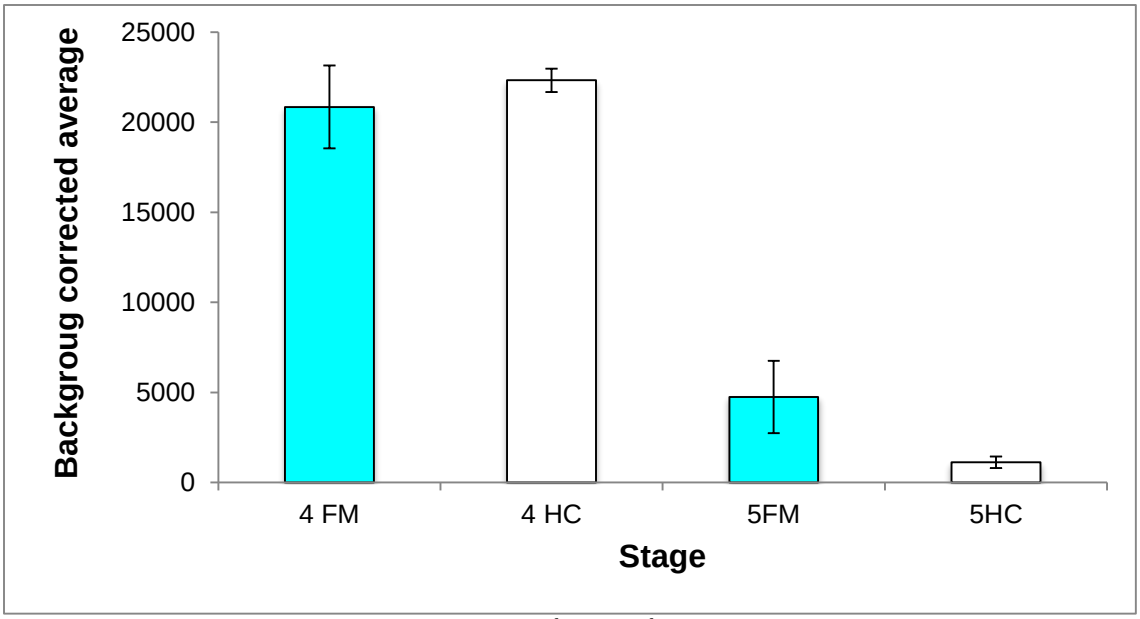

Figure D1. Whole body denseotometry in 4th and 5th instar *M. sexta.*

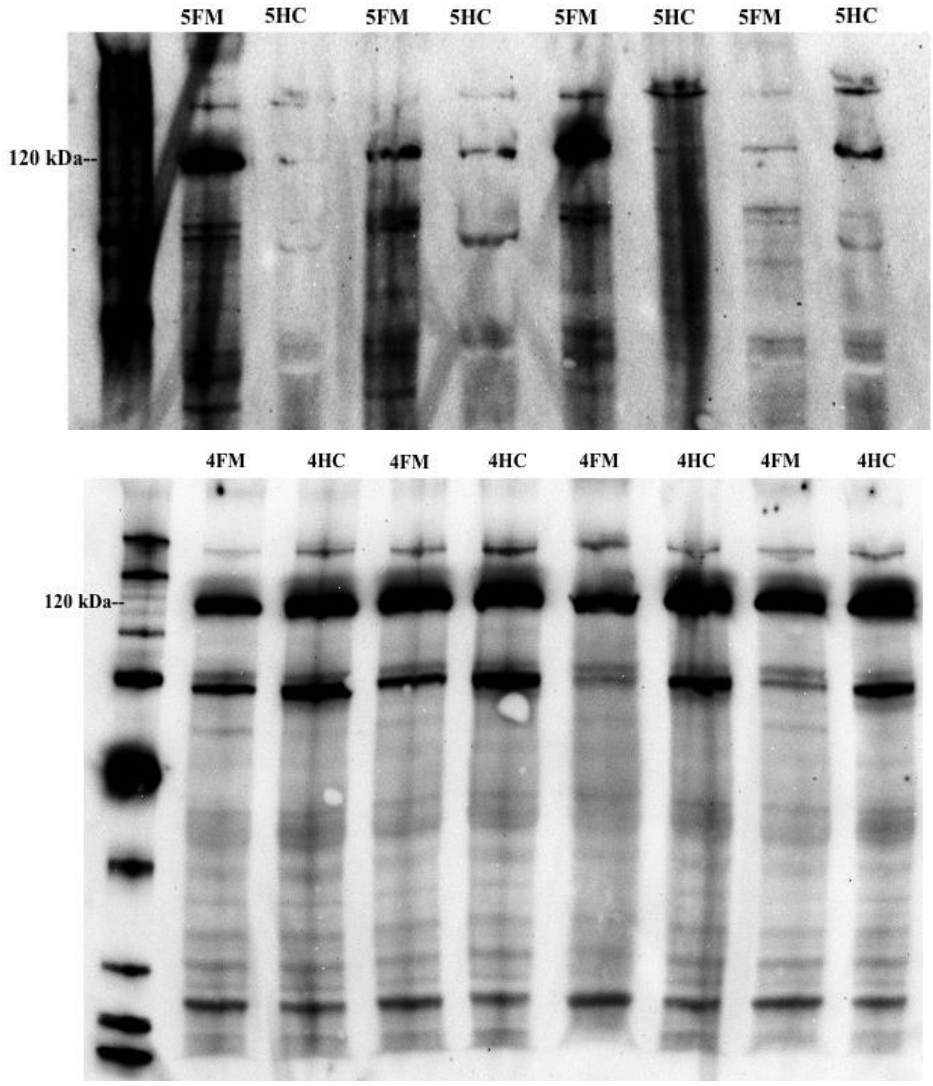

