METAMORPHIC DEVELOPMENT OF *MANDUCA SEXTA*: AN *IN VIVO* INTEGRATIVE APPROACH TO STUDYING WHOLE ANIMAL PHYSIOLOGY

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

in Partial Fulfillment of the Requirements for the Degree of

Doctorate of Philosophy

by

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August 2013

ABSTRACT

METAMORPHIC DEVELOPMENT OF *MANDUCA SEXTA*:

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Cornell University 2013

Multicellular organisms have evolved specialized tubular structures to transport gases and liquids throughout the body. For vertebrates, such structures include the trachea, bronchi, bronchioles and blood vessels. The genesis of these elaborate tubular structures, known as tracheogenesis for trachea and angiogenesis for blood vessels, has received a great deal of attention in the last decade in many fields, including developmental biology and oncology. It has become increasingly important to understand how the genesis of these structures is regulated to produce a functional organ system where the transport capacity matches the physiological needs of the organism. In particular, investigators have asked when do new branches arise, what determines the direction of growth, what specifies the formation of the next generation of branches, and how do tubular networks fuse to create functional organs.

This PhD. dissertation research attempts to address some of the above questions in the context of tracheogenesis, using a unique animal model, a moth *Manduca sexta*. During a relatively short, on average 19 day, cycle of development known as pupal metamorphosis, the respiratory system of this invertebrate remodels completely to accommodate new adult organ systems such as an extensive tracheal network and thoracic fight muscles. The goal of this research is to understand and establish the

dynamics of tracheogenesis and organ development by conducting a longitudinal study of pupal metamorphosis, *in vivo*, using minimally invasive diagnostic imaging technology of micro-computerized tomography (Micro-CT). Interestingly, our animal model, the moth, is also capable of surviving conditions of anoxia that would be lethal for humans. As a result, another important aim of this research was to establish the role of unusual structures and adaptations specific to the pupal respiratory system of *Manduca* (e.g., airsacs) during metamorphosis via Micro-CT imaging and flow respiratory.

This dissertation also describes the successful application of the information on tissue morphogenesis acquired from Micro-CT images to construct an efficient protocol for implantation of MEMS probes into *Manduca* pupae. This project aimed at creating insect bioborgs where the flight capacity of insects, some of nature's best fliers, was harnessed by surgically integrating micro actuators inside or on an insect body.

In my aim to design inquiry based lessons and low-cost experimental protocols to enhance the science curriculum for the CLIMB GK-12 education program, I combined advanced imaging systems such as MCT with traditional bio laboratory methods of bioinquiry and respirometry, to teach students important concepts on developmental biology, anatomy, ecology and evolution. By posing real life examples and problems, such as global warming and resulting changes in physiology, ecology and habitat of insect pests, I attempted to link what we learn in a classroom to dynamic physiological phenomenon occurring in organisms in our surrounding environment.

BIOGRAPHICAL SKETCH

Ayesa Kaur was born in Sambalpur, Orissa, India. She graduated with a Masters degree in Environmental Biology from Utkal University, India in 2001. She graduated with her second M.S degree in Natural Resources under the guidance of Dr. Bernd Blossey at Cornell University in December 2007. Her dissertation project involved mounting passive radar tags on to the biocontrol weevil, *Hylobius transversovittatus*, and track its migration patterns and habitat preference in the wetlands of upstate NY. During her Masters degree, she was actively involved in the Insect Hybrid MEMS (HIMEMS) program, a DARPA-MTO project that aimed at creating insect bioborgs to use as micro aerial vehicles. She, under the mentorship of Dr. Amit Lal and guidance of Dr. John Ewer, developed and patented a novel "early metamorphic surgical insertion technology" using *Manduca sexta* pupa as the model organism. Development of this technology laid the ground to successfully insert fabricated implants into the body cavity without any bio rejection and lead the way towards creating viable insect bioborgs. She continued on to her doctoral studies in Molecular Integrative Physiology under the guidance of Dr. Robert F. Gilmour Jr., Spring 2008 at Cornell University. While continuing on to complete the HIMEMS program her primary research focus has been to investigate the metamorphic development of the tracheal system in a Lepidopteran pupa *Manduca sexta*, *in vivo,* during the metamorphic stage using Micro-CT imaging and investigate its respiratory physiology during this dynamic stage.

To,

my son Vilokin and husband Rupinder Singh

ACKNOWLEDGMENTS

As I stand at the end of my graduate studies, I see the true meaning of the saying: “Its not the destination but the journey that counts”. And this spectacular journey would not be possible without the support of the extraordinary people I have met along the way.

First and foremost I would like to thank my advisor, Prof. Robert F. Gilmour Jr., to whom I am eternally grateful. It has been an honor to be your student. Your wise guidance, unwavering support and your faith in me paved my path towards becoming an independent research scientist. With your confidence in me, I could sail through the rough patches with grace. You are my mentor and everyday I aspire to be like you.

I would also like to thank my committee members, Prof. Amit Lal, Prof. Cole Gilbert and Prof. Chris Schaffer, for their valuable advice and support throughout my PhD career at Cornell.

Prof. Amit Lal, I am forever indebted to you for granting me the opportunity to work with you on your extraordinary vision on Hybrid-Insect-Micro-electro-mechanical Systems (HIMEMS) program. You placed your confidence in me at a crucial turning point of my career and for that I am forever thankful. I would not be where I am today without your guidance.

The reason why I pursued Insect Physiology, as my field for scientific research, was because of the extraordinary teacher who opened up the world of insects for me in the very first class I attended at Cornell University. Thank you Prof. Cole Gilbert! Your undying enthusiasm and unfathomable knowledge for these beautiful animals has kept me, and forever will, in state of absolute bliss and constant intrigue towards them.

Dr. Chris Schaffer, you have been the portal that truly opened up the world of STEM education for me. I thank you for granting me this opportunity to be trained as a forerunner in this movement.

I would like to express my sincere thanks to my lab mates at the Gilmour Lab, SonicMEMS Lab, Boyce Thompson Institute and to my colleagues and friends in Ithaca for their support and to the numerous faculty members who have shared their precious time and knowledge with me.

We often take for granted the people in our lives that most deserve our gratitude. I take this opportunity to thank my dear father Prabhas Krishna Sinha, my wise mother Dr. Banani Roy and my doting brother Siddhartha Shankar Sinha for their constant support, encouragement and undying confidence in me. My sincere gratitude to my father-in-law, Jagdish Singh, whose serene presence and support during the chaos of thesis writing has been nothing short of a divine blessing.

Finally, I thank my husband Rupinder Singh and my son Vilokin who I owe lots and lots of fun hours. This thesis would not be possible without you Rupinder. You have been my number one encourager and your wisdom helped keep things in perspective. You are the one who stuck to his guns making me believe in myself even when I thought it was impossible to continue. Thank you for being my rock.

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CHAPTER 1

INTRODUCTION

1.1 INSECTS AS THE BIOLOGICAL MODEL TO INVESTIGATE TRACHEOGENESIS

Insects show remarkable adaptations to unfavorable environmental conditions. Their resilience contributes to their abundance in this living world. They are the most diverse and abundant multicellular life forms on this planet (>50% of all described species). Their inter species diversity lies not only in their varied morphology but also in the diverse habitats they occupy. Their keen ability to survive a wide range of environmental conditions is by adapting to these living situations. A prime example is the diversity in their respiratory systems. May it be a terrestrial or an aquatic one, insects adapt by forming specialized respiratory organs. These modifications are not only between species in different habitats, but also between different life stages of a single insect. A larva molts to accommodate growth in body size eventually morphing into a pupa where the entire respiratory network may be scrapped and then remodeled to sustain organ development and at the same time lay down a tubular network that would eventually support a highly metabolic and specialized adult life form.

Most multicellular organisms have evolved specialized tubular structures to transport gases and liquids throughout the body. In the case of vertebrates, such structures include the trachea, bronchi, bronchioles and blood vessels. In insects, these tubular structures are less diverse and consist of primarily the invertebrate trachea, an extensive tubular network of trachea and tracheoles connected to the environment via

spiracles. A common feature of these structures in both vertebrate and invertebrate systems is their branched tubular nature, where epithelial cells are used as building blocks. These tubular structures share structural and developmental homology where growth of epithelial cells in tracheogenesis and angiogenesis is governed by similar developmental principles (Manning 1993). But, resistance and recovery from hypoxia sets insects apart from veterbrates making them an excellent model to study how respiratory system works and since has received a great deal of attention in the last decade (Ghabrial 2003, Horowitz 2008). The major questions in this developing field have addressed how the process of tracheogenesis is regulated as to produce a functional organ system whose transport capacity matches the physiological needs of the organism. In particular, investigators have asked how do the new branches arise, what determines the direction of tubular growth, what specifies the formation of the next generation of branches and how do tubular networks fuse to create these 3D structures. *Drosophila melanogaster,* due to the structural simplicity of its respiratory system and readily accessible genetics, has emerged as a paradigm of branching morphogenesis. Almost all research conducted on *Drosophila* to identify the molecular pathways that control the major morphogenetic events of tracheal development has been conducted during embryogenesis, primarily because of the almost-transparent egg and embryonic stage that allow dynamic *in vivo* imaging studies (such as fluorescence, confocal and two-photon microscopy) to quantitatively observe growth of flurophore tagged tracheal placodes/tubes/cells, along with parallel gene and protein expression assays involved in tracheogenesis. However, an important aspect of tracheal development that remains almost completely unexplored

at the molecular level is the formation of the adult respiratory system during metamorphosis.

