Direct effects of hypoxia and nitric oxide on ecdysone secretion by insect prothoracic glands

A thesis presented

by

Leon J. DeLalio

to
The Department of Biology
In partial fulfillment of requirements for the degree of

Master of Science

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Boston, Massachusetts

July 8, 2013
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ABSTRACT OF THESIS

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ABSTRACT

Insect metamorphosis is controlled by steroid-hormone signaling events. The primary molting hormone, ecdysone, is secreted by the prothoracic glands, sequentially promoting commitment of fifth stage *Manduca sexta* larvae to pupae and then to adult development. A recent study suggests that tracheal growth limits tissue oxygenation, which comprises the size sensing mechanism responsible for triggering molt timing in a given insect stage. Hypoxia-reared fifth (last) larval stage *Manduca sexta* initiate molting at lower weights than normoxic larvae. Further, in *Drosophila*, the signaling gas nitric oxide (NO) appears to be required for normal developmental timing. Knockdown of nitric oxide synthase (NOS) in the prothoracic glands prolongs larval feeding, increases lipid storage, and blocks metamorphosis. As observed in *Drosophila*, NO signaling targets the nuclear hormone receptor βFTZ-F1, a regulator of ecdysone production and metamorphic tissue progression. Additionally, low intracellular oxygen supplies have been shown to enhance NO signaling cascades. Therefore, we set out to directly examine the effects of hypoxia and NO on ecdysone secretion using glands from feeding fifth (last) larval stage *M. sexta*. We postulated that each agent would have a stimulatory effect on ecdysone secretion. Our results have indicated that, on the contrary, treatment of prothoracic glands with hypoxia (2% oxygen) or the NO donor DETA-NONOate (10mM) significantly inhibit ecdysone secretion relative to controls. Protein markers of glandular activity (reduced 4EBP and increased pERK) were measured by Western blotting. Results are in keeping with an initial inhibition measured by a respective increase in non-p4EBP and decrease in pERK following exposure to hypoxia and NO. Levels of gene expression, including *Manduca* hormone receptor 3 (MHR3), *Manduca* βFTZ-F1, nitric oxide synthase (NOS), and the *Manduca* PTTH receptor Torso, were quantified using
real-time PCR. Overall, NO treatment increased mhr3 expression, suggestive of increased ecdysone steroidogenic capacity, and decreased nos expression, suggestive of negative feedback by NO. By contrast, hypoxia significantly increased MHR3 transcript after 2 hours, but decreased transcript after 12 hours. Both NO and hypoxia had small effects on βftz-f1 expression. Moreover, NO and hypoxia strongly increased torso transcription. Our results suggest that oxygen and NO modify the steroidogenic capacity and sensitivity of the PG rather than directly influence ecdysone secretion.
This thesis is dedicated to Leonard, Janice, and Jessica

For all your love and support
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>2</td>
</tr>
<tr>
<td>Dedication</td>
<td>5</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>6</td>
</tr>
<tr>
<td>List of Figures</td>
<td>9</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>10</td>
</tr>
<tr>
<td>Introduction</td>
<td>11</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>16</td>
</tr>
<tr>
<td>Results</td>
<td>21</td>
</tr>
<tr>
<td>Discussion</td>
<td>32</td>
</tr>
<tr>
<td>References</td>
<td>41</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

**Figure 1.** Proposed mechanism of NOS-mediated NO control of E75 and DHR3 on \( \beta ftz-f1 \) expression in *Drosophila*.

**Figure 2.** Direct effects of 2 hour and 12 hour low oxygen tension (2% oxygen: 98% nitrogen) on basal ecdysone secretion by *M. sexta* prothoracic glands.

**Figure 3.** Direct effects of 2 hour and 12 hour 10mM DETA-NONOate stimulation on basal ecdysone secretion by *M. sexta* prothoracic glands.

**Figure 4.** Trypan blue (0.4%) cell viability assay of 12 hour 10mM DETA-NONOate and 12 hour hypoxic (2% oxygen: 98% nitrogen) *M. sexta* prothoracic glands.

**Figure 5.** Western blot analysis of non-phospho4EBP and phospho-ERK after 2 hour hypoxia and 10mM DETA-NONOate.

**Figure 6.** Western blot analysis of non-phospho4EBP and phospho-ERK after 12 hour hypoxia and 10mM DETA-NONOate.

**Figure 7.** Relative gene expression of molt-related transcription factors after 2 and 12 hour 10mM DETA-NONOate.

**Figure 8.** Relative gene expression of molt-related transcription factors after 2 and 12 hour hypoxia.
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>βFTZ-F1</td>
<td>beta-ftz transcription factor 1</td>
</tr>
<tr>
<td>DHR3</td>
<td><em>Drosophila</em> hormone receptor 3</td>
</tr>
<tr>
<td>4EBP</td>
<td>eukaryotic translation initiation factor 4E-binding protein</td>
</tr>
<tr>
<td>EcR</td>
<td>ecdysone receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MHR3</td>
<td><em>Manduca</em> hormone receptor 3</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of Rapamycin</td>
</tr>
<tr>
<td>NF-kB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol-(3,4,5)-triphosphate</td>
</tr>
<tr>
<td>PG</td>
<td>prothoracic glands</td>
</tr>
<tr>
<td>PTTH</td>
<td>prothoracicotropic hormone</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative real-time PCR</td>
</tr>
<tr>
<td>Ras</td>
<td>Ras GTP-binding protein</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>USP</td>
<td>ultraspiracle</td>
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Introduction

Hormonal regulation of molting and metamorphosis

Holometabolous insects undergo complete metamorphosis involving morphologically distinct larval and adult stages (Nijhout, 1994). Under ideal conditions, the molting cycles leading up to metamorphosis, and the onset of metamorphosis itself, occur in a timely and predictable manner (Nijhout, 1994). However, when conditions change, the timing of molt cycles and the onset of metamorphosis also change. This observation has been correlated with the presence of a physiological size-sensing mechanism thought to be responsible for responding to environmental cues and appropriately timing the decision to initiate molting and metamorphosis (Nijhout & Williams, 1974).

