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The effect of within-instar development on tracheal diameter and hypoxia-inducible factors α and β in the tobacco hornworm, *Manduca sexta*

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Abstract

As insects grow within an instar, body mass increases, often more than doubling. The increase in mass causes an increase in metabolic rate and hence oxygen demand. However, the insect tracheal system is hypothesized to increase only after molting and may be compressed as tissues grow within an instar. The increase in oxygen demand in the face of a potentially fixed or decreasing supply could result in hypoxia as insects near the end of an instar. To test these hypotheses, we first used synchrotron x-ray imaging to determine how diameters of large tracheae change within an instar and after molting to the next instar in the tobacco hornworm, *Manduca sexta*. Large tracheae did not increase in diameter within the first, second, third, and fourth instars, but increased upon molting. To determine if insects are hypoxic at the end of instars, we used the presence of hypoxia-inducible factors (HIFs) as an index. HIF- α and HIF- β dimerize in hypoxia and act as a transcription factor that turns on genes that will increase oxygen delivery. We sequenced both of these genes and measured their mRNA levels at the beginning and end of each larval instar. Finally, we obtained an antibody to HIF- α and measured protein expression during the same time. Both mRNA and protein levels of HIFs were increased at the end of most instars. These data support the hypothesis that some insects may experience hypoxia at the end of an instar, which could be a signal for molting.

1. Introduction

Insects exhibit two types of growth, continuous and discontinuous. Continuous growth occurs in soft structures and is evidenced by measures of body mass, which can increase

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Competing interests

The authors declare no competing interests.

Author contributions

RA sequenced HIF- α and HIF- β and wrote methods for those processes. JK conducted qPCR and Western blotting and their analyses, assisted with sequence analysis, and edited methods. TL conducted qPCR and western blotting studies, X-ray experiments, and wrote the first several drafts of this manuscript. KJG designed the experiments, obtained funding, performed statistical analyses, and made figures. All authors edited the manuscript.

200- to 10,000-fold from hatching to the penultimate instar in some species (Goodman et al., 1985; Greenlee and Harrison, 2004). Discontinuous growth occurs in structures that are formed from the hardened exoskeleton and only increase in size after molting (Chapman, 1998). The juvenile life stage is punctuated with periodic molts during which the exoskeleton, including the large airways of the respiratory system, are enlarged and replaced. The major tracheae are thought to increase in size only after a molt (Clarke, 1957a; Snelling et al., 2011)

One consequence of having a fixed respiratory capacity is that oxygen supply may not keep up with increases in oxygen demand from increased tissue mass (Callier and Nijhout, 2011; Clarke, 1957b; Greenlee and Harrison, 2004, 2005). In addition, increasing masses of internal tissues with fixed external structures could compress internal, air-filled spaces. Indeed, in grasshoppers, *Schistocerca americana*, oxygen delivery does not match demand as they progress within an instar, and grasshoppers exhibit decreased tolerance to hypoxia (Greenlee and Harrison, 2004), decreased jump performance, and compressed air sacs (Kirkton et al., 2012). Caterpillars (*Manduca sexta*) also exhibit signs of mismatches between oxygen supply and demand, as they show decreased hypoxia tolerance at the end of an instar compared to the beginning of the instar (Greenlee and Harrison, 2005). Tracheal system volumes have been found to decrease within an instar in two species of grasshopper (Clarke, 1957a; Lease et al., 2006) and in the 3rd and 4th instars in *M. sexta* (Callier and Nijhout, 2011). This has led researchers to hypothesize that oxygen demand outstripping oxygen supply within an instar may be a signal for molting (Callier and Nijhout, 2011; Greenberg and Ar, 1996; Greenlee and Harrison, 2004, 2005).

To test the hypothesis that tracheal system volumes are fixed or even decreasing within an instar, several studies have measured tracheal system volume during this time period. However, potential problems with these studies arise from the methods that were used to measure tracheal system volume, potentially leading to over- or under-estimation of tracheal volume. For example, previous measures of tracheal system volume have used vacuum pressure to fill tracheae with fluid, which can lead to over- or under-filling of the tracheae (Callier and Nijhout, 2011; Clarke, 1957a).