1.2 METAMORPHIC DEVELOPMENT OF THE RESPIRATORY SYSTEM

Respiration in insects is accomplished primarily by an extensive cuticularized tubular network, the trachea, which opens to the outside environment at the spiracles and branches throughout the animal, transporting gases to and from the various metabolizing tissues. The chitinous lining of these tracheal tubes is regenerated at every molt as an insect sheds its old structures to accommodate growth. At the pupal molt, the insect enters a metamorphic stage to develop into a highly metabolic adult life form. During metamorphosis the pattern of tracheal ramification dramatically remodels to support delivery of oxygen to developing adult organ systems. This rapid tissue development also depends on the distribution of hemolymph, the primary circulatory fluid, to transport nutrients and remove waste products in an open arthropod circulatory system. Recently, there have been a few, but very interesting, reports on adult tracheal development during pupal metamorphosis in *Drosophila* (Weaver 2008, Sato 2002). These studies have not only shed light on how expression of a chemical mitogen, FGF Branchless, at the site of developing adult tissue acts as a chemoattractant for tracheal ramification, but they also have revealed that pupal/adult tracheal tissue progenitors can arise by early allocation of multipotent cells (imaginal discs) and by facultative stem cells (pre-existing differentiated tracheal cells that reenter the cell cycle). Unfortunately, all of these studies are *in vitro* studies because visualizing the adult trachea is difficult through the opaque pupal cuticle.

In vitro studies are invaluable for understanding developmental molecular pathways, but this approach is unable to elucidate the dynamic relationship between changes in tracheal topography and function during metamorphic development. In order to conduct a complete study of adult tracheal reorganization during pupal metamorphosis, there is a need for an *in vivo*, non-invasive and nearly continuous imaging method to track tracheal remodeling, in concert with parallel assessments of its functional physiology and expression profiles of the key genes during tracheogenesis.

1.3 NOVEL IN VIVO APPROACH TO STUDY MORPHOGENESIS

Traditional methods to study anatomy and morphology of insects, using histological preparations, dissection and SEM/TEM or even corrosion casts, involves killing the animal. These techniques prevent us from looking at the dynamic changes in a developmental life stage over a span of time in the same animal. A combination of morphological and behavioral variations results in a great variety of respiratory patterns and an overall plasticity of the respiratory system. And because of these variations, our understanding of the patterns and processes of the respiratory system as a function of body size, phylogeny, development and life history remains incomplete. New imaging techniques such as MRI, X-ray synchrotron and Micro-CT allow us to look inside an intact insect and observe anatomical variations such as tracheal shape and size, arborization patterns and modifications in tracheal conductance under varying environmental situations over a period of time.

In my attempt to understand how a structurally simple, yet diverse respiratory system works, I chose *Manduca sexta* as my biological model with my focus on the rarely studied pupal metamorphic stage. My aim was to track the development of the respiratory system, thoracic flight muscles: the power compartment of a flying adult moth, and its nervous system during the entire pupation period. My novel approach involved using Micro-CT to image a single pupa thoughout its entire metamorphic developmental time.

1.4 DISSERTATION OUTLINE

Chapter 2 of this thesis describes the application of this novel *in vivo* approach of using Micro-CT to study metamorphic development of the tobacco hornworm moth, *Manduca sexta* (Lepidoptera: Sphingidae). The chapter also reports the implementation of a stop flow respirometer in parallel with Micro-CT scans that enabled me to visualize the development as well as the functional respirometric physiology of the tracheal system at the same time.

The information gathered from Micro-CT study also allowed me to construct an efficient probe implantation design for the DARPA Hybrid Insect MEMS (HIMEMS) project described in detail in Chapter 3 of this thesis. The aim of this project was to create insect *bioborgs* where the flight capacity of an efficient flier was harnessed by surgically integrating micro actuators in or on the insect body. From my tissue development data, I was able to determine the most effective developmental stage, site for implantation and the appropriate dimension and design of MEMS probe to be implanted for creating the most efficient tissue-machine interface. This technology

was named Early Metamorphic Insertion technology (EMIT). This chapter also reports the integration of the implanted probes and its fate in the adult flight tissue using 3D, 4D and fluorescence Micro-CT scanning methods.

Chapters 4 and 5 report the implementation of my research in the NSF GK-12 education program. In these chapters I describe the collaborative work with my partnered teachers to enhance the science curriculum by developing inquiry-based modules for 6^{th} and10^{th} grade (Regents) Science classes in New York state.

REFERENCES

Ghabrial, A., Luschnig, S., Metzstein, M. M., Krasnow, M. A. (2003). "*Branching morphogenesis of the Drosophila tracheal system*". Annual Review of Cell and Developmental Biology, Volume 19, 623-647.

Horowitz, A., Simons, M. (2008). "*Branching Morphogenesis*". Circulation Research, Volume 103, 784-795.

Manning, G. and Krasnow, M.A. (1993). Chapter 11: "*Development of the Drosophila tracheal system*". In: The development of *Drososphila melanogaster*, by Bate and Martinez, Cold Spring Harbor Laboratory Press, Volume 1, 609-685.

Sato, M., Kornberg, T.B. (2002). "*FGF is an essential mitogen and chemoattaractant for the airsacs of the Drosophila tracheal system*". Developmental Cell, Volume 3, 195-207.

Weaver, M., Krasnow, M.A. (2008). "*Dual origin of tissue-specific progenitor cells in Drosphila tracheal remodeling*". Science, volume 321, 1496-1499.

CHAPTER 2

AN *IN VIVO* STUDY OF MORPHOGENESIS OF THE RESPIRATORY SYSTEM IN *MANDUCA SEXTA* USING RESPIROMETRY AND MICRO COMPUTED TOMOGRAPHY

2.1 ABSTRACT

A GE-120 Micro-CT with 25um resolution was used to visualize the 3D morphology of *Manduca sexta* pupae during metamorphosis. This *in vivo* approach involved following an individual pupa throughout its metamorphic developmental period to study the dynamic changes in its internal morphology. Stacks of tomographic images over 19 days of pupation were used to reconstruct 4D images of the developing adult tissue systems, primarily the thoracic flight muscles and the respiratory system. By using a stop flow respirometer to generate a metabolic profile of pupal metamorphosis and integrating the results with the 4D Micro-CT images, we were able to visualize the dynamics of tissue development in *Manduca* pupa non-invasively. This diagnostic methodology, in combination with spiracular occlusion studies, shed light on the morphology and function of the rarely studied airsacs found in the abdomen of *Manduca* pupae.

2.2 INTRODUCTION

Insects respire primarily through an extensive cuticularized tracheal network, which opens to the outside environment at the spiracles and branches inside the animal transporting gases to and from the various metabolizing tissues (Figure 2.1). This

chitinized tubular network is regenerated at every molt when the insect sheds its old exoskeleton to accommodate growth (Figure 2.2). At a pupal molt, the insect enters a metamorphic stage to develop into a highly metabolic adult life form. During metamorphosis the pattern of tracheal ramification dramatically remodels to support and permanently establish the delivery of oxygen to developing adult organs. This rapid tissue development also depends on the distribution of hemolymph, the primary circulatory fluid, to transport nutrients and remove waste products in an open arthropod circulatory system (Wasserthal 1996).

The direction of tracheal growth, similar to angiogenesis in vertebrates, is determined by release of fibroblast growth factors, FGF Branchless, expressed at sites of oxygen starved developing tissues (Sato 2002). As insect tracheal development is governed by similar developmental principles as in vertebrate angiogenesis (Manning 1993), morphogenesis of these elaborate tubular structures has received a great deal of attention in the last decade (Ghabrial 2003, Horowitz 2008). The major questions in this field have addressed how this process is regulated to produce a functional organ system whose transport capacity matches the physiological needs of the organism. In particular, investigators have asked how do new branches arise, what determines the direction of growth, what specifies the formation of the next generation of branches and how do tubular networks fuse to create the 3D structures?

Drosophila melanogaster, due to the structural simplicity of its respiratory system and readily accessible genetics, has emerged as a paradigm of branching morphogenesis. Almost all research conducted on *Drosophila* to identify the molecular pathways that control the major morphogenetic events of tracheal development has been conducted

during embryogenesis, primarily because of the almost-transparent egg and embryonic stage that allows dynamic *in vivo* imaging studies (such as fluorescence, confocal and two-photon microscopy) to quantitatively observe growth of flurophore tagged tracheal placodes/tubes/cells, along with parallel gene and protein expression assays involved in tracheogenesis. However, an important aspect of tracheal development that remains almost completely unexplored at molecular and at morphogenetic levels is the formation and the functional profile of the adult respiratory system.