Fluctuating levels of hormones control insect development (Nijhout, 1974). The initiation of each larval molt cycle begins with the secretion of prothoracicotropic hormone (PTTH), a neuropeptide hormone produced by the brain. PTTH then binds to the PTTH receptor Torso, a receptor tyrosine kinase that activates pathways in the prothoracic glands (PG) involved in the production of steroid molting hormones known as ecdysteroids (Rewitz et al., 2009). The PG are a pair of endocrine organs composed of a single steroidogenic cell type that respond to PTTH through Ca2+/calmodulin signaling (Smith & Rycbczynski, 2011). Each individual gland subsequently secretes ecdysteroids at a similar rate (Gilbert et al., 2002). The main ecdysteroid, ecdysone, is converted to its active form, 20-hydroxyecdysone (20E), by peripheral target tissues (Gilbert et al., 2002). The 20E then coordinates the orderly expression of genes necessary for growth and development.

Molts from one larval stage to another are controlled by a single peak of PTTH and ecdysone just before the end of each larval stage. However, during the final larval stage,
multiple peaks of ecdysone regulate the more complex metamorphic molt (Nijhout, 1994). An initial ecdysone peak, known as the commitment peak, causes behavioral and transcriptional changes that vary with species. Later, a second pulse of PTTH and subsequent round of ecdysone secretion stimulate retraction of the epidermis (apolysis) and deposition of new cuticle, defining the larval-pupal molt (Nijhout, 1994; Gilbert et al., 2002; Riddiford et al., 2003; Gilbert, 2012).

The experimental insect used in the proposed study is the tobacco hornworm, Manduca sexta, which initiates metamorphosis (from larvae to pupae) in the fifth larval stage (instar). M. sexta larvae release a small pulse of PTTH about 3 days after molting to the fifth instar, which in turn stimulates the small (commitment) peak of ecdysone (Bollenbacher et al., 1981). Overall, the timing of PTTH secretion and the initiation of metamorphosis are determined by a size characteristic described as the critical weight. The critical weight is a size threshold at which PTTH secretion becomes independent of further nutrition (Nijhout & Williams, 1974). This observation suggests the presence of a molecular control mechanism, which allows larvae to sense the external environment, relay size information to metamorphic stimulating hormones such as PTTH, and decide the appropriate time to initiate molting and metamorphosis (Nijhout, 1994).

Regulation of hormone production by nitric oxide and hypoxia

One cellular signal that has recently been suggested to regulate PG function is nitric oxide (NO). NO is a product of nitric oxide synthase (NOS) and is a diffusible second messenger that influences a variety of functions in vertebrates, as well as insects (Koch et al., 1994; Davies, 2000; Alderton et al., 2001; Yamanaka & O'Connor, 2011). NO commonly
binds the heme moieties of soluble guanylyl cyclase, stimulating the synthesis of cyclic GMP (cGMP), which in turn activates cGMP-activated kinases (Arnold et al., 1977). NO can also inhibit cytochrome c oxidase in the mitochondria and form posttranslational modifications through S-nitrosylation, G-glutathionylation, and tyrosine nitration (Martínez-Ruiz et al., 2011). In vertebrates, a common end result is vasodilation (Priviero & Webb, 2010). In insects, NO has been implicated in the regulation of fluid production, cell proliferation, synapse formation, and innate immunity (Davies, 2000; Yamanaka & O'Connor, 2011).

In Drosophila, the PG were recently shown to contain high levels of NOS expression, and knockdown of NOS in the PG disrupted the production of ecdysone-stimulating genes and the initiation of metamorphosis (Cáceres et al., 2011). Unlike the more commonly observed cGMP-mediated effects of NO, in the PG of Drosophila melanogaster, NO appeared to have novel effects. Specifically, NO has been observed to directly bind the heme moiety of the nuclear transcription factor E75, a regulator of its heterodimer partner Drosophila hormone receptor 3 (DHR3) (Reinking et al., 2005). The DHR3 gene product enhances transcription of the βfitz-f1 gene, the insect homolog of the vertebrate steroidogenic factor 1, which, in vertebrates, is a regulator of steroidogenic enzyme transcription (Asahina et al., 2000; von Hofsten & Olsson, 2005; Yamanaka & O'Connor, 2011) (Figure 1).

In insects, βFTZ-F1 controls expression of ecdysone synthetic enzyme genes, which enhance ecdysone production in the last larval instar of Drosophila (Broadus et al. 1999). Thus, βFTZ-F1 is a key competence factor for the unidirectional progression through metamorphosis (King-Jones & Thummel, 2005; Yamanaka & O'Connor, 2011). The novel effects of NO signaling on βFTZ-F1 production observed in Drosophila (Caceres et al., 2011) were not directly linked to ecdysone production. The observed phenotypes (extended feeding
and increased fat deposition) do not prove that reduction of NOS causes a reduction in ecdysone steroidogenesis. Therefore we set out to measure the direct effects of NO, using a model system in which ecdysone secretion can be more practically assessed.

Figure 1. Proposed mechanism of NOS-mediated NO control of E75 and DHR3 on βftz-f1 expression in Drosophila. The pink box represents events in the PG and the yellow boxes represents events in the peripheral tissues. Reproduced from Yamanaka & O'Connor (2011).

While we may not think of oxygen as a signaling gas, like NO, oxygen can influence cellular function through a cascade of signaling events. In vertebrates, cells respond to low oxygen tension through hypoxia-inducible transcription factor 1 (HIF-1). HIF-1 is a heterodimeric transcription factor consisting of constitutively active (beta) and oxygen-regulated (alpha) subunits (Semenza, 2010). The availability of HIF-1α induces changes in gene expression that mediate responses to low oxygen, including angiogenesis, altered metabolism, apoptosis, and migration (Semenza, 2010). Similarly, invertebrates respond to low oxygen through a conserved HIF-1 signaling pathway (Gorr et al., 2006).
In insects, the tracheal system carries oxygen from the atmosphere into body tissues. In *M. sexta* the total tracheal volume is fixed throughout each instar, which causes an increase in oxygen demand and a restriction in larval respiration rates (Callier & Nijhout 2011). These authors demonstrated that when *M. sexta* larvae are reared under hypoxic conditions to simulate growth-induced hypoxia, they initiate molting at a lower weight than normoxic controls (Callier & Nijhout, 2011). The results suggest that cellular oxygen tension may serve as a size-sensing mechanism in *M. sexta*.