If tracheal volumes are fixed or decreasing within an instar, the mismatch between oxygen supply and demand may become so severe that insects could become hypoxic as they reach the end of an instar, in turn initiating the hypoxia-inducible-factor (HIF-1) signaling pathway. HIF-1 is an important transcription factor and a key regulator of cellular responses to hypoxia in mammals, triggering many physiological pathways that maintain energy homeostasis, such as angiogenesis, erythropoiesis, glucose and iron transport, glycolysis, tricarboxylic acid cycle, cell proliferation, and apoptosis pathways (Ke and Costa, 2006; Prabhakar and Semenza, 2012; Semenza et al., 1991). HIF-1 is composed of two protein subunits, HIF-1 α and HIF-1 β . HIF-1 α is continuously expressed, ubiquitinated, and degraded in normoxia (Gorr et al., 2006). HIF-1 β is also continually expressed in normoxia, but not degraded (Huang et al., 1998; Pugh et al., 1997). In hypoxia, HIF-1 α degradation is inhibited, whereupon it dimerizes with HIF-1 β , forming the HIF-1 complex (Wang and Semenza, 1995). Levels of HIF-1 protein have been shown to increase during hypoxia in many animals, including invertebrates such as *Caenorhabditis elegans* (Jiang et al., 2001;

Powell-Coffman et al., 1998), *Callobruchus maculatus* (Ahn et al., 2013), *Daphnia magna* (Gorr et al., 2004), *Drosophila melanogaster* (Ma and Haddad, 1999; Nambu et al., 1996; Ohshiro and Saigo, 1997; Sonnenfeld et al., 1997), and *Melitaea cinxia* (Marden et al., 2013).

In this study, we tested the hypothesis that the tracheal system supply is fixed or even decreasing within an instar, which in turn leads to insects that experience hypoxia near the end of an instar. We first determined how diameters of large tracheae change from the beginning to the end of an instar, using the tobacco hornworm caterpillar, *M. sexta*. We used synchrotron X-ray imaging, because it is non-invasive and allowed us to visualize living, breathing insects. We measured diameters of large tracheae in the head and hind segments of caterpillars at the beginning and end of the first, second, third, and fourth instars. To determine whether caterpillars become hypoxic and initiate the HIF-1 pathway at the end of an instar, we measured gene expression of the hypoxia-inducible factor subunits, HIF1- α and - β , and protein expression of HIF1- α at the beginning and end of the first, second, third, fourth, and fifth instars.

2. Materials and methods

2.1 Animals

Manduca sexta (Linnaeus) caterpillars were purchased as eggs (Carolina Biological Supply, Burlington, NC, USA) or collected from adult moths reared at North Dakota State University (Fargo, ND, USA). Larvae were reared as previously described, with ad libitum access to a wheat germ-based diet under a 16L:8D photoperiod at 25°C (Vishnuvardhan et al., 2013). Fresh food was provided every two days. Caterpillars were observed daily to determine age within an instar, as indicated by the presence or absence of a slipped head capsule and fresh exuvia. A head capsule is the sclerotized exoskeleton of the head, which becomes too small as the animal grows within an instar. The head capsule appears to slip forward on the anterior of the caterpillar prior to molting, indicating the end of an instar and impending molt. Once the head capsule and exoskeleton were shed, animals were considered to be at the beginning of the next instar.

2.2 Synchrotron X-ray imaging

To determine how the diameters of large tracheae change from the beginning to the end of an instar, we used synchrotron phase-contrast X-ray imaging as previously described (Greenlee et al., 2013; Kirkton et al., 2012; Socha et al., 2007). Caterpillars of different ages were transported to Argonne National Laboratory (Argonne, IL, USA) with ad libitum access to artificial diet and maintained at 25°C. Daily, caterpillars were weighed on an analytical balance to the nearest 0.01 mg (model 245, Mettler, Columbus, OH, USA), and body length and head width were measured using digital calipers to the nearest 0.01 mm (Fisher Scientific, Waltham, MA, USA).

For imaging, caterpillars were placed in an acrylic or aluminum chamber with Kapton® windows on a remote-controlled stage. Chambers were perfused with either 21 kPa or 1 kPa O₂ ranging from 300 to 5000 ml per min, depending on the animal's size. Caterpillars at the

beginning and end of each instar were imaged. The goal was to have $n = 10$ for each instar and stage within the instar. However, due to availability of animals of the right ages during our time at Argonne and the resulting quality of x-ray video images for a few caterpillars, sample sizes of each group varied (1st instar: beginning $n = 8$, end $n = 7$; 2nd instar: beginning $n = 7$, end $n = 9$; 3rd instar: beginning $n = 9$, end $n = 6$; 4th instar: beginning $n = 9$, end $n = 9$; 5th instar: beginning $n = 10$, end $n = 0$). Because the field of view is only about $2 \text{ mm} \times 2 \text{ mm}$, caterpillars at the end of the 5th instar were too big for imaging. Thus, comparisons of the beginning versus end of that instar are lacking. Focal tracheae in the head and hind region (Fig. 1) were recorded for at least 1 min each during exposure to each gas mixture. Resulting video was converted to image sequences using ImageJ (Rasband, 1997–2011) and analyzed frame by frame for maximal (open) and minimal (compressed) tracheal diameter, as we have done previously (Greenlee et al., 2013). For caterpillars that did not exhibit tracheal compression (i.e., all those in normoxia and younger caterpillars), open and compressed diameters were the same. In insects that exhibited tracheal compression, compression frequency was calculated.