Unlike vertebrates, where circulation and respiration are tightly coupled, insects do not rely on oxygen delivery by respiratory pigments in the hemolymph (Wigglesworth 1972) and the link between cardiac function of the dorsal vessel and respiration has been largely ignored, more so in pupae than in adults (Wasserthal 2003a). Oxygen delivery occurs directly to the tissues via tracheoles. The circulation of hemolymph distributes nutrients required for tissue growth during larval and pupal morphogenesis. Carbon dioxide along with other metabolic wastes is removed by the hemolymph from tissues in a dissolved acid state. Hemolymph circulation in larvae occurs by posterior to anterior pumping of the dorsal vessel and peristaltic movement of the gut and abdominal segments. Periodic heart beat reversal is observed in pre-pupae. During metamorphosis, the dorsal vessel remodels into an abdominal heart-tube and a thoraco-cephalic-aorta for the adult. Pupae show long periods of cardiac inactivity and the functional significance of these long pauses in unknown (Wasserthal 2003b). Also, specific morphological adaptations of the dorsal vessel or any internal structure for pupal circulatory requirements are unknown. Consequently, the question arises as to how circulation of hemolymph occurs during pupal metamorphosis to support adult

tissue development and dramatic organogenesis, given that transport of essential nutrients to tissues and removal of respiratory waste products is critical for cell proliferation.

It is observed that the trachea in most winged adult insects is further modified into distended airsacs that develop only after adult eclosion and are described primarily as adult structures, i.e., not observed in pupae or larvae (Wigglesworth 1972). These adult airsacs participate in the circulation of hemolymph by alternately inflating and deflating the airsacs in the abdomen and thorax compartments (Figure 2.3). Airsacs in hawkmoth pupae such as *Manduca sexta* are an exception (Wasserthal 2003a & b), but the function and structural development of these structures in pupae is unknown. Presence of these tracheal modifications during a developmental life stage may indicate an adaptive feature of physiological significance in hawkmoths. These structures have not been reported in other pupae. But if these structures are dynamic, i.e. the shape is inconsistent through metamorphic period; a histological dissection of a specimen at a single time point may fail to reveal its possible presence. There is need of further research in order to investigate the presence and purpose of these developmental structures.

Tracheal structures have been studied extensively using histological serial sections obtained at discrete time points but this approach is unable to elucidate the *dynamic* relationship between changes in tracheal topography and function during development. Also, a combination of morphological, behavioral and developmental variations results in a great variety of respiratory patterns and an overall plasticity in the insect gas exchange system. To address this issue, studies in which tracheogenesis

and gas exchange are monitored simultaneously and over the entire period of pupal development are needed. Diagnostic imaging technologies such as x-ray synchrotron imaging, x-ray Computed Tomography, Magnetic Resonance Imaging and Ultrasound imaging are a few popular whole animal imaging technologies that allow us to virtually dissect and visualize the internal morphology and dynamics of an organism. But, *in vivo* studies using these technologies have been a challenge due to the inability to visualize and resolve between soft tissue types in the absence of a contrast agent and in a fluid filled, highly dynamic internal environment.

In this study, we have used micro-computerized tomography (Micro-CT) in conjunction with conventional flow respirometry to establish the functional relationship between the development of key components of the respiratory system (i.e., trachea, spiracles and air sacs) and gas exchange in *Manduca sexta* pupae at different stages of metamorphosis leading up to adult eclosion. Micro-CT provides high-resolution 25μm images at multiple times points, noninvasively and in the same animal *in vivo*, thereby permitting nearly continuous tracking of respiratory system development. The respirometric analysis conducted in parallel generates a metabolic profile of pupal metamorphosis. We test the hypothesis that structural reorganization of the pupal respiratory system occurs to support metamorphosis: where arborization and direction of tracheal growth is initiated as a response to localized hypoxia created in regions of rapid cellular growth and structural modifications of pupal trachea into inflated airsacs occurs to support discontinuous ventilation by serving as internal O_2 reservoirs and to aid in circulation of hemolymph by regulating the internal hydrostatic pressure.

2.3 METHODS AND MATERIALS

Insect Colony

A colony of the tobacco hornworm, *Manduca sexta* (Lepidoptera: Sphingidae), was maintained at Boyce Thompson Institute, Ithaca NY on artificial diet (Bell 1976, Ojeda 2003) at 26^0C, at 80% relative humidity, exposed to a 12:12h light: dark photoperiod. The pupae collected for the experiments were tagged with tape looped around their proboscis and labeled with personal identification numbers and housed in covered wooden boxes in order to maintain a dark, under-soil condition, at 27^0C and 80% relative humidity until adult emergence.

Micro-CT

The Micro-CT scans were performed using a GE CT-120 (GE Healthcare, Ontario CA) without the use of any contrast agents on the specimens. The *in vivo* scans of the thoracic muscles and airsacs were conducted at 360 projections in 10 minutes (2 frame signal averaging) with a resulting resolution of 50μm pixel for 2D images and voxels of 25-50 μm^3 for the 3D volumetric images. The 4D scans to observe *in vivo* arborization of the tracheal network throughout metamorphosis were conducted at 1200 projections obtained at 0.3^0 intervals using 80keV, 32ma and 25μm x-y-z resolution. The 3D scans used to generate the dynamic images of the pupae, 24 hours prior to eclosion, used the 50μm resolution protocol for every 15 minutes until adult emergence. In *Manduca sexta*, emergence of adults is protandrous where males emerge before females. The fluorescence x-ray mode of the CT-120 was used to visualize the internal dynamics of the pulsatile foregut in real time. The images were processed, using OsiriX imaging software (Pixmeo, UCLA, Los Angeles), to visualize

the major tissue types: the respiratory system comprising of tracheal network and abdominal airsacs, thoracic flight muscles, and the foregut of the digestive system.

Spiracular Occlusion

Manduca sexta pre-pupae were removed from the larval tubes at the green pupal stage (Schwartz and Truman 1983) and were stored in covered containers. When the pupal cuticle had hardened and turned brown at 48hours, the metamorphic stage labeled as Day1, the pupae were removed and anesthetized on ice for 15minutes. The spiracles were then sealed using orthodontic dental wax (Dental Wax, Othromechanics, Oklahoma, USA) melted and applied using a low temperature medical cauterizer (Medical Cautery Kit, Coastal Wholesale Medical, LaVergne, Tennessee, USA). Two separate spiracular occlusion experiments were conducted.

One involved sealing the seven pairs of the abdominal spiracles only and the delay in metamorphic time due to the anatomic intervention was recorded. In this experiment, a set of 30 pupae, 15 each of males and females, was sealed each day for the 19 day metamorphic period (N=570). The pupae were weighed everyday to record hygric loss and were also checked for leaks in the wax seal (Figure 2.4). The readings were continued past adult eclosion where mortality was evaluated 24 hours post emergence and fecundity readings were based on the adult's ability to fly, feed, mate and, in females: lay eggs. Only healthy adults with inflated and functional wings were recorded as alive.

The second occlusion experiment involved partial and complete sealing of pupal spiracles. Four female pupae were used for this experiment. The experimental animals consisted of one normal pupa and three pupae with sealed spiracles at Day 1 of

metamorphosis. The first pupa had a sealed pair of thoracic spiracles, second pupa had seven pairs of sealed abdominal spiracles and the third pupa had all of its spiracles including the anal pore sealed with dental wax. These pupae were used to investigate the respiratory function of the airsacs and to create a metabolic profile of the pupae during metamorphosis using a stop flow respirometer. The pupae were weighed everyday to record the hygric loss and were CT scanned every three days in order to investigate changes in airsac topology. Observations for these pupae were continued until adult eclosion.

Respirometry

Respiratory profile during metamorphosis in *Manduca sexta* pupae was determined by stop-flow respirometry (Sable Systems, Nevada, USA). . The experiment was conducted at 27°C at low light conditions to minimize pupal movement thus avoiding any confounding effects on the metabolic rate. The experiments were conducted for 15 hours, 1replicate/hour/day and everyday, throughout the metamorphic period until adult eclosion. Each pupa, normal and sealed, was transferred to individual 60ml disposable open syringes 1 hour prior to analysis and kept in an incubator in order to acclimatize to the experimental conditions. After the initial acclimatization period of 30 minutes, the syringes were filled with air that was scrubbed of carbon dioxide by soda-lime columns and water vapor by DriRite. The experimental set of syringes also included an empty syringe filled with air scrubbed of CO_2 and water vapor to serve as the control for drift in the baseline measures for both CO_2 and O_2. The rate of respiration was measured after a 30 minute incubation period. The incubated air volume of pupal respiratory gases was injected into a Sable Systems SS3 Gas

Analyzer Sub-sampler with an FCA-10A CO_2 and a FC O_2 Analyzer through Ascarite and magnesium-perchlorate columns at a flow rate of 57ml/min. The CO_2 and the O_2 analyzers were calibrated every day with a 50ppm CO_2 and a 20.9% O_2 gas. The respiratory gas contents, CO_2 and O_2 , were analyzed using the data acquisition software ExpeData (Sable Systems) .

Statistics

We used JMP software for the statistical analysis where ID for each pupa was entered as random effect and the treatment used was time and its interaction terms. Multiple comparisons between treatments at each time point was then calculated using a Bonferroni correction for multiple comparisons. Multi level (mixed) model was used for the statistical analysis because each pupa was repeatedly measured over time.

2.4 RESULTS

Normal Ontogeny

The 4D Micro-CT scans, where the same pupa was scanned everyday throughout metamorphosis, created a physical map to visualize the development of the adult tissues and tracheal arborization. Tracheal network in a pre-pupa is similar to the larval network but is largely degenerated and confined at the larval spiracular region (Figure 2.6). But it is observed that the trachea start to regenerate significantly within 48 hours of pupation. The virtual 3D sections of the *Manduca* pupa indicate the presence of prominent distended airsacs that are located between each pair of abdominal spiracles (Figure 2.4). These lobular structures are observed to appear on Day 2 and grow in volume over the first half of the metamorphic period (Figure 2.5).