The above studies (NO signaling in *Drosophila* and tissue oxygenation in *M. sexta*) bring to light observations of two phenomena that may play critical roles in molt signaling and size-sensing. Linking these observations, oxygen limitation has been observed in other studies to not only control body size in insects (Kaiser et al., 2007), but also influence NO signaling (Gess et al., 1997; Palmer et al., 1998; Wingrove & O'Farrell, 1999; O'Farrell, 2009; Ducsay & Myers, 2011). We reasoned that molt initiation, prior to the attainment of normal critical weight, may occur through changes in oxygen supply and nitric oxide synthase (NOS) activity in the PG. Therefore, we measured *in vitro* levels of ecdysone secretion after direct stimulation with NO or low oxygen tension (2% oxygen; 98% nitrogen). To further analyze possible changes in PG ecdysteroidogenic activity, modulated through NOS, both the expression of NOS and the expression of NO-responsive transcription factors (MHR3 and βFTZ-F1) thought to play a role in *Drosophila* ecdysone steroidogenesis were examined using qRT-PCR. We reasoned that if MHR3 and BFTZ-F1 are similarly expressed in DETA-NONOate and hypoxia-treated PG, and if hypoxia can modulate increases in NOS expression, then oxygen tension might influence ecdysone steroidogenesis through NO signaling. In
addition, we measured relative expression of the PTTH receptor torso, as a first step in examining the effects of NO or hypoxia on PTTH response.

**Materials and Methods**

*Animals and Prothoracic glands*

*Manduca sexta* eggs were obtained from Carolina Biological Supply (Burlington, NC). *Manduca* larvae were reared on standard artificial laboratory diet (Bell & Joachim, 1976) at 25°C on a long-day 16hr:8hr light:dark cycle. Larvae were transferred to fresh diet at the time of ecdysis to the fifth (final) larval stage (day 0). Late day two fifth instar animals (4.5-5.3 grams) were anesthetized in water for 10 minutes and their prothoracic glands were removed under lepidopteron saline (Riddiford et al., 1979). Larvae were age/weight matched and prothoracic gland pairs were separated to ensure control and treated samples contained a single gland from each organism.

*Nitric oxide/Hypoxia incubation*

*Manduca sexta* larval prothoracic glands were incubated for 2 hours or 12 hours in Grace’s insect medium (2 glands/100µl droplet) at room temperature under one of the following conditions: Grace’s medium alone under normoxic conditions (20% oxygen), 10mM DETA-NONOate sodium salt (Sigma-Aldrich, St. Louis, MO) under normoxic conditions, or Grace’s medium under hypoxic conditions (2% oxygen: 98% nitrogen). Hypoxic conditions were generated by placing samples in an airtight modular incubator chamber (Billups-Rothenberg, Del Mar, CA). Prior to sealing the chamber, 2% oxygen: 98% nitrogen was pumped into the chamber for 10 minutes at a rate of 15 psi. Oxygen levels were
monitored with a ToxiRAE II oxygen sensor (Rae Systems, San Jose, CA) before being sealed for 2 hours or 12 hours. Control and treated sample groups contained a single prothoracic gland from each organism. All prothoracic glands were removed from incubation media after 2 hours or 12 hours. For Western blots, prothoracic glands (4 glands/sample) were held at 90°C for 3 minutes in 25µL 2X SDS sample buffer and subsequently stored at −20°C. For RNA extractions, prothoracic glands (12 glands/sample) were flash frozen, and stored at −80°C. For measurements of ecdysone secretion, incubation media was collected in 0.5 mL centrifuge tubes and stored at -20°C for radioimmunoassay.

Viability Assay

Trypan blue cell viability assays were conducted after 12 hour treatment with either 10 mM DETA-NONOate, 2% oxygen: 98% nitrogen, or Grace’s medium alone. Prothoracic glands were incubated directly in 0.4% trypan blue (Beckman Coulter, Brea, CA) for 2 minutes and 5 minutes. Non-viable controls were generated by exposing prothoracic glands to ultraviolet light for 5 minutes post incubation. The presence of trypan blue reagent within cells denotes non-viability.

Western blot analysis

Proteins were separated by gel-electrophoresis using 4-20% gradient Mini-PROTEAN® TGX acrylamide gels (BIO-RAD, Hercules, CA) according to the manufacturer’s protocol. Gels were transferred to nitrocellulose membranes (BIO-RAD, Hercules, CA) at 4°C, blocked with 3% BSA or 5% milk for one hour, washed with 1X TTBS for 15-minutes, and placed in primary antibody overnight at 4°C. Primary antibodies included
anti-non-phospho 4EBP, anti-pERK, and anti-actin (Cell Signaling Technologies, Danvers, MA). Blots were rinsed for 15 minutes in 1X TBS and incubated in secondary anti-rabbit antibody with attached HRP or anti-mouse antibody with attached HRP (Cell Signaling Technologies, Danvers, MA) for 75 minutes. Blots were then treated with Pierce® ECL Western blotting substrate (Thermo Fisher Scientific, USA), exposed on blue- sensitive autoradiography film (Midsci, St. Louis, MO), and developed using Kodak® GBC fixer and developer (Kodak, USA). All blots were scanned and analyzed using Image J software (Abramoff et al., 2004). Protein bands were normalized to actin as a loading control.