Figure D2. Whole body protein expression of 4th and 5th instar *M.sexta.*

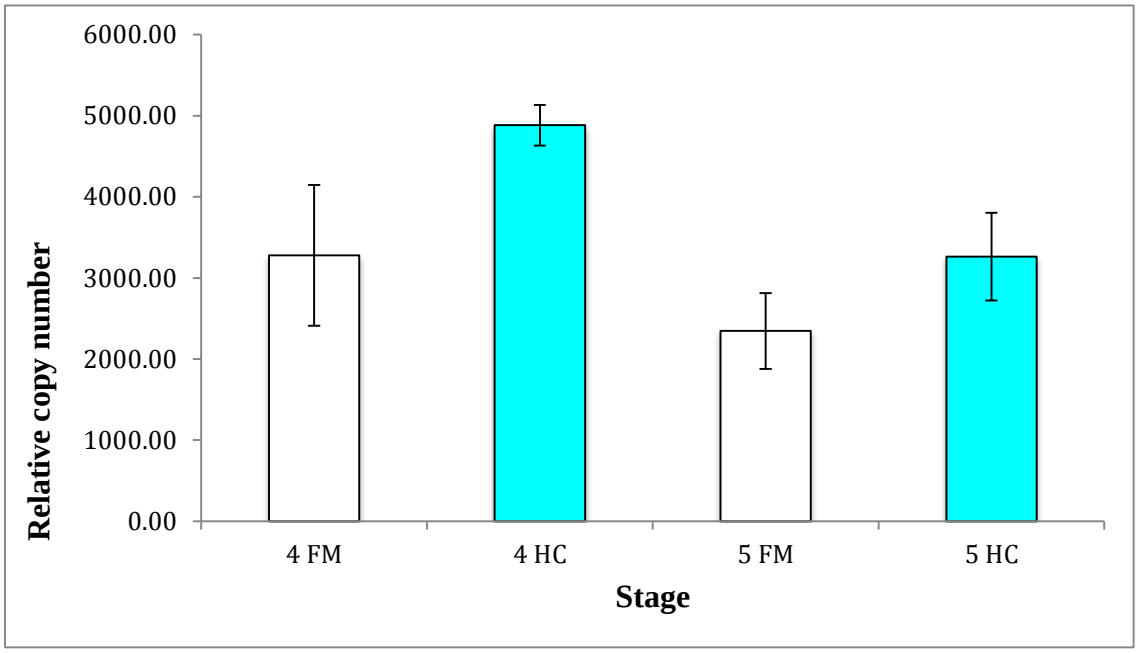

Figure D3. Whole body mRNA epression in 4th and 5th instar *M. sexta.*