2.3 Measurement of HIF- α and HIF- β

To determine if insects are hypoxic at the end of an instar, we used the presence of hypoxia-inducible factors (HIFs) as an index of hypoxia. However, before we could measure HIF expression at the beginning and end of each instar, we first had to sequence both of those genes. Finally, we obtained an antibody to HIF- α and measured protein expression at the same times. For all molecular biology kits or reagents used, we followed the manufacturer's protocol and only note exceptions to that throughout the rest of this section.

2.3.1 Cloning and sequencing of HIF- α and HIF- β —To ensure that we had adequate expression of HIF- α and HIF- β for sequencing, we exposed caterpillars to 4 hours of anoxia. Fifth instar caterpillars at the beginning of the instar were used to ensure adequate amounts of tissue for extracting mRNA. A mass flow meter (Sierra Instruments, Monterey, CA, USA) and a mass flow controller (MFC-4, Sable Systems, Inc., Las Vegas, NV, USA) were used to control the flow of 100% N_2 through the chamber, a glass jar. The jar lid was fitted with hose barbs and three-way valves, flushed with N_2 for one minute every hour, and sealed for the remaining time. Caterpillars were immediately flash frozen in liquid N_2 and kept at -80°C prior to dissection and RNA extraction.

Thawed caterpillars were cut open dorsally and the guts removed. Tracheae and fat body were removed to an Eppendorf tube and diluted 1:10 with Trizol reagent for RNA total extraction (ThermoFisher, Waltham, MA, USA) with all the centrifugation steps performed at room temperature. DNA contamination was removed from total RNA using *DNase I*, amplification grade (Invitrogen, Carlsbad, CA, USA). RNA quality was determined on a denatured 1.2% agarose gel. Total RNA (1–2 μg) was used to synthesize 5' and 3' RACE cDNA each using Clontech SMARTER™ RACE cDNA amplification kit (Takara BIO USA, Mountain View, CA, USA).

Sequences for HIF- α and HIF- β from *Danaus plexippus*, *Bombyx mori*, *Tribolium castaneum*, *Ades aegypti*, and *Meghachile rotundata* were aligned with the *M. sexta*

genome (www.agripest/manduca). Aligned sequences of ~150–200 bp were used to design primers to extend the 5' and 3' ends of HIF- α and HIF- β . Touchdown PCR on 5' and 3' ends was carried out using 5' and 3'-RACE cDNA as a template with reverse gene-specific primers and UPM to extend 5' and forward gene-specific primers and UPM to extend the 3' end. Touchdown PCR generated multiple fragments, and a second round of PCR was carried out using the touchdown PCR product as a template with nested reverse gene-specific primers and UPM, forward gene-specific primers and UPM. The second round of PCR generated single 5' and 3'-RACE fragments. Single fragments were cloned and sequenced (Tufts University Core Facility, Boston, MA, USA). Five' and 3'-RACE fragment sequences were searched against databases and found to be homologous with insect HIF-1 α and HIF-1 β sequences.

All PCR reactions were carried out in a final volume of 50 μ l, each containing 1 μ l of 10 mM ultrapure dNTPs mix, 1 μ l of forward or reverse primer at the concentration of 10 μ M, UPM primer A mix (per manufacturer instructions), 1–2 μ l of 50 fold diluted 3' and 5' RACE cDNA for touch down PCR. Thermocycler parameters were as follows: 5 cycles of 94°C for 30 sec, 72°C for 3 min, followed by 35 cycles of 94°C for 30sec, 70°C for 30 sec, and 72°C for 3 min. PCR reactions were kept in the thermocycler at 4°C until removed. The PCR product was diluted 10–50 fold with tricine-EDTA buffer and used as template for touch down 3' and 5' of RACE PCR.

The PCR products were gel-electrophoresed in 1 $\times$ TAE buffer. The PCR bands were gel-purified using Wizard SV gel and PCR clean-up system (Promega, Madison, WI, USA), cloned into pGEM^T-easy vector system II (Promega, Madison, WI, USA) and transformed into JM109 chemically competent cells (Promega, Madison, WI, USA). Recombinant DNA was isolated using QIAprep spin miniprep kit (Qiagen, Germantown, MD, USA) with samples centrifuged for 15–20 minutes to separate the DNA from cell debris. The presence of the insert in plasmid was confirmed by *Eco*R1 restriction digestion (Promega, Madison, WI, USA) after incubating for 2–3 h at 37°C. Recombinant clones were sequenced with universal M13 forward and M13 reverse primers at the Tufts University Core Facility (Boston, MA, USA).

Resulting sequences were compared to those from other insects as we have previously described (Vishnuvardhan et al., 2013). Homologous sequences were aligned and assembled using Clustal W2 and Geneious Pro software (Geneious version 4.02). The amino acid sequence was deduced using Expasy translation tool. Sequences are available at National Center for Biotechnology Information (accession numbers to be submitted upon publication).