Quantitative real time PCR (qPCR) analysis of gene expression

*Manduca sexta* βfiz-1 (*GenBank: AF288089.1*), mHR3 (*GenBank: X74566.1*), nos (*GenBank: AF062749.1*), and torso (*Manduca base*) gene expression profiles were analyzed with quantitative real-time PCR (qRT-PCR) using the Applied Biosystems 7000® detection system (Applied Biosystems, USA). To ensure that measureable amounts of RNA were collected, total RNA was isolated from samples containing 12 paired prothoracic glands using PureLink® RNA Micro Kit (Invitrogen, USA) according to manufacturer’s protocol. RNA was spectrophotometrically tested for purity and concentration using NanoDrop® analyzer (Thermo Fisher Scientific, USA). 700ng total RNA was reverse transcribed with random hexamer primers using Superscript III First Strand Synthesis System® (Invitrogen, USA) according to manufacturer’s protocol. Gene specific primers for qRT-PCR analysis were designed using Primer Express software (Applied Biosystems, USA) and CLC Main Workbench (CLC bio A/S, Denmark). Primer sequences were as follows:
$\beta$ftz-1 forward 5’-TGACACCAAGGACGTGATCG-3’
$\beta$ftz-1 reverse 5’-GATTCGCATGTGAGCAGGC-3’

$mhr3$ forward 5’-TCACCCACACCACGTATTTC-3’
$mhr3$ reverse 5’-AGCGTTGAGATCGAATTC-3’

$nos$ forward 5’-TGCGGTCTTCGCTTTAGGG-3’
$nos$ reverse 5’-TGCCGCTACCATCATCGC-3’

$torso$ forward 5’-CGCAATATTCTGGTCACAGGG-3’
$torso$ reverse 5’-CTCCTAGTAAGCGACTCCAGCG-3’

$rp49$ forward 5’-GAGGAATTGGCGTAAACCTAGAG-3’
$rp49$ reverse 5’-TGACGCGTCTTCGCTTTAGGA-3’

Dilution curves generated by cDNA serial dilutions were used to validate reverse transcription efficiency and to calculate primer amplification efficiencies. Primer efficiencies were within 100±10%. qRT-PCR reactions were performed using SYBR Green Master Mix® (Applied Biosystems, USA) in a 25µl reaction mixture containing 12.5µl 2X SYBR Master Mix®; 200nM of each $Bftz$-1, $mHR3$, $nos$, and $torso$ gene specific primer; and 150ng of reverse transcribed total RNA. Samples were analyzed in triplicate and expression levels were normalized to the $Manduca$ ribosomal gene $rp49$ (900nM) after correcting for differences in amplification efficiency (Pfaffl, 2001). All quantitative cycling conditions consisted of 1 cycle at 50°C for 2 minutes, 1 cycle at 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, and 60°C for 1 minute. Non-template controls and non-reverse transcribed RNA were included to account for contamination. qRT-PCR products were subject to melting curve analysis (1 cycle
of 95°C for 15 seconds, 60°C for 20 seconds, and 95°C for 15 seconds), gel-electrophoreses on 2% agarose gel to check PCR product size, and amplicon sequencing to ensure PCR product specificity. Fold changes were calculated using the $2^{-\Delta \Delta Ct}$ method (Schmittgen & Livak, 2008). All comparisons were made between controls and their biological treatments.

**Ecdysone radioimmunoassay**

Radioimmunoassays were used to measure secreted ecdysone in samples of medium from prothoracic gland incubations described above. 10µl of sample were analyzed by procedures as previously described (Warren et al., 1984; Walsh & Smith, 2011). 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). [3 H] labeled ecdysone (60mCi/mmol) was obtained from GE Healthcare.

**Statistics**

Statistical analyses were performed using Student’s t-test with $p \leq 0.05$ denoting statistical significance. All experiments were repeated at least three times to ensure reproducibility of the results, and data are expressed as the mean ± S.E.M.
Results

*Nitric oxide and low oxygen tension inhibit ecdysone secretion.*

To explore the direct effects of hypoxia (2% oxygen: 98% nitrogen) on ecdysone secretion in *M. sexta*, prothoracic glands (PG) were isolated from late day two fifth-instar larvae. It was our goal to analyze PG just prior to the attainment of critical weight. Based on the observation that hypoxic intact *M. sexta* larvae molt at smaller weights, hypoxia was expected to directly enhance ecdysteroid secretion by isolated prothoracic glands. On the contrary, basal ecdysone secretion was significantly reduced (p≤0.0001) following 2-hour hypoxia treatment compared to normoxic controls (Figure 2a). Similarly, 12-hour hypoxia treatment significantly (p≤0.0001) reduced basal ecdysone secretion (Figures 2b). These observations indicate that hypoxia has an inhibitory, rather than a stimulatory, effect on ecdysone secretion.

To test the effect of nitric oxide (NO) on secretion, which again was postulated to be stimulatory, isolated PG were incubated in droplets of Grace’s growth media supplemented with 10mM of an NO donor (DETA-NONOate) for 2 hours or 12 hours. After 2 hour or 12 hour NO treatment, basal ecdysone secretion was significantly (p≤ 0.005 and p≤0.0001) reduced as compared to controls lacking NO supplemented media (Figure 3 a, b). Lower doses of DETA-NONOate (0.1 mM and 0.001mM) had no effect on basal ecdysone secretion (data not shown). My results indicate that direct exposure to NO inhibits basal ecdysone secretion.
Figure 2. Direct effects of 2 hour and 12 hour low oxygen tension (2% oxygen: 98% nitrogen) on basal ecdysone secretion by *M. sexta* prothoracic glands. Pairs of prothoracic glands in Grace's insect media were supplemented with nitric oxide donor, incubated under hypoxic conditions (2% oxygen: 98% nitrogen), and then ecdysone was measured by radioimmunoassay using 10µl samples. Error bars represent ± S.E.M. Asterisks indicate significant differences as compared to controls (atmospheric oxygen, 20%). **A)** 2 hour incubation (*n*=24; *p*≤0.001). **B)** 12 hour incubation (*n*=17; *p*≤0.001).
Figure 3. Direct effects of 2 hour and 12 hour 10mM DETA-NONOate stimulation on basal ecdysone secretion by M. sexta prothoracic glands. Pairs of prothoracic glands in Graces insect media were supplemented with nitric oxide donor, and then ecdysone was measured by radioimmunoassay using 10µl samples. Error bars represent ± standard S.E.M. Asterisks indicate significant differences as compared to controls (atmospheric oxygen, 20%). A) 2 hour NO treatment (n=24; p≤0.005). B) 12 hour NO treatment (n=17; p≤0.001).
PG cell viability after 12-hour hypoxia and 12 hour DETA-NONOate treatment.