The volume change in the airsacs can be very dynamic as observed in the Micro-CT scans performed during the last 24 hours prior to adult eclosion (Figure 2.7, 2.8) and the fluorescent real-time scans to observe the pulsatile movement of the foregut (Figure 2.16). The pulsatile oscillations and distortion of the pupal foregut caused distortion of the abdominal airsacs. The abdominal airsacs deflate gradually as the pupa gets ready to eclose and completely collapse a few hours before adult emergence (Figure 2.9 & 2.10a). Pharate adults that are completely devoid of inflated airsacs (Figure 2.9a) are observed to increase their abdominal movement and retract the adult abdomen from the pupal exoskeleton (Figure 2.9b). The reduction in airsac volume occurs earlier in males than in females (Figure 2.7) and this corresponds to early male moth emergence recorded in *Manduca* sp. The scans for the thoracic flight muscles, primarily the dorso-longitudinal (DL) and the dorso-ventral (DV) muscles, showed marked development after seven days of pupation (Figures 2.10 & 2.11) and this increase in tissue morphogenesis corresponds with the increase in tracheal arborization in the thoracic compartment. Pronounced tracheal ramification in the head compartment is observed in the last two days of metamorphosis (Figure 2.5).

Ontogeny with spiracular occlusion

Relative to control pupae, spiracular occlusion of the abdominal spiracles significantly prolonged ($p<0.001$) the time to complete metamorphosis and the pigmentation on the pupal wing pads (Figure 2.13). This also resulted in subsequent delay in adult emergence. Delay in emergence was not protrandic except on D14 and D18 where males with sealed spiracles took significantly longer time to eclose than sealed

females (Figure 2.13). The pupae that were sealed on Day 8 to Day 11 showed the maximum delay in metamorphosis: 14 days compared to control pupae. Females with sealed spiracles during latter half of the metamorphic period showed higher delay in adult emergence than males (Figure 2.13). The reduction in body weight due to respiratory water loss was observed to be similar for both control and occluded pupae (Figure 2.14) except in sealed males on D14. Mortality in pupae with occluded abdominal spiracles, in spite of delayed adult emergence, was less than 20%.

Metabolic profile

The metabolic profile of *Manduca* pupae (Figure 2.15) during metamorphosis shows that the rate of respiration gradually increases from Day 7 onwards. This is followed by a sharp increase on Day 18. These two shifts in metabolic rate correspond to rapid tissue development, specifically thoracic flight muscles after Day 7 (Figure 2.10), and then upon the reduction in tracheal volume due to deflation of the abdominal airsacs on Day 18. Minor release of carbon dioxide was observed until Day 9 in pupa with complete seal. This implies that a small fraction of carbon dioxide is exchanged through the pupal cuticle. Sealing of thoracic and abdominal spiracles caused a delay of three and six days respectively. In spite of delay in eclosion and an overall reduction in the metabolic rate, the pupa with sealed thoracic or abdominal spiracles followed the respiratory pattern similar to the normal pupa until Day 19. A sharp reduction in the respiratory rate was observed after Day 19. Pupa with sealed thoracic spiracles regained its respiratory metabolism 24 hours before eclosion. This trend was observed but was shallower in the case of pupa with sealed abdominal spiracles.

2.5 CONCLUSION

In this study we demonstrate Micro-CT imaging as a non-invasive diagnostic technology to study tissue development *in vivo*. Using this method, we were able to show the developmental time line of tracheal ramification, regeneration of the respiratory network and muscle tissue development during pupal metamorphosis in *Manduca sexta* by following individual specimens throughout their metamorphic ontogeny. When we compare the micro-CT images with the respirometric profile of the pupa we see that the increase in tracheal ramification occurred midway during a metamorphic period of 19 days. From our Micro-CT study, our observations indicate that this timeline was congruent with the development and compartmentalization of the thoracic muscles and inflation of the abdominal airsacs. We also report, for the first time, the dynamic morphology of airsacs in the pupal abdomen where these modified tracheal structures appear during the first three days of metamorphosis. Our speculation on the function of the pupal airsacs, based on the diagnostic analysis via CT imaging, indicates that these structures may play a vital role in maintaining the internal hydrostatic pressure to aid in circulation of the hemolymph during a phase of heightened tissue development. These airsacs showed marked increase in volume until the last quarter of the metamorphic period. This period, through metabolic data, also marked a gradual increase in the respiratory rate. This increase in airsac volume may result in increasing the internal hemolymph pressure and compensate for the regenerative period. Foregut pulsations may also add to hemolymph circulation directly by pulsatile movements or indirectly by pushing and causing distortions on

the airsacs (Figure 2.16). This developmental phase was followed by a sharp decline in tracheal volume due to deflation in the airsacs on Day 18 of pupation and this period was congruent with an accelerated increase in metabolic rate. This was the final developmental stage when the pharate adult prepared for eclosion. Increased respiratory rates yet low respiratory water loss during this time period may also be indicative of reduced spiracular activity, which may be due to the presence of inflated airsacs acting the pupa's internal oxygen reservoirs. Our experiments on spiracular occlusion support our hypothesis of airsacs as oxygen reservoirs where the hygric loss in normal pupae and sealed pupae with prolonged metamorphic period are similar to each other. Sealing of abdominal spiracles did not reduce the percentage of respiratory water loss. This implies that thoracic spiracles may regulate maximum respiratory water exchange while abdominal spiracles maintain the airsac structure. Pupae with reduced number of available abdominal conduits may further regulate the opening frequency of its thoracic spiracles to avoid excessive water loss.

Insects are the most diverse and one of the most abundant life forms on this planet. Their interspecies diversity lies not only in their varied morphology but also in the diverse habitat they occupy. They show remarkable adaptation to these diverse habitats and this adaptive capability has prompted the development of distinct organs to better exploit their living conditions. This is the case for the respiratory system of arthropods. The diversity in the respiratory system varies not only between species but also between the different life stages of an individual insect. Our approach, to understand the different mechanisms involved in tracheogenesis of *Manduca sexta* pupae, has attempted to address the need of an *in vivo* and integrative technology to

study insect physiology. Our method of diagnostic radioentomology using Micro-CT, in combination with other physiological, behavioral and genetic assays, may contribute towards addressing a series of big and unresolved questions in insect biology. For example, transcriptomic analysis of the genes involved in the development of the respiratory system during metamorphosis in combination with diagnostic imaging of the same, during normal and modified environmental and/or sensory conditions as well as in wild and laboratory raised population of insects, will help identify the molecular basis of regeneration of the respiratory system, tracheal ramification and also the development of adult tissues. Similar studies may also be constructed to address the behavioral plasticity in insects when exposed to varied ecological conditions, effect of pathological load, evolution of adaptive mechanisms and on how insects buffer physiological stress in response to the changing environment.

2.6 ACKNOWLEDGEMENT

We thank Mark Riccio for his assistance with the Micro-CT scans and Dr. Marjolein Schat at Boyce Thompson Institute Insect facility for the maintenance of the *Manduca sexta* colony. This work was supported by DARPA-MTO for Hybrid Insect MEMS program. The facilities used for this research include the Cornell Imaging Center at Weill Hall and Boyce Thompson Institute, Ithaca-NY.

2.7 FIGURES

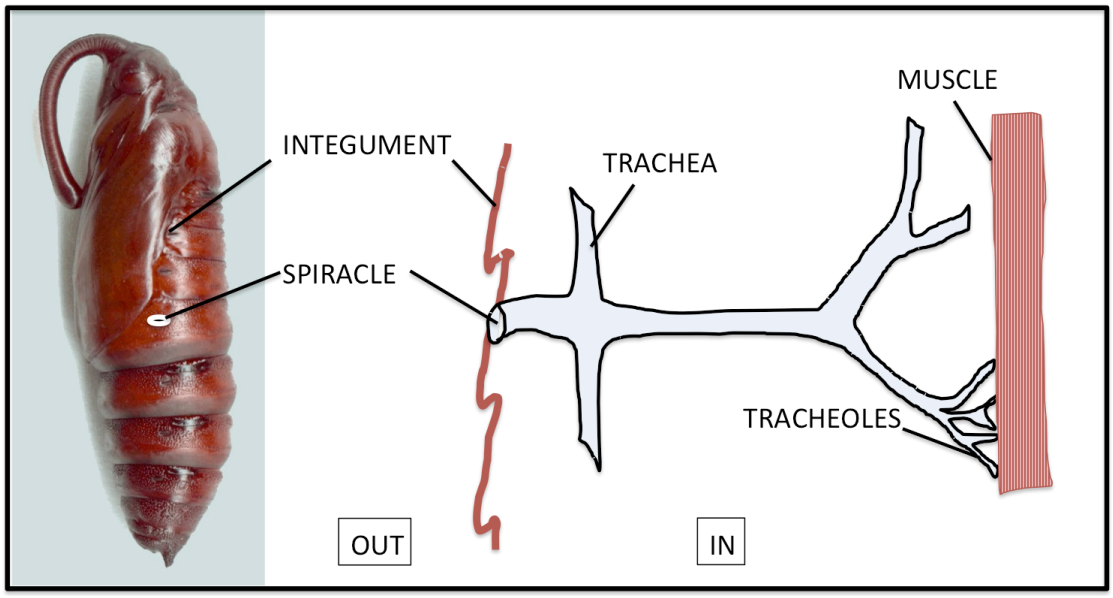


Figure 2.1: Schematic representation of the tracheal conduit in *Manduca* pupa. Seven pairs of spiracles on the abdominal segments and one the thorax open the tubular network to the atmospheric air. Tracheae divide into fine tracheoles that supply oxygen directly to the tissues and are sites for respiratory gas exchange.