2.3.2 Tissue collection—To determine if caterpillars increase expression of hypoxia-inducible factors near the end of an instar, samples were collected at the beginning and the end of each of the five larval instars. Because insects varied in size throughout their juvenile instars, tracheae were collected differently depending on the caterpillar age. Larvae from 1st–3rd instars were collected and RNA or protein was extracted from the whole larva, because they were too small for dissecting out large amounts of tracheae. First instar larvae were pooled in groups of 10 to ensure enough material for analysis. Tracheae from 4th and

5th instar larvae were easily dissected out and abundant, so RNA and protein were extracted from isolated tracheae in those instars. For quantitative PCR, each sample (individual or pooled) was placed in 500 µl of Trizol (TRI Reagent, Fisher Scientific, Waltham, MA, USA) per 500 mg of sample, placed on dry ice, and stored at –80°C before until RNA isolation. Dissections took a maximum of 1 min per sample. Samples for Western blotting were stored at –20°C until protein isolation.

As a positive control for our ability to detect changes in HIF- α expression and to validate that hypoxia results in increased HIF expression, third instar caterpillars that were at the end of the instar were submerged in water for 0, 10, or 30 minutes or 1, 4, or 8 h. We opted to use submersion as an ecologically relevant event that exposes insects to low oxygen. Ten caterpillars were used for each time point. Water was covered with plastic to ensure they remained submerged for the entire time period. At the end of the time period, caterpillars were removed from the water, the gut was dissected out and the carcass was immediately frozen on dry ice for either RNA or protein isolation.

2.3.3 Quantitative PCR—HIF1- α and HIF1- β mRNA levels were measured using quantitative PCR, as previously described (Bergan et al., 2013). Samples were collected as described above from caterpillars at the beginning and end of the 1st (n = 10 and 7 respectively), 2nd (n = 9 and 10 respectively), 3rd (n = 10 and 10 respectively), 4th (n = 8 and 9 respectively), and 5th (n = 10 for both stages) instars. RNA was extracted and pellets were re-dissolved in 30–100 µl RNase-free deionized water. Each of the samples was diluted 1:30, and RNA was 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 was made from mRNA using an RT-cDNA synthesis kit (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).

HIF-1 α and HIF-1 β 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-1 α (F-GTGATGCGTCTAGCTATATCC, R-ACAAATGGCTGAACTCGAAC) and HIF-1 β (F-CATGAAAGCTTTGAGAGGTA, R-GGTCGTATAAACATGACGAA) (Integrated DNA Technologies, Skokie, IL, USA) were synthesized and validated using PCR. Quantitative PCR reactions for both HIF-1 α and HIF-1 β 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 of a 96-well plate 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 et al., 2013). The data were normalized with the RpS3 housekeeping gene (F-CCGCATCCGCGAGTTG, R-CGGACTGTTCCGGGATGTT) (Jiang et al., 1996). From that correction, copy number was calculated using the relative standard curve method and used for statistical analysis.

2.3.4 Western blotting—To determine whether HIF protein levels were increased at the end of an instar, we used Western blotting. Protein expression was only determined for HIF- α , because no antibody was available for HIF- β . Samples were collected at the beginning and end of the 1st, 2nd, 3rd, 4th, and 5th instars (n = 4 for each instar and stage within the instar).

Each sample was homogenized using an electric tissue homogenizer (IKA, Wilmington, NC, USA) in 100–500 μ l of cell lysis buffer (Cell Signaling, Danvers, MA, USA). Then, samples were centrifuged at $12,000 \times g$ 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 (Bio-Rad Laboratories, Hercules, CA, USA) using 10 μ l of sample or standard and 200 μ l of reagent. 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).

Isolated proteins were separated by molecular weight using electrophoresis. Fifty μ g of each sample was loaded into each well of a Bio-Rad 10% pre-cast TGX gel 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 (TransBlot Turbo Kit, Bio-Rad; Hercules, CA, USA) and 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. The membrane was washed 3 times for 5 min with TBS-T and then probed with the primary antibody (rabbit-anti-Sima antibody used in (Marden et al., 2013) that was a gift from P. Wappner at a 1:1,000 dilution in (40 μ l of anti-HIF-1 α in 40 ml of 5% bovine serum albumin) at 4°C overnight on a shaker. Then, the membrane was washed 3 times for 5 min with TBS-T and incubated with goat anti-rabbit IgG secondary antibody with HRP conjugate (Thermo Scientific, Rockford, IL, USA) at a 1:5,000 dilution (8 μ l 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 membrane was washed 3 times for 10 min with 1X TBS-T. Proteins were visualized using the West-Femto kit (ThermoFisher, Grand Island, NY, USA) with exposure times of 20 – 30 min on a chemi-imager (Alpha-Innotech, Miami, FL, USA).