Because both hypoxia and DETA-NONOate strongly reduced ecdysone secretion, PG viability was assessed using a trypan blue assay. PG were dissected and incubated in normoxic conditions, hypoxic conditions, or normoxic conditions with DETA-NONOate for 12 hours, followed by direct incubation with 0.4% trypan blue for 2 minutes (not shown) or 5 minutes. For all conditions, no stain was detected within cells, indicating that the cells remained viable for 12 hours (Figure 4b, 4c). By contrast non-viable cells created by exposure to ultraviolet light permitted entry of trypan blue reagent after 5-minute exposure (Figure 4d).

Figure 4. Trypan blue (0.4%) cell viability assay of 12 hour 10mM DETA-NONOate and 12 hour hypoxic (2% oxygen: 98% nitrogen) M. sexta prothoracic glands. Pairs of prothoracic glands were dissected under saline and incubated in 100μl droplets of Grace’s growth media. PG were removed from growth media, directly treated with 0.4% trypan blue for 5 minutes, and viewed under a microscope. A) PG incubated in Grace’s media only for 12 hours. B) PG incubated in 2% oxygen for 12 hours. C) PG incubated in 10mM DETA-NONOate for 12 hours. D) PG incubated in Grace’s media for 12 hours and subject to 5-minute UV exposure.
Non-phospho4EBP and pERK levels fluctuate with NO or hypoxia

As a means of further assessing the influence of hypoxia and NO on glandular function, Western blot analysis of two diagnostic proteins, non-phospho4EBP (non-p4EBP) and phosphoERK (pERK) was conducted. Specifically, non-p4EBP has been linked to reductions in PG activity, particularly under conditions of starvation (Walsh & Smith, 2011). Conversely, enhanced glandular content of pERK has been linked to increased steroidogenic output (Rybczynski et al., 2001). Following 2 hour hypoxia-treatment, the amount of non-p4EBP increased significantly (p≤ 0.05) while the amount of pERK decreased, though not significantly, relative to controls (Figure 5a). Similar results were also observed following 2-hour NO exposure (Figure 5b). These results are in keeping with reductions in steroidogenesis.

However, after 12 hours of hypoxia or DETA-NONOate treatment, non-p4EBP and pERK equalized between experimental and control groups (Figure 6 a, b). In the case of NO, a trend toward reduced non-p4EBP was observed. Taken together, the similar responses of the PG to both hypoxia and NO suggest a shared gas-sensing control point in the mTOR and MAPK signaling pathways. These results also suggest that the observed change, from an inhibitory to a neutral status, may reflect a reacquisition of steroidogenic capacity after 12 hour exposure to hypoxia or NO.
Figure 5. Western blot analysis of non-phospho4EBP and phospho-ERK after 2 hour hypoxia and 10mM DETA-NONOate. Blots show representative samples. Each sample contains four glands from age- and weight- matched larvae. Quantification of Western blot band pixel density was done using Image J software. A Student’s t-test was used to calculate significance. Asterisks denote significant differences. Error bars represent ± S.E.M. A) 2 hour hypoxia (n= 6; p≤0.05). B) 2 hour 10mM DETA-NONOate (n=8; p≤0.005).
Figure 6. Western blot analysis of non-phospho4EBP and phospho-ERK after 12 hour hypoxia and 10mM DETA-NONOate. Blots show representative samples. Each sample contains four prothoracic glands from age- and weight- matched larvae. Quantification of Western blot band pixel density was done using Image J software. A Student’s t-test was used to calculate significance. Error bars represent ± S.E.M. A) 12 hour hypoxia (n=5). B) 12 hour DETA-NONOate (n=6).
Expression levels of βftz-f1, mhr3, nos, and torso

The activation of novel NO signaling, in Drosophila, begins when ecdysone binds the ecdysone receptor complex and activates transcription of the early response gene E75. In previous work, the ability of E75 to bind and control other transcription factors was found to be modulated by interactions of monoatomic gases with a ligand binding domain heme moiety (Reinking et al., 2005). In Drosophila, E75-mediated repression of DHR3 was alleviated by the activation of NOS (Cáceres et al., 2011). Together, this signaling cascade controls the timely expression of βFTZ-F1, a metamorphic competence factor that in addition to controlling stage specific responses, also affects expression of two ecdysone steroidogenic enzymes (Parvy et al., 2005). The present study investigated whether transcription factors proposed to have novel control over molting and metamorphosis in Drosophila (MHR3, βFTZ-F1, and NOS) were present in M. sexta PG and whether these transcription factors respond to NO in a manner similar to that observed in Drosophila.

Additionally, the recent connections between hypoxia and the enhancement of NOS expression led us to hypothesize that hypoxia-induced acceleration of molting may function through novel NO-responsive transcription factor signaling in the PG. We expected to observe increased expression of nos, mhr3, and βftz-f1 under hypoxic conditions. Lastly, since our Western blot analysis demonstrated a possible reacquisition of steroidogenic capacity after 12-hour treatments, we tested the effects of NO and hypoxia on the mRNA transcription levels of Torso, the putative PTTH receptor.

Gene expression was measured in groups of 12 PG and analyzed by qRT-PCR. Levels of expression in PG exposed to NO were compared to levels of expression in controls from the same larvae (matched controls). Exposure to NO (10mM DETA-NONOate) significantly
increased mhr3 expression at 2 and 12 hours (p≤0.05) (Figure 7). A trend toward increased βftz-f1 expression was also observed at 2 and 12 hours; however, these levels did not reach significance. Increases in mhr3 and βftz-f1 are in keeping with a trend of enhanced steroidogenic capacity. Moreover, NO treatment increased torso expression at 2 hours and significantly increased expression at 12 hours (p≤0.05) (Figure 7). In contrast, a negligible decrease in nos expression was detected at 2 hours, with a significant decrease occurring at 12 hours (p≤0.05). Our results suggest a potential negative feedback by long-term NO on nos transcription. It seems that the influence of NO on mhr3, βftz-f1, and torso may be enhancing steroidogenic capacity and PG sensitivity.