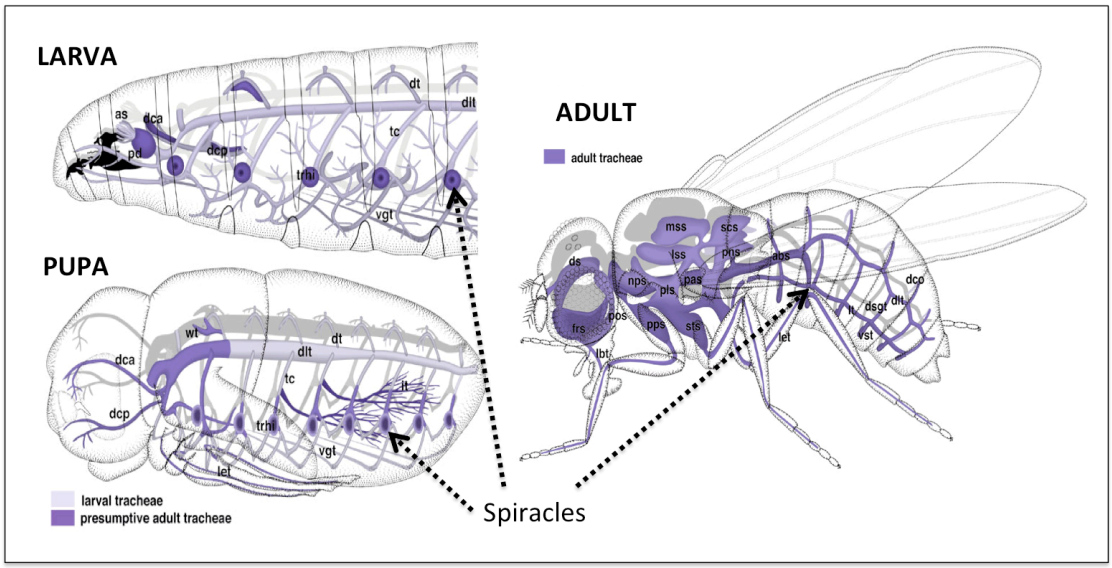


Figure 2.2: Tracheal network in the *Drosophila* larva, pupa and adult (Hartenstein 1993).

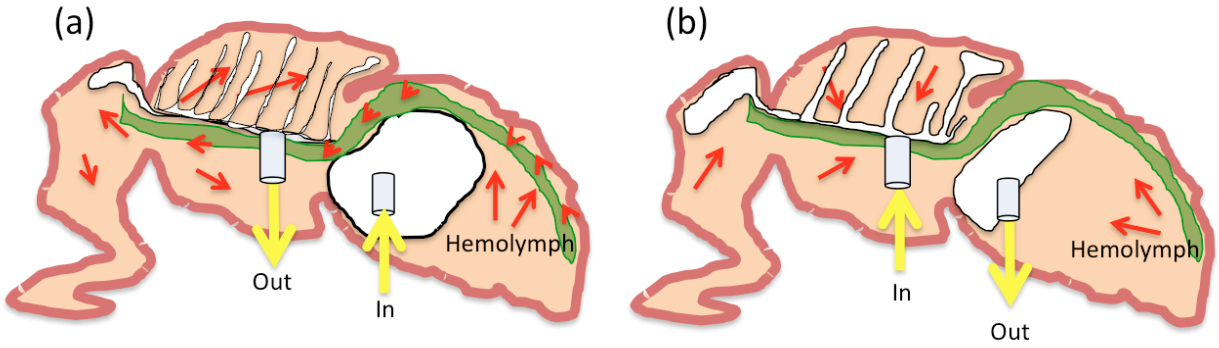


Figure 2.3: Function of airsacs in adult insects (Redrawn from Wasserthal 1996). Airsacs, by changing their volume, are known to aid in the circulation of the hemolymph. (a) Inflation of the abdominal airsac causes hemolymph to be pushed to the anterior body compartments, (b) inflation of anterior and wing airsacs returns the hemolymph back to the posterior compartment.

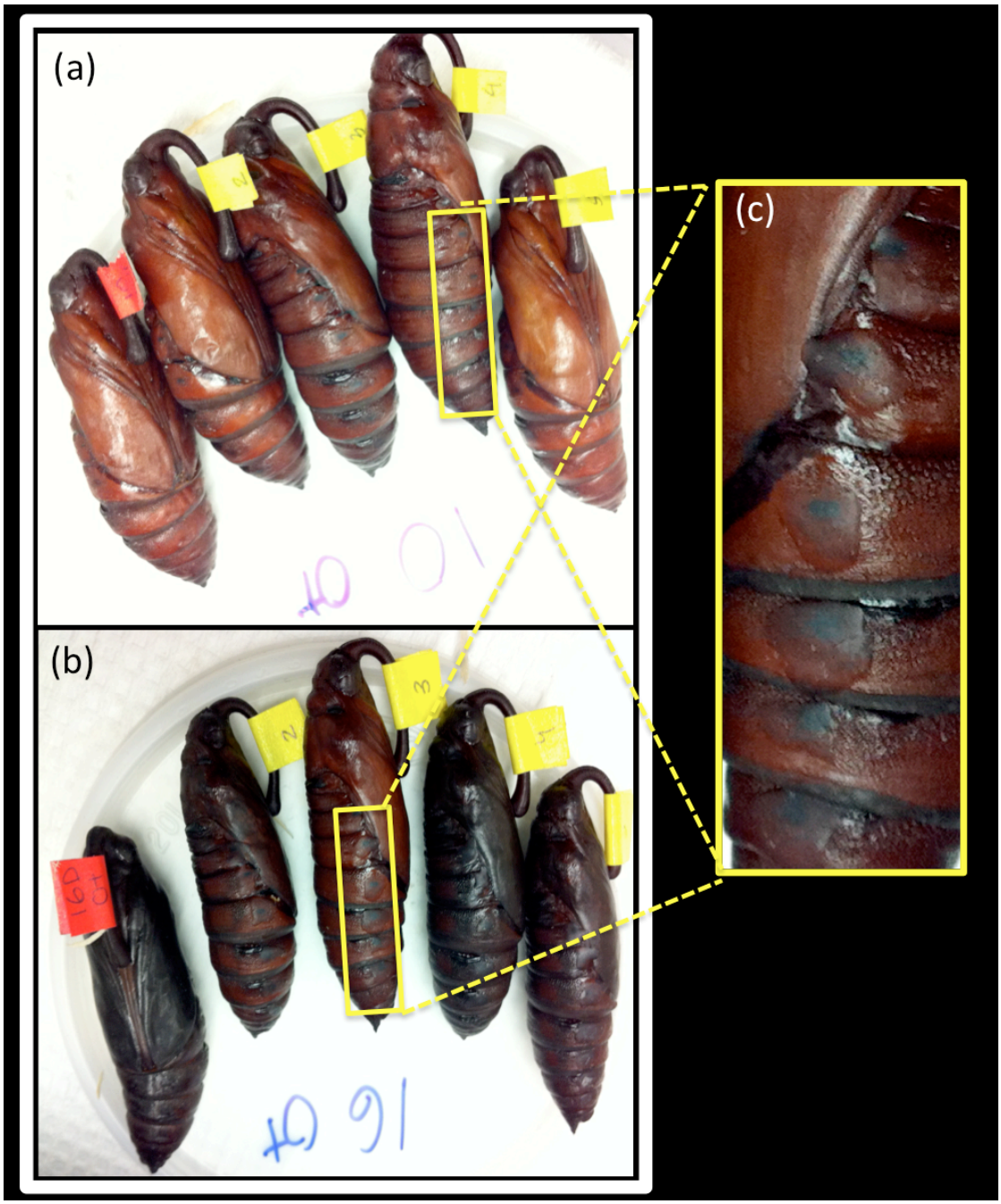


Figure 2.4: Spiracular occlusion in *Manduca sexta* pupae: The abdominal and thoracic spiracles were sealed shut using melted dental wax (c). These seals were checked for leaks throughout the metamorphic period: (a) sealed pupae at Day 10 and the same pupae on Day 16 (b).