2.6 Statistical analysis

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 within an instar (referred to as stage) as the factors. Repeated measures ANOVA was used to detect differences in tracheal diameters, with location (i.e., head or hind segment) and oxygen as the within subjects factor and instar and stage within

an instar (referred to as stage) as between subjects factors. Linear regression was used to find relationships between log-transformed mass and diameters. Non-parametric Jonckheere-Terpstra test for ordered alternatives was used to identify differences in gene and protein expression in submerged caterpillars and protein expression throughout development. Non-parametric Kruskal-Wallis tests were used to identify differences in gene expression among instar and the stage within each instar. Means $\pm$ S.E.M. are shown throughout.

3. Results

3.1 Morphological measurements

Body mass increased both across and within each instar (Fig. 2A, stage $\times$ instar interaction, $F_{3,73} = 6.13$, $p < 0.002$). In contrast, head width increased only after each molt, remaining constant within each instar (Fig. 2B, effect of instar, $F_{4,67} = 318.7$, $p < 0.001$, effect of stage, $F_{1,67} = 1.2$, $p = 0.28$). For example, body mass increased more than 3-fold during the 2nd instar (0.0076 ± 0.0004 g to 0.0346 ± 0.002 g), although head width did not change (1.38 ± 0.08 mm to 1.30 ± 0.06 mm). However, upon molting to the 3rd instar, head width increased by 60% to 2.07 ± 0.09 mm.

Diameter of open tracheae varied differently with O_2 depending on the caterpillar's instar (Fig. 3A and B, effect of $O_2 \times$ instar, $F_{4,65} = 4.54$, $p < 0.01$). For example, upon molting from the 1st instar to the 2nd instar and from the 4th to the 5th instars, tracheal diameters nearly doubled, while molts from 2nd to 3rd and 3rd to 4th instars resulted in smaller increases in diameter. The effect of instar also depended on the location of the trachea (effect of location $\times$ instar, $F_{4,65} = 4.7$, $p < 0.01$). For example, upon molting from the 4th to the 5th instar, head tracheae increased 95% in diameter, while hind tracheae increased in diameter only 75%. There was no effect of stage on diameter at any oxygen level or at either location ($F_{1,65} = 2.6$, $p = 0.11$). Across all caterpillars, tracheal diameter scaled isometrically with body mass (i.e., slope = 1/3 for a linear dimension (Calder, 1996); Fig. 4, 95% CI for hind diameter = 0.282–0.328 and for head diameter = 0.302 – 0.346).

3.2 Respiratory responses

Only caterpillars past the end of the third instar exhibited abdominal pumping and tracheal compressions in hypoxia (Fig. 5). Tracheae in the head compressed more frequently than tracheae in the last abdominal segment (effect of location, $F_{1,8} = p < 0.03$). Among instars that exhibited compression, there was no effect of either instar ($F_{1,8} = 3.86$, $p = 0.59$) or stage ($F_{1,8} = 3.03$, $p = 0.647$) on compression frequency, which averaged 11 ± 0.8 per min for the head and 6 ± 0.6 for the hind segment. Diameter of compressed tracheae varied differently with O_2 depending on the caterpillar's instar and the location of the trachea (Fig. 3C and D; effect of $O_2 \times$ instar, $F_{4,65} = 56.1$, $p < 0.001$; effect of $O_2 \times$ location, $F_{1,65} = 6.34$, $p < 0.02$). In 5th instar caterpillars, head tracheae compressed to 42% of their open diameter and hind tracheae compressed to 48% of their open diameter. There was no effect of stage or its interaction with any other factors on closed diameter.

3.3 HIF1- α and HIF1- β mRNA expression

The partial sequence we identified for HIF- α is 1450 base pairs, with a translated protein sequence of 434 amino acids (Fig. 6A). The translated sequence includes the predicted bHLH and PAS domains and PAC motif (Fig. 6A). The partial HIF- β nucleotide sequence contains 917 base pairs, with a translated protein sequence of 183 amino acids that includes predicted a bHLH and PAS domains (Fig. 6B). The partial sequences were aligned and showed identity to HIF- α and HIF- β proteins in other insects (Table 1).

As a positive control for our ability to detect changes in HIF- α expression and to validate that hypoxia results in increased HIF expression, caterpillars were submerged in water for varying durations. Submersion of larvae increased expression of HIF- α mRNA (Fig. 7A) but not protein (Fig. 7B). Expression of mRNA varied significantly with submersion time ($\chi^2 = 2.77$, $p < 0.01$), with the highest expression level occurring after 4 hours of submersion (Fig. 7A). Expression of HIF- α mRNA nearly doubled from time 0 to 4 and 8 hours post-submersion ($t_0 = 3376 \pm 353$, $t_4 = 6167 \pm 1088$, $t_8 = 5359 \pm 631$). There was no significant effect of submersion time on protein levels of HIF- α ($\chi^2 = 0.89$, $p = 0.376$).