The same genes were measured following exposure to hypoxia. Overall, hypoxia had a disparate effect on mhr3 expression. At 2 hours, levels of mhr3 expression were significantly increased, similar to observations to NO treatment. However, at 12 hours, mhr3 expression was negligibly affected. It is unknown at this time what caused these differences. In keeping with results observed after NO treatment, hypoxia significantly increased overall torso expression at 12 hours (p≤0.05) (Figure 8). In contrast, βftz-f1 and nos expression showed little change in response to hypoxia. The fact that nos transcription was unaffected provides indirect evidence that hypoxia does not strongly alter glandular levels of NO. The absence of changes in βftz-f1 transcription, suggest that hypoxia has little effect, or is ultimately inhibitory, of steroidogenic enzyme activity, but may have novel influences on early MHR3 expression. Overall, increased torso expression indicates that hypoxia may enhance sensitivity to PTTH.
Figure 7. Relative gene expression of molt-related transcription factors after 2 and 12 hour 10mM DETA-NONOate. RNA was collected from samples containing twelve-pooled, fifth-instar day two PG and reverse transcribed using random hexamer primers. 150ng cDNA was analyzed using quantitative real time PCR. Primer efficiencies fell within 100±10%. Expression levels were normalized *Manduca* ribosomal protein (RP49) transcript. The relative change in gene expression ($2^{-\Delta\Delta CT}$) of target genes was calculated from the $\Delta\Delta CT$ value after normalization using the Livak method (Livak & Schmittgen, 2001). Each bar represents the mean ± S.E.M of paired assays. A Student’s t-test was used to calculate significance as indicated by asterisks, * $p \leq 0.05$. 
Figure 8. Relative gene expression of molt-related transcription factors after 2 and 12 hour hypoxia. RNA was collected from samples containing twelve-pooled fifth-instar day two PG and reverse transcribed using random hexamer primers. 150ng cDNA was analyzed using quantitative real time PCR. Primer efficiencies fell within 100±10%. Expression levels were normalized Manduca ribosomal protein (RP49) transcript. Transcription levels of bftz-f1, mhr3, nos and torso were measured compared to normoxic (20% oxygen) controls. The relative change in gene expression ($2^{\Delta \Delta C_T}$) of target genes was calculated from the ΔΔCT value after normalization using the Livak method (Livak & Schmittgen, 2001). Each bar represents the mean ± S.E.M of paired assays. A Student’s t-test was used to calculate significance as indicated by asterisks, * $p \leq 0.05$. 
Discussion

*Effects of hypoxia on ecdysone secretion and 4EBP*

The coordination of growth signals and the initiation of ecdysteroid synthesis are critical for the normal timing of development (Nijhout, 1994). The initiation of molting and metamorphosis are controlled by the synthesis of ecdysone molting hormone by the prothoracic glands (PG). In fifth-instar *M. sexta*, achievement of the critical weight, a developmental checkpoint whereby no further nutrition is needed for normal timing to metamorphosis (Nijhout & Williams, 1974), marks the period just prior to the first ecdysone peak (Baker et al., 1987; Riddiford et al., 2003). Activation of PG during the first ecdysone peak irreversibly triggers synchronous signaling cascades responsible for molting and metamorphic changes. This investigation examined isolated PG during the developmental period just prior to the achievement of critical weight to investigate whether direct stimulation by hypoxia (2%) or a NO donor (10mM DETA-NONOate) directly enhance ecdysteroid secretion.

In early instar *M. sexta* larvae, intratracheal oxygen levels can be maintained as low as 5 kPa (approximately 5% oxygen) (Greenlee & Harrison, 2008). However, as the size of larvae increase so does the demand for more oxygen. Low oxygen tension has been observed to accelerate the appearance of molting and metamorphic markers (dorsal heart exposure and wandering behavior) in *M. sexta* fifth-instar larvae reared under hypoxic conditions (Callier & Nijhout, 2011). The presence of these physiological and behavior markers, at lower weights than normoxic larvae, indicates that oxygen content may have a role in the size-sensing metric that determines when molting is initiated (Nijhout & Williams, 1974; Callier & Nijhout, 2011). Additionally, hypoxia has been reported to increase the expression of nitric oxide
synthase, an enzyme that produces nitric oxide gas (Fagan et al., 2001; Monau et al., 2009; O'Farrell, 2009). Control of NO-responsive genes is thought to regulate the timing and progression of ecdysteroid secretion (Cáceres et al., 2011). We reasoned that hypoxia-induced early molting might be controlled at the level of the PG through NO responsive transcription factors, augmenting brain (PTTH)-gland regulatory stimuli (Smith & Rybczynski, 2011).

The present study first investigated whether hypoxia could directly enhance ecdysteroid secretion in *M. sexta* larval PG. This critical variable was not reported in previous reports (Callier & Nijhout, 2011; Cáceres et al., 2011). We hypothesized that hypoxia would enhance steroidogenesis in vitro. On the contrary, radioimmunoassay of ecdysone at low oxygen tension (2%), revealed significant inhibition of steroidogenesis in response to short-term (2 hour) and long-term (12 hour) hypoxia. While our observations do not reveal a mechanism for steroidogenic inhibition, the results do agree with observed inhibitory effects of hypoxia on vertebrate steroidogenesis (Raff et al., 1996; Bruder, 2002; Nishimura et al., 2006; Jiang et al., 2011). Hypoxia may directly inhibit oxygen-sensitive steroidogenic enzymes belonging to the cytochrome P-450 family due to the necessity for molecular oxygen in enzyme-catalyzed reactions (Lieberman & Lin, 2001). In *insects*, P-450 mediated oxidation steps are required for the synthesis of ecdysone precursor molecules in the PG (Gilbert et al., 2002; Warren et al., 2009). In comparison, the production of vertebrate steroid hormones similarly requires the biochemical conversion of cholesterol to the intermediate pregnenolone in the inner mitochondrial membrane using cytochrome enzymes (Simpson, 1979; Stocco, 2001). In one account, hypoxia was shown to reduce ACTH-stimulated aldosterone, cAMP, and corticosterone synthesis, and the expression of P-450scc and P-450aldo enzymes (Bruder, 2002). Additionally, in bovine primary corpus luteum cells, progesterone production,
pregnenolone content, and P450scc mRNA expression were reduced in LH- and non-LH-stimulated hypoxic cells (Nishimura et al., 2006). Lastly, short-term hypoxia led to a decrease in aldosterone synthesis through decreased expression of the P-450c11AS enzyme in adult rats (Raff et al., 1996) and a small yet significant reduction in cAMP stimulated steroidogenesis in hypoxic pre-natal rats (Raff et al., 2000). However, hypoxia was seen to have little effect on neonatal aldosterone secretion (Raff et al., 1999). On balance, studies on vertebrate steroidogenesis indicate that hypoxia inhibits steroidogenesis at the level of the P-450 enzymes. Future experiments are needed to clarify the role of P-450 enzymes and their activity in fifth-instar *M. sexta* PG under hypoxia.