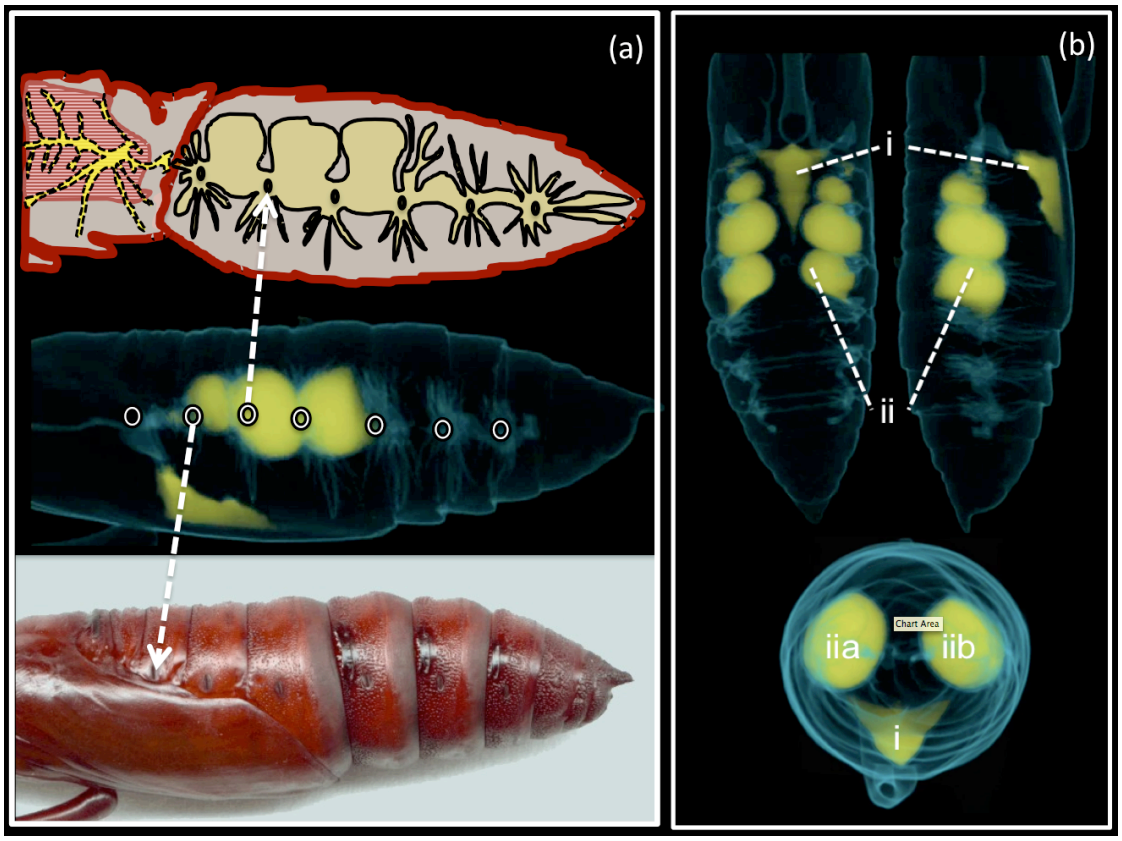


Figure 2.5: Internal morphology of the tracheal system in *Manduca sexta*. (a) The schematic representation of the tracheal network of an adult *Manduca sexta* (top) shows the position of abdominal spiracles and adult airsacs. These airsacs are modified respiratory structures and are also found at the same location in *Manduca* pupa (bottom) as observed by the Micro-CT scan of the same (middle). (b) The abdominal airsacs are lobular and bilaterally positioned (ii a & b) in between the adjoining abdominal spiracles. A large triangular airsac is also observed under the wing pads of the developing pupa (i).

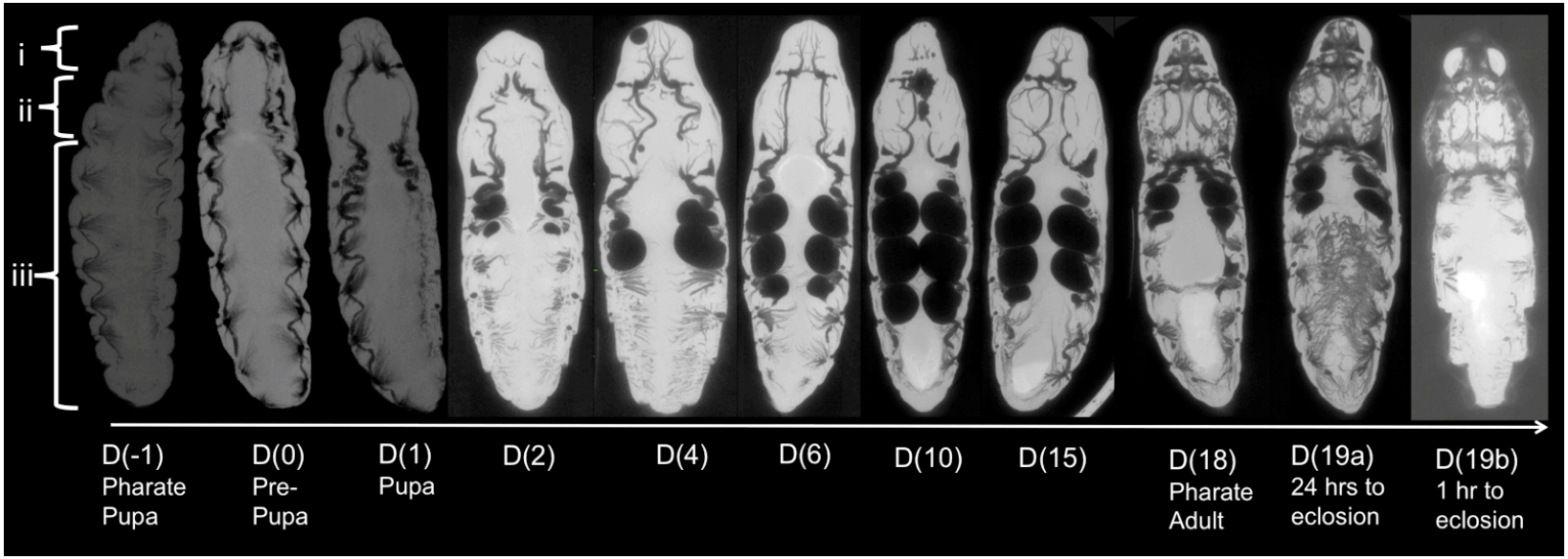


Figure 2.6: Development of the tracheal system during pupal morphogenesis where (i) head, (ii) thoracic and (iii) abdominal compartments of the pupal body. The Micro-CT scans reveal the increasing ramification of the tracheal network in the pupa, especially in the thoracic compartment and the head. The airsacs are observed to increase in volume from Day2 of pupation until Day 10 following which the airsacs begin to collapse until adult eclosion on Day19.

Figure 2.7: Micro-CT images of a pre-pupa (later view) at time zero (left) where the tracheal arborization is vastly reduced and is primarily confined to the larval spiracular (arrows) regions and the same pupal specimen on the right (dorsal view) after 48hours showing preliminary regeneration of the pupal tracheal network extending from the larval tracheal scaffold.

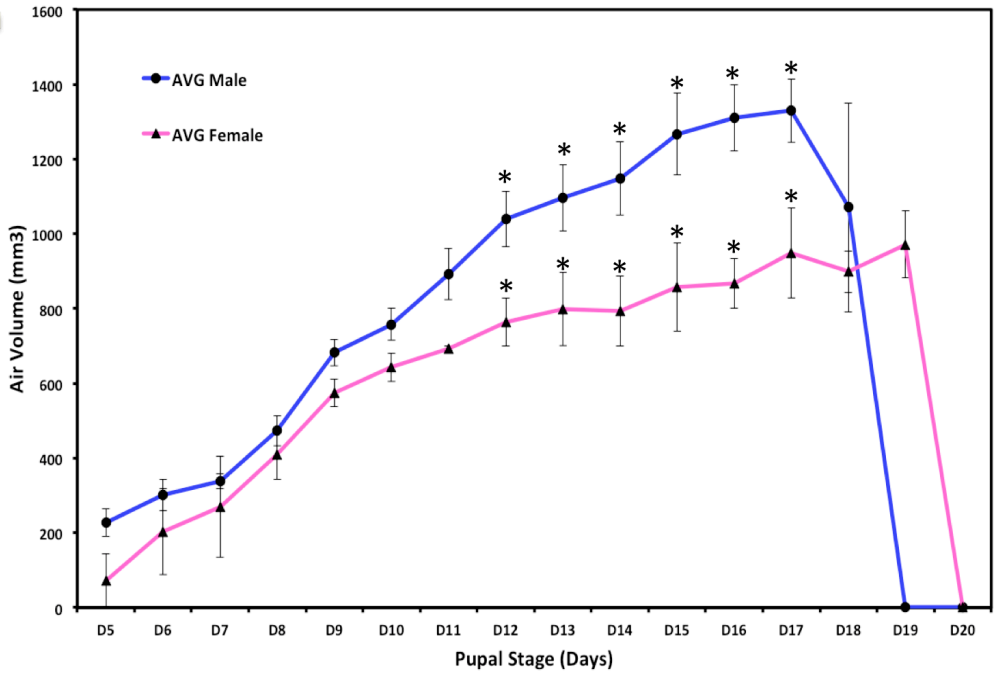


Figure 2.8: The volume of the developing respiratory system increases due to the inflation of the abdominal airsacs. The airsacs in males were larger than females from D12- D17 ($p<0.001$). This was followed by a sharp decrease in the total respiratory volume when the pupa approached eclosion. The sharp decline was observed earlier in male pupae than in females and this corresponds with protandrous male eclosion in general.