Expression of HIF-1 α mRNA varied differently with instar and stage ($\chi^2 = 22.84$, $p < 0.001$; Fig. 8A). For example, in the first instar, there was no difference from beginning to the end of the instar ($p = 0.72$), while in the 3rd instar, HIF-1 α expression was higher at the end compared to the beginning of the instar ($p < 0.001$; Fig. 8A). Post-hoc tests indicated that HIF- α expression was higher at the end of only the 2nd and 3rd instars. HIF-1 β gene expression also varied with instar and stage ($\chi^2 = 65.64$, $p < 0.001$; Fig. 8B). As with HIF-1 α , in the first instar, there was no difference in expression from the beginning to the end of the instar, while in the 2nd, 3rd, and 4th instars, HIF-1 β expression was higher at the end compared to the beginning of the instar (HIF-1 β : 2nd instar, $p < 0.01$; 3rd instar, $p < 0.001$; 4th instar $p < 0.01$).

HIF-1 α protein (120 kDa) was detected at the end of all of the instars, and densitometric measurements indicated significant differences in expression among the instar-stage groups (Fig. 8C; Jonckheere-Terpstra test for ordered alternatives, $\chi_2 = 2.57$, $p < 0.02$). Post hoc comparisons identified differences between the beginning and the end of instars 3 ($p < 0.05$) and 4 ($p < 0.02$). In contrast to our prediction however, fifth instar caterpillars, HIF- α protein expression was more than 4 times higher at the beginning of the instar compared to the end of the instar (Fig. 8C).

4. Discussion

This work supports the hypothesis that oxygen delivery in insects may be constrained within an instar until they can molt. We showed for the first time that *M. sexta*, at least in some instars, are hypoxic near the end of an instar, as suggested by the increased expression of HIF- α and HIF- β mRNA and HIF- α protein. In addition, we show that diameters of large tracheae do not change within an instar, using X-ray data of tracheae. Together, these data contribute to the growing body of research investigating regulation oxygen dependent induction of molting (Kivela et al., 2016a; Kivela et al., 2016b) and lend support for the hypothesis that the mismatch between oxygen supply and demand near the end of an instar

may be a trigger for molting (Callier and Nijhout, 2011; Greenberg and Ar, 1996; Greenlee and Harrison, 2004, 2005).

To date, several studies have shown static or decreased tracheal system capacities within instars in various insect species, including *Locusta migratoria* (Clarke, 1957a), *Schistocerca americana* (Greenlee and Harrison, 2004; Kirkton et al., 2012; Lease et al., 2006), and *Manduca sexta* (Callier and Nijhout, 2011; Greenlee and Harrison, 2005). These studies have used a variety of methods to measure tracheal system capacity, including volume displacement, critical PO₂, gas chromatography, and x-ray imaging. Although these methods each have their limitations, together they lend strong support to the idea that oxygen delivery capacity of the tracheal system decreases within instars. Ideally, microCT imaging should be used in conjunction with measures of tracheolar density to get a more accurate measurement of how the tracheal system changes within an instar.

The effects of a fixed or decreased respiratory capacity within an instar may be compounded by increases in metabolic tissue mass during this same time (200 – 400% in this study and 300 – 700% in a study by Goodman et al. 1985). Indeed, absolute metabolic rates increase within an instar by 2 – 5 fold depending on the instar (Callier and Nijhout, 2011; Greenlee and Harrison, 2005) from beginning to end of instars. Together, these studies provide strong support for the hypothesis that there is a mismatch between oxygen supply and demand, which together may lead to functional hypoxia at the end of an instar. Hypoxia can initiate tracheal system remodeling and branching of tracheoles (Centanin et al., 2008; Centanin et al., 2010; Jarecki et al., 1999). Thus, low oxygen at the tissue level might stimulate tracheal growth in an effort to maintain tissue oxygen PO₂ levels, since diameters of large tracheae (Fig. 3) and tracheal volumes (Callier and Nijhout, 2011; Clarke, 1957a; Lease et al., 2006) do not increase during that time.

In this study, we used expression of hypoxia inducible factor (HIF) as an indicator of hypoxia. HIF is a heterodimeric transcription factor known to increase expression of genes that code for proteins involved in increasing oxygen supply (reviewed in Prabhakar and Semenza, 2012, 2015; Semenza, 2012). HIF is composed of the subunits, HIF- α and HIF- β , which are both constitutively expressed. Under normoxic conditions, HIF- α is continuously ubiquitinated and degraded. In hypoxia, HIF- α is stabilized and dimerizes with HIF- β . Although much of the regulation of HIF occurs via post-translational regulation, several studies have shown that hypoxia increases HIF- α mRNA expression (BelAïba et al., 2007; Catron et al., 2001; Chen et al., 2006; Wang et al., 1995; Wiener et al., 1996; Yu et al., 1998). Interestingly, we found increases in mRNA expression of HIF- α at the end of the 2nd and 3rd instars and increases in HIF- β mRNA expression at the end of the 2nd, 3rd, and 4th instars. Although the presence of lactate is considered to be a better indicator of hypoxia, the increase in HIF expression suggests that these insects are attempting to compensate for hypoxia at the molecular level. Measurements of lactate would be an excellent complement to these data.