Previous studies have revealed characteristic changes in phosphoprotein content in active PG. Specifically, steroidogenically active PG show increased levels of phosphorylated ERK, a member of the MAPK pathway required for ecdysone secretion (Rybczynski and Gilbert, 2003; Walsh and Smith, 2011). Active PG also display increased phosphorylation of the translation inhibitor 4EBP, and decreased amounts of non-phosphorylated 4EBP, with a net effect of enhanced translation, which is also necessary for secretion (Walsh and Smith, 2011). In the present study, we used pERK and non-p4EBP as markers to follow intracellular alteration in PG activity. Our results revealed that short-term (2-hour) hypoxia significantly increased levels of non-p4EBP and decreased levels of pERK, both in keeping with the inhibition of translation and reduced steroidogenesis. In vertebrates, low oxygen tension has been observed to reduce protein synthesis (Kraggerud et al., 1995). Inhibition of translation has been reported in tumor models where large shifts in 4EBP dephosphorylation were observed after hypoxia (Connolly et al., 2006). Further, siRNA knockdown of 4EBP in immortalized breast epithelial cells uncouples hypoxia from inhibition of translation.
(Connolly et al., 2006). Also, overexpression of non-p4EBP protein in muscle cells induces a hypoxia-like decrease in the number of mitotic cells and a decrease in muscle-specific proteins (Hidalgo et al., 2012). These studies indicate that hypoxia can inhibit protein synthesis via increased 4EBP content (Connolly et al., 2006). The results of the present study indicate that effects of NO and hypoxia on intracellular signaling proteins in the PG are short-lived. In particular, after 12 hours of NO or hypoxia, both non-p4EBP and pERK are similar to controls. These results suggest that short-term and long-term hypoxia have differential effects on glandular function.

Effects of nitric oxide on ecdysone secretion and 4EBP

We initially predicted that DETA-NONOate treatment of isolated PG would increase basal ecdysone secretion. Our results conversely revealed significant reductions in basal ecdysone secretion for both short-term (2 hour) and long-term (12 hour) incubations. Our results support observations of inhibitory effects of NO on vertebrate endocrine cells (Lal & Dubey, 2012), and may further indicate functional similarities between hypoxia and NO steroidogenic inhibition through heme moieties of cytochrome P-450 enzymes (Del Punta et al., 1996). NO inhibition has previously been reported to inactivate the P-450 enzyme aromatase involved in vertebrate steroid hormone synthesis, specifically by the nitrosylation of cysteine amino acids in the enzyme’s active site (Snyder et al., 1996). In porcine granulosa cells, basal and GnRH-stimulated estradiol secretion and P-450 aromatase activity decreased after treatment with a NO donor (Masuda et al., 1997). Inhibition of NOS, conversely increased estradiol production (Masuda et al., 1997; Masuda et al., 2001). Differences in the extent of steroidogenic suppression changed as follicular growth progressed (Masuda et al.,
This may indicate that at some point in tissue growth, NO repression becomes ineffective. In buffalo granulosa cells, reduced estradiol-17β and progesterone secretion were also detected in the absence and presence of follicle stimulating hormone (FSH) following treatment with the nitric oxide donor SNAP (Shanmugam et al., 2013). These studies indicate that NO may inhibit steroidogenesis through P-450 enzyme modulation. Further investigation of P-450 enzyme activity is needed in M. sexta PG to examine whether NO modulates effects on ecdysone steroidogenesis through similar mechanisms.

Interestingly, NO and hypoxia had similar effects on PG levels of non-p4EBP and p-ERK. Most notably, non-p4EBP content was significantly increased following short-term NO treatment, while long-term NO treatment modestly reduced non-p4EBP levels compared to controls. In vertebrates, net effects of NO on translation have been reported as inhibitory or stimulatory, and the mechanisms underlying effects on protein synthesis are not clear. For example, in vertebrate skeletal muscle, inhibition of translation by LPS/IFNγ is accompanied by an elevation in NOS activity, an inhibition of Akt phosphorylation by NO, and an enhancement of AMP-K phosphorylation of the mTOR1 mediator Raptor (Frost et al., 2009). By contrast, in breast epithelial cancer cells, NO was observed to enhance translation, via interactions with both MAPK and PI3K/AKT signaling pathways (Pervin et al., 2007). Our results suggest that short-term and long-term effects of NO on translation may differ. NO initially increases non-p4EBP and later decreases non-p4EBP content. Further research must be done to investigate the role of NO on the modulation protein translation in fifth-instar M. sexta PG that may, over time, alter ecdysone secretion, molting, and metamorphosis.
Nitric oxide and hypoxia: βFTZ-F1, MHR3, and NOS mRNA expression

βFTZ-F1 is a stage specific competence factor that controls expression of late genes that encode expression of ecdysone synthetic enzymes (Hiruma & Riddiford, 2001; Parvy et al., 2005; Ruaud et al., 2009). In Drosophila, the transcription of βFTZ-F1 is stimulated by the nuclear receptor DHR3, which is in turn repressed by the nuclear receptor E75. This transcriptional cascade is further stimulated by NOS activity (Caceres et al., 2011) and is thought to comprise a regulatory mechanism that drives progression of metamorphic events in the PG (Yamanaka & O’Connor, 2011).