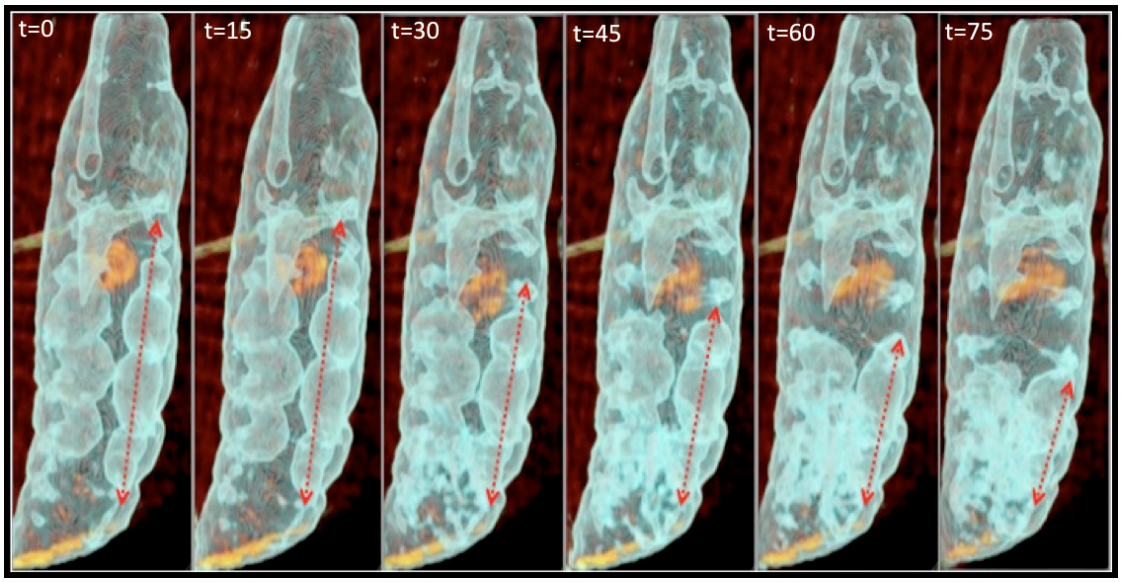


Figure 2.9: The panel of Micro-CT images indicates the dynamic reduction of the abdominal airsac volume (red arrow) observed on Day 18 of pupation. These images were taken every15 minutes.

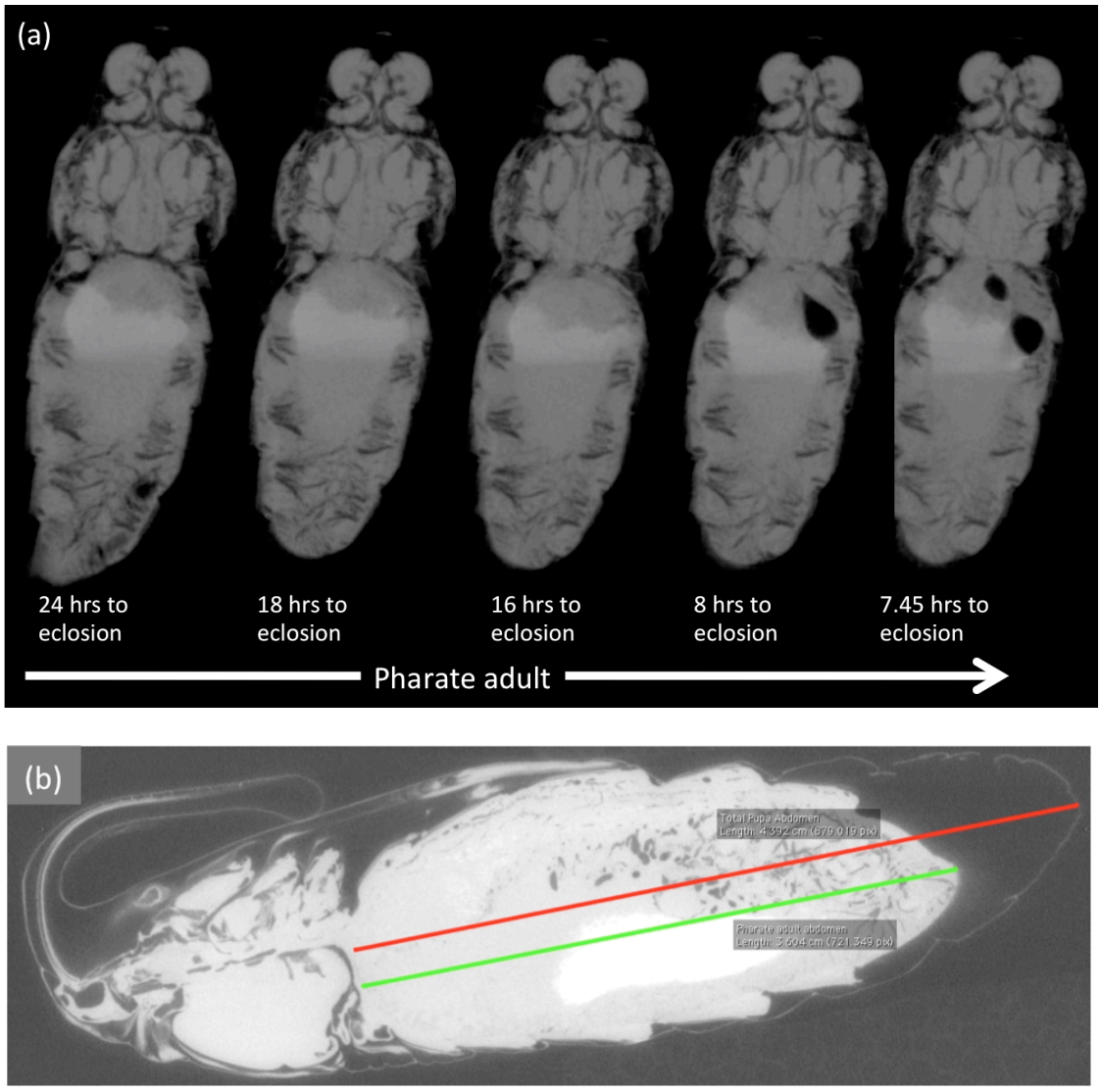


Figure 2.10: The abdominal airsacs are invisible due to complete deflation of these structures (a) in the pharate adult on the 19th day metamorphosis. This phenomenon occurs in parallel with heightened movement of the pharate adult and retraction of the body (b) from the pupal exoskeleton (red line: length of the abdominal pupal exoskeleton, green line: abdominal length of the pharate adult) and is indicative of an impending adult eclosion within 24 hours.

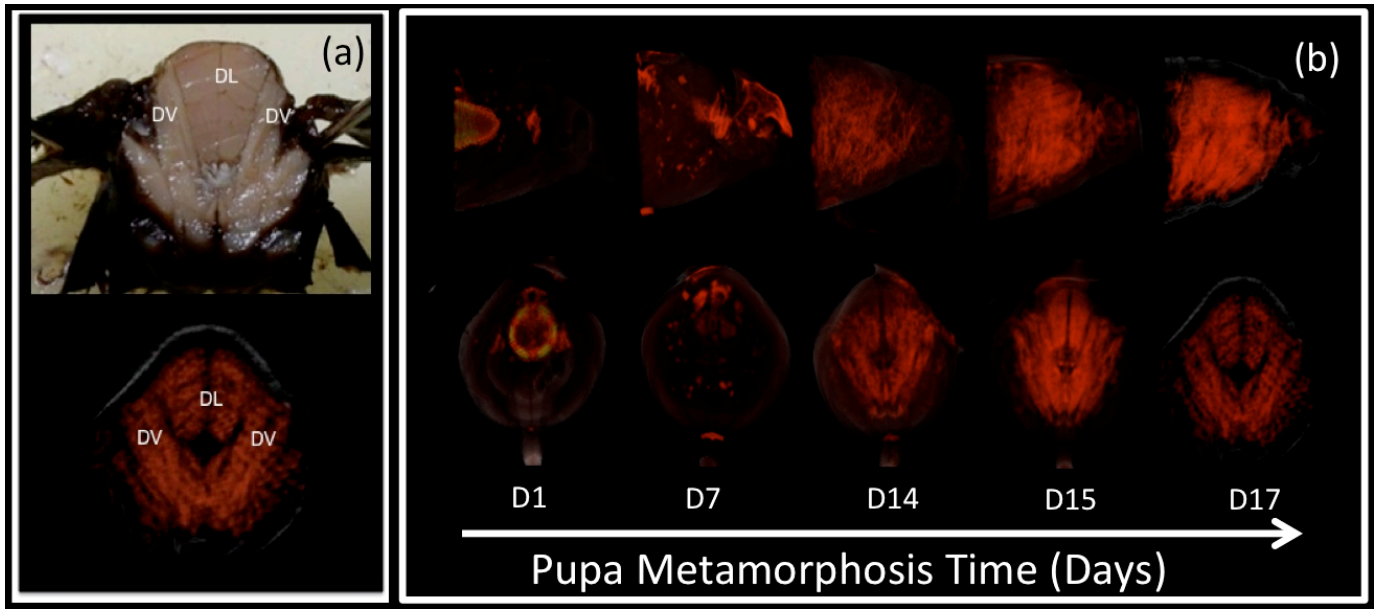


Figure 2.11: Development of thoracic upstroke (DV) and downstroke (DL) flight muscles during pupal metamorphosis. (a) Photograph (top) and Micro-CT image (bottom) of cross section of thoracic flight muscle in *Manduca* sexta. (b) In *vivo* micro-CT scans of *Manduca* pupa shows the appearance of the flight muscles (top: Longitudinal section and bottom: cross section of thorax) after Day7 and tissue compartmentalization into DV and DL muscles at Day 14.