The finding that either mRNA expression or protein expression of HIF- α was increased in 2nd, 3rd, and 4th instars, suggests that hypoxia may occur only at the end of some instars. HIF has been shown to increase tracheal branching in response to hypoxia and is implicated

in tracheal system remodeling (Centanin et al., 2008; Centanin et al., 2010; Jarecki et al., 1999). First instar larvae maybe small enough and have such a low oxygen demand that they do not experience hypoxia at the end of an instar or that diffusion is always sufficient to supply oxygen at this life stage. Our findings are partially in alignment with previous work in *M. sexta* showing that critical PO₂ was higher at the end of the 3rd and 5th instars, but not 1st (Greenlee and Harrison, 2005) and that tracheal volumes decrease within the 3rd and 4th instars (Callier and Nijhout, 2011). However, in this study, animals at the end of the 5th instar did not have increased HIF-1 α mRNA or protein expression. Initially, we were concerned that this discrepancy was because of our methods of extracting mRNA from whole animals for smaller caterpillars and from isolated tracheae for larger animals. To address this issue, we homogenized whole animal samples of caterpillars from the 4th and 5th instars and measured HIF- α mRNA and protein expression (Supplemental Fig. 1). This experiment resulted in mRNA expression patterns similar to those from 4th and 5th instar tracheae only (Fig. 8), suggesting that this is a biologically relevant result and not just a result of different sampling methods. However, this pattern matches with the increased tracheal volumes found within the 5th instar in two previous studies (Callier and Nijhout, 2011; Helm and Davidowitz, 2013).

These findings suggest that caterpillars in the 5th instar are just not hypoxic (Fig. 8). The decrease in HIF protein later in the 5th instar supports this hypothesis. Perhaps in the 5th instar caterpillars increase tracheal volume enough to prevent hypoxia, a hypothesis supported by the work of Helm and Davidowitz (2013), who showed that both tracheal mass and volume increase in the 5th instar. In addition, mass specific metabolic rates were higher at the end of the 5th instar (Greenlee and Harrison, 2005). Because caterpillars during the 5th instar are preparing for metamorphosis and possible overwintering, this instar may be completely different than other juvenile instars. Indeed, other studies have also found that the final larval instar differed from other instars in its metabolic response to aging within an instar, similar to what has been found in *M. sexta* (Kivela et al., 2016b). Clearly more work is needed to understand how tracheae are remodeled during metamorphosis in preparation for the adult life stage.

HIF-1 β expression was not expected to change in hypoxia, since it is continuously expressed in normoxia. Although some studies have found that hypoxia does not increase expression of HIF- β gene expression (Huang et al., 1998; Pugh et al., 1997), other studies have shown that HIF- β gene expression increases in hypoxia (Catron et al., 2001; Wang et al., 1995; Wood et al., 1996). However, in our study, we found increases that matched expression of HIF-1 α . It is possible that the increase in HIF-1 α results in upregulation of HIF-1 β to increase the amount of that subunit available for binding to create the heterodimeric HIF-1. Alternatively, both HIF- α and HIF- β are regulated by pathways involving growth and development, including insulin and TOR pathways (Biswas et al., 2013; Dekanty et al., 2005; Masoud and Li, 2015; Treins et al., 2002), so it is possible that the increased gene expression of these is related to general growth and not just decreases in available oxygen.

Determining whether insects become hypoxic near the end of an instar is important, because it has been proposed that decreased oxygen availability is a signal for molting (Callier and Nijhout, 2011; Greenberg and Ar, 1996; Greenlee and Harrison, 2004, 2005). Indeed, some

support for this has been shown in several moth species, and this phenomenon has been coined as oxygen-dependent induction of molting (ODIM) (Kivela et al., 2016a; Kivela et al., 2016b). Identifying what triggers molting is critical for improving our understanding of the control of body size and development in insects and will have implications ranging from life history evolution to integrated pest management.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Summary Statement

As caterpillars grow within an instar, major tracheae do not increase in size, while metabolic demand increases. At the same life stages, caterpillars increased expression of hypoxia inducible factors, suggesting that they become hypoxic near the end of an instar.

Highlights

- Large tracheae remain constant in diameter within the first, second, third, and fourth instars and increase in diameter after molting.
- Increased expression of hypoxia inducible factor alpha suggests that insects may experience functional hypoxia at the end of these instars.
- Within the 5th instar, levels of hypoxia inducible factor alpha decreased, suggesting that something very different happens at this stage.