Therefore, the present study examined mRNA expression levels of key transcription factors required for the regulation of molting and metamorphosis (Cáceres et al., 2011). Using qRT-PCR, this study assessed relative expression of mhr3 and βftz-f1 in isolated PG. We hypothesized that similar to Drosophila, NO treatment would increase expression of both mhr3 and βftz-f1. Indeed, both short-term and long-term NO treatments increased mhr3 and βftz-f1 expression (although βftz-f1 expression levels did not reach significance). These results suggest that the regulation of mhr3 and βftz-f1 in Manduca are similar to that seen in Drosophila, but that such changes are not responsible for the enhanced production of ecdysone in isolated PG. We expected to see very little expression of mhr3 and βftz-f1 in glands that were not stimulated with NO. This is because mhr3 is transcriptionally regulated by the binding of ecdysone to the ecdysone receptor (EcR), in conjunction with the nuclear receptor/heterodimer Ultraspiracle (USP). While no ecdysone was added to our in vitro PG cultures, the glands themselves may have secreted enough ecdysone to facilitate basal transcription of downstream nuclear receptors.
In addition to measuring steroidogenesis-related transcription factors, we measured the expression of *nos* in the PG as a means of further monitoring glandular response to NO. In *Drosophila*, NOS activity has been shown to be limited through NO feedback inhibition (Martínez-Ruiz, 2005). In vertebrates, NO has been observed to inhibit NF-kB, a pro-inflammatory transcription factor that increases transcription of endothelial NOS genes (Grumbach et al., 2005; Park et al., 2007). Our results reveal, as expected, that NO has a negative effect on NOS expression in the PG. The mechanism underlying this response remains to be determined.

The present study further investigated whether genes regulated by NO were also regulated by low oxygen tension. Our results revealed little to no change in β-ftz-f1 expression after 2 hours or 12 hours of hypoxia. With regard to *mhr3*, short-term hypoxia significantly increased gene expression, while long-term hypoxia moderately decreased expression. *Mhr3* has effects unrelated to steroidogenesis (Carney et al., 1997; Montagne et al., 2010). The non-steroidogenic effects of *mhr3* may, with hypoxia, lead to changes in gene expression that were not measured in this study. With regard to *nos*, little change was seen following hypoxia. These results suggest that if hypoxia is enhancing NO, as proposed in other systems (Gess et al., 1997; Wingrove & O'Farrell, 1999; O'Farrell, 2009), it is not doing so to the extent that NOS transcripts are reduced, as observed with 10mM DETA-NONOate treatment.

*Torso mRNA expression*

Our results indicate that NO treatment can enhance the expression of genes related to steroidogenesis, yet inhibit basal ecdysone secretions. We reasoned that, particularly after 12-hour treatment, significant increases in *mhr3* and corresponding trends in β-ftz-f1, along with
the reacquisition of translational markers, might have positive effects on other aspects of PG function. Using qRT-PCR analysis, we quantified the relative expression of *M. sexta torso*. Torso is a receptor tyrosine kinase, and the putative PTTH receptor (Rewitz et al., 2009). Moderate up-regulation in *torso* transcript was observed in both short-term and long-term NO treatment relative to biological controls. We additionally tested *torso* expression levels in short-term and long-term hypoxic PG. Again to our surprise, and similar to NO treatment, long-term hypoxia significantly up-regulated *torso* transcription. The mechanism for this change is not known, but is likely to involve transcriptional regulation via HIF-1α, which in conjunction with its partner HIF-1β, binds to hypoxia-regulated-DNA elements (HRE). Genes with HRE are responsible for adaptive cellular responses to hypoxia (Semenza, 2010). Both hypoxia and NO have been reported to affect the nuclear content of HIF-1α (Semenza, 2003). HIF-1α is controlled by the rate of protein degradation, which occurs more slowly in hypoxic than in normoxic cells (Semenza, 2010). NO also stabilized HIF-1α, in this case by enhancing its mRNA expression (Metzen, 2003; Brune & Zhou, 2007). This study is the first to report on enhanced Torso transcription by hypoxia and NO in isolated *M. sexta* PG. At this time, more research is necessary to understand what causes increases in Torso transcription in *M. sexta*. It is plausible that Torso possesses HRE gene sequences that sensitize the PG to NO and hypoxia. It would be interesting to see if inhibitors of HIF-1α attenuate hypoxia- and NO-induced Torso up regulation.

In conclusion, our results demonstrate that hypoxia and NO signaling directly inhibit ecdysteroid secretion in isolated *M. sexta* PG, but at the same time, may enhance PG responsiveness. Results point to regulatory triggers outside of the PG as initiators of the secretion of development-promoting peaks of ecdysone. We propose that as oxygen becomes
limited by growth outpacing tracheal capacity, hypoxia and NO alter glandular and non-
glandular functions. In the PG, P-450 enzymes are initially inhibited, transiently decreasing
steroidogenesis. Further, translation is impaired through up regulation of 4EBP. Over time,
hypoxia and NO then continue to prime the PG, i.e. to enhance aspects of glandular
preparedness to secrete ecdysone, including expression of MHR3 in the case of NO, and to
respond to PTTH. Translational capacity is restored, and up-regulated torso expression
facilitates PTTH-responsiveness. Outside of the PG, one would postulate that the net
stimulatory effects of hypoxia and NO on the peripheral regulation of glandular function
might enhance the release of PTTH and other prospective regulatory factors. This model
would explain observations in *Drosophila* and *Manduca*. In *Drosophila*, knockdown of *nos* as
would deter the propensity of larvae to metamorphose, prolonging the larval feeding. In
*Manduca*, one would observe a decrease in critical weight in hypoxia larvae due to premature
acquisition of optimal PTTH responses at lower weights.

Clearly, additional studies are needed to probe the steroidogenic consequences of
Torso up-regulation. The mechanisms underlying changes in Torso mRNA expression are
also intriguing, for example, our results suggest the presence of HRE in the promoter region
of Torso gene. Analysis of this promoter region will be of general interest in identifying other
potential regulators of this important receptor. The results of the present, and future studies
will provide a useful bridge in reconciling the cellular effects of signaling gases, such as NO
and hypoxia, with observed effects on insect development. Such studies may also provide
novel insights into the regulation of developmentally important steroids across animal groups.
References