Fig. 1. X-ray image of a second instar *M. sexta* (body mass 0.044 g) illustrating the head and hind tracheae (white arrowheads) that were used to determine tracheal diameters across and within instars.

Fig. 2.

Box and whisker plots showing body mass (A) and head width (B) as a function of instar. Open boxes represent caterpillars at the beginning of the instar, and filled boxes represent the end of the instar. Mass data are missing for the end of 5th instar, and head width is missing for end of 3rd instar. The lower and upper boundaries of boxes show the 25th and 75th percentiles respectively. Whiskers represent the 10th and 90th percentiles, and black points indicate outliers.

Figure 3. Diameter of tracheae at the beginning (b) and end (e) of each instar. Open tracheae (A, B) and compressed tracheae (C, D) in the head (A, C, circles) and the hind (B, D, triangles) segments in 21 (black) and 1 kPa (gray) PO_2 .

Fig. 4.

Across instars, diameters of tracheae in the head and hind segments scaled isometrically with body mass. However, within each instar, tracheae did not differ as body mass increased. Instars are color-coded: 1st (pink), 2nd (lavender), 3rd (green), 4th (blue), and 5th (yellow).

Fig. 5. Compression frequency of tracheae in hypoxia. Open symbols represent caterpillars from the beginning of the instar, while filled symbols represent those at the end of the instar.

A. HIF- α partial amino acid sequence

```

1   DCVHIAKFHN SVSYHKNEM SSKPANQKRR NNEKRKEKSR VAARCRRTKE MQIFSELTAA
61  LPAKKEEVEQ LDKASVMRLA ISYLRARDVV SMLPDVDKEQ PKVQNPKGYE ELSELSYMK
121 ALDGFVMVLS QQGDIVYCSE NIADHLGVSQ MEIMGQSVFE FSHLCDHEEI REALRPNGGG
181 RRDLLRLKLC TLTSKGRNVH LKSASYKVIH ITGHMLMPTN GKERNNNKED KKIDDDKKAS
241 GASLVAVGRP IPHPSNIEIP LDSKTFLSKH SLDMKFTYVD EALLNTLGFE PDELVGRSLY
301 DYHHAADSAS MIQQFKSLFS KGQCETGQYR FLAKAGGYAW VQTQATVITG KQOKPISVVC
361 VNYVISGIEC KDEVFAAHQV QHADLKPPVA VTAPVPAPAP LCAPVAPPAN GAIVAAPPSD
421 DERPIPVTEL IFAP

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B. HIF- β partial amino acid sequence

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1   MEEDNIQDEE RFASRENHCE IERRRRNKMT AYITELSDMV PTCSALARKP DKLTILRMAV
61  AHMKALRG TG NTSTDGTYKP FFLTDQELKH LI LEAADGFL FVVSCTGRV IYVSDSIAPV
121 LNYSQGEWYS SCLYDQVHPD DVEKVREQLS TQEPQNTGRI LDLKTGTVKK EGHQCKRHKS
181 VVL

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Fig. 6.

Amino acid sequences translated from the identified partial nucleotide sequence for HIF- α (A) and HIF- β (B). Highlighted sequences indicate bHLH domains (yellow) and PAS domains (blue) in both, and the PAC motif (underlined) only in HIF- α .

Fig. 7.

Expression of HIF-1α mRNA (A) and protein (B) as a function of submersion time.

Fig. 8.

Expression levels of HIF-α (A) and HIF-β (B) mRNA levels. Representative lanes from Western blots (C) and HIF-α protein levels calculated from densitometry of Western blots (D) across and within instars. Open symbols are beginning and filled symbols are end of instar. Densitometry analysis was performed on the band that migrated between 150 and 100 kD, because the predicted size of HIF-α protein in *M. sexta* is 120 kD.

Table 1

Percent identity matrices created by Clustal 2.1 using translated amino acid sequences from *Manduca sexta* for HIF- α compared to protein sequences from *Bombyx mori*, *Helicoverpa zea*, and *Drosophila melanogaster* and HIF- β compared to protein sequences from *Bombyx mori*, *Papilio polytes*, and *Drosophila melanogaster*.

Translated amino acid sequence				
HIF-1 α				
	<i>Drosophila</i>	<i>Bombyx</i>	<i>Manduca</i>	<i>Helicoverpa</i>
<i>Drosophila</i>	100	34.78	48.02	35.57
<i>Bombyx</i>	34.78	100	82.1	77.16
<i>Manduca</i>	48.02	82.1	100	82.51
<i>Helicoverpa</i>	35.57	77.16	82.51	100
HIF-1 β				
	<i>Drosophila</i>	<i>Papilio</i>	<i>Manduca</i>	<i>Bombyx</i>
<i>Drosophila</i>	100	76.34	83.06	61.89
<i>Papilio</i>	76.34	100	92.9	89.07
<i>Manduca</i>	83.06	92.9	100	93.44
<i>Bombyx</i>	61.89	89.07	93.44	100