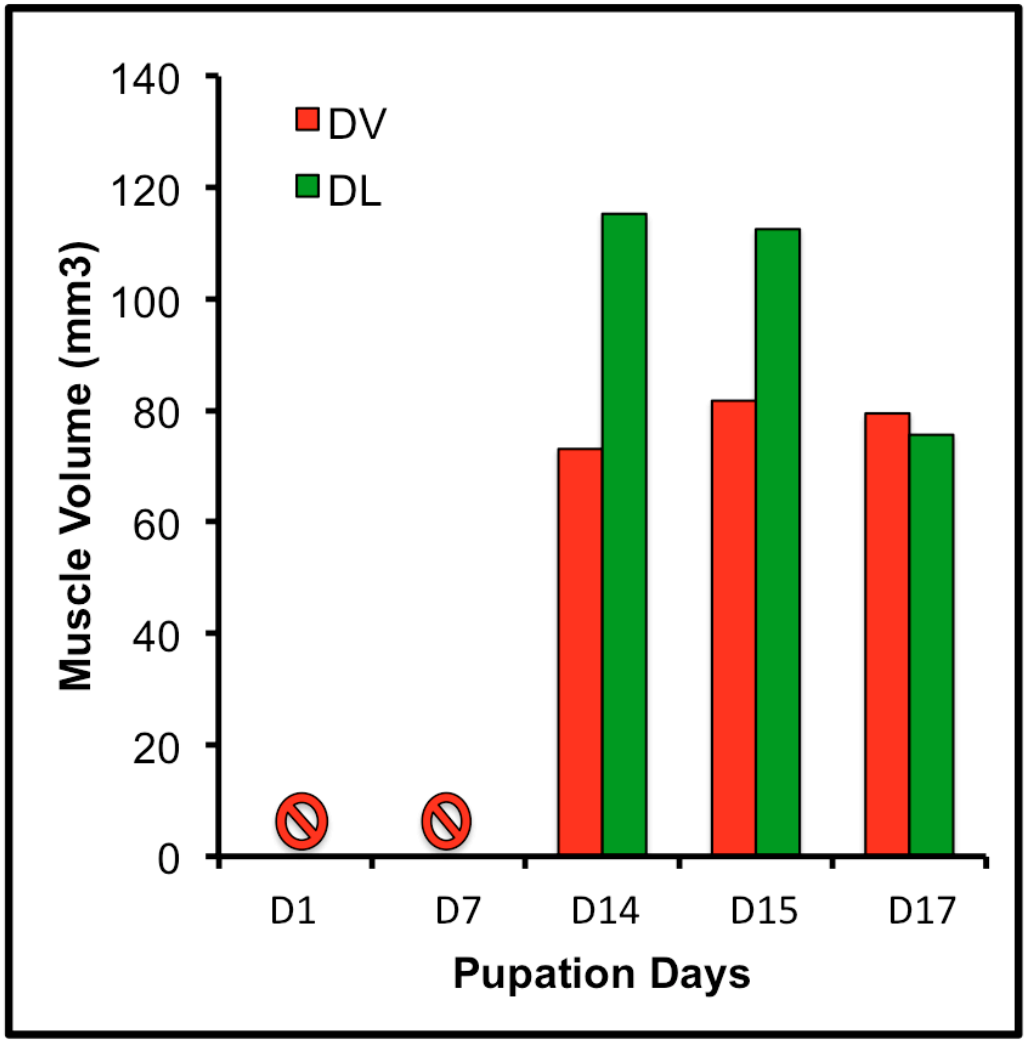


Figure 2.12: DL muscles aggregate earlier than DV muscles in the thorax and become more compact when the pupa is close to adult eclosion.

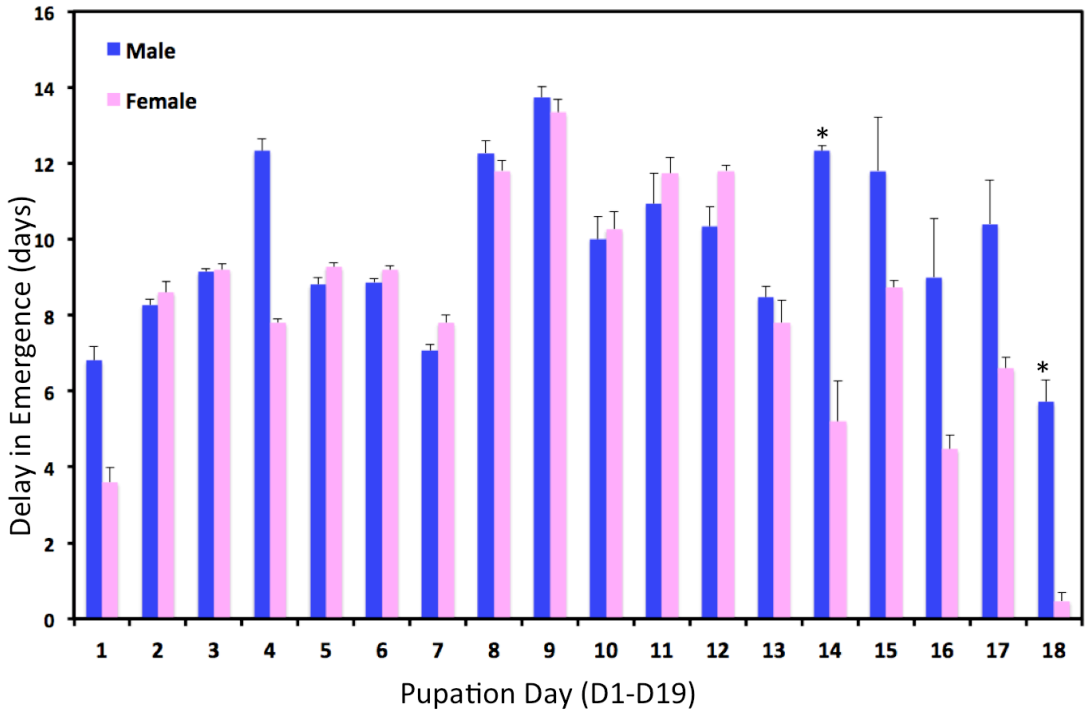


Figure 2.13: Developmental delay in pupae with occluded abdominal spiracles. Spiracular occlusion significantly prolonged pupation time compared to controls ($p<0.001$) for all except in females sealed on D18. Delay in emergence days between males and females were significantly different on D14 and D18 (*, $p<0.001$).

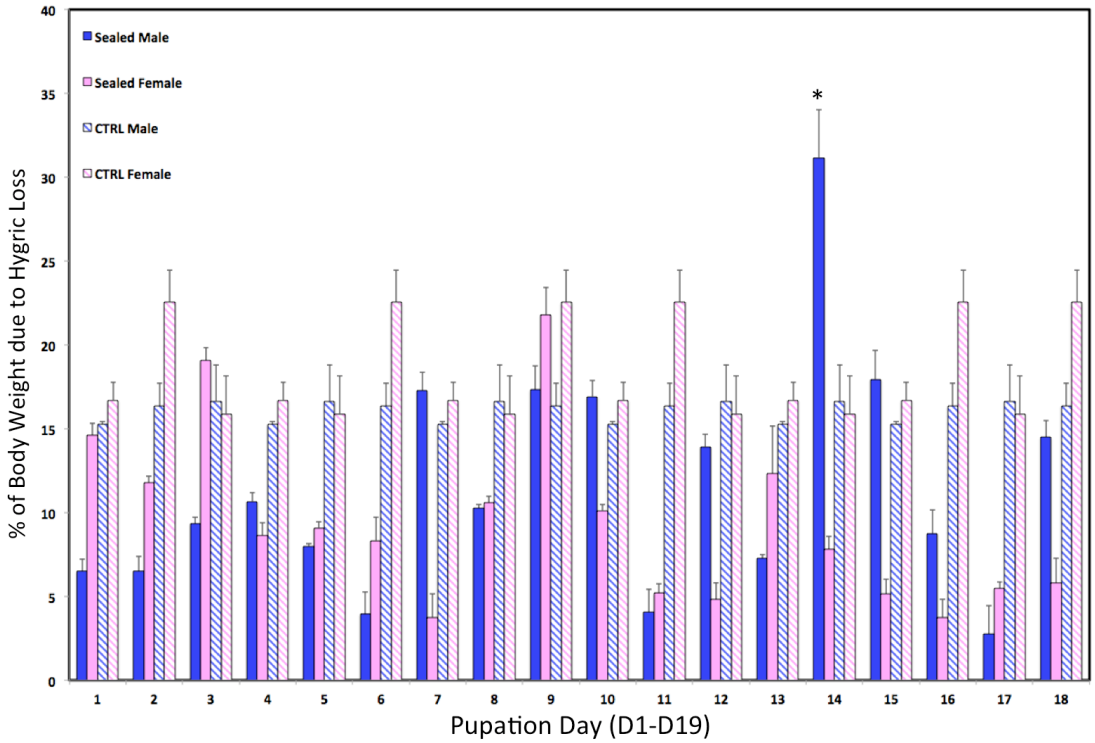


Figure 2.14: Hygric loss in pupae with occluded abdominal spiracles. Control males and females (N=76 each), Sealed males and females (N=304 each). Loss in body weight was less in sealed specimens compared to the control unsealed pupae except at D14 in sealed males where hygric loss was significantly higher (*, $p<0.001$) than control and sealed pupae and sealed females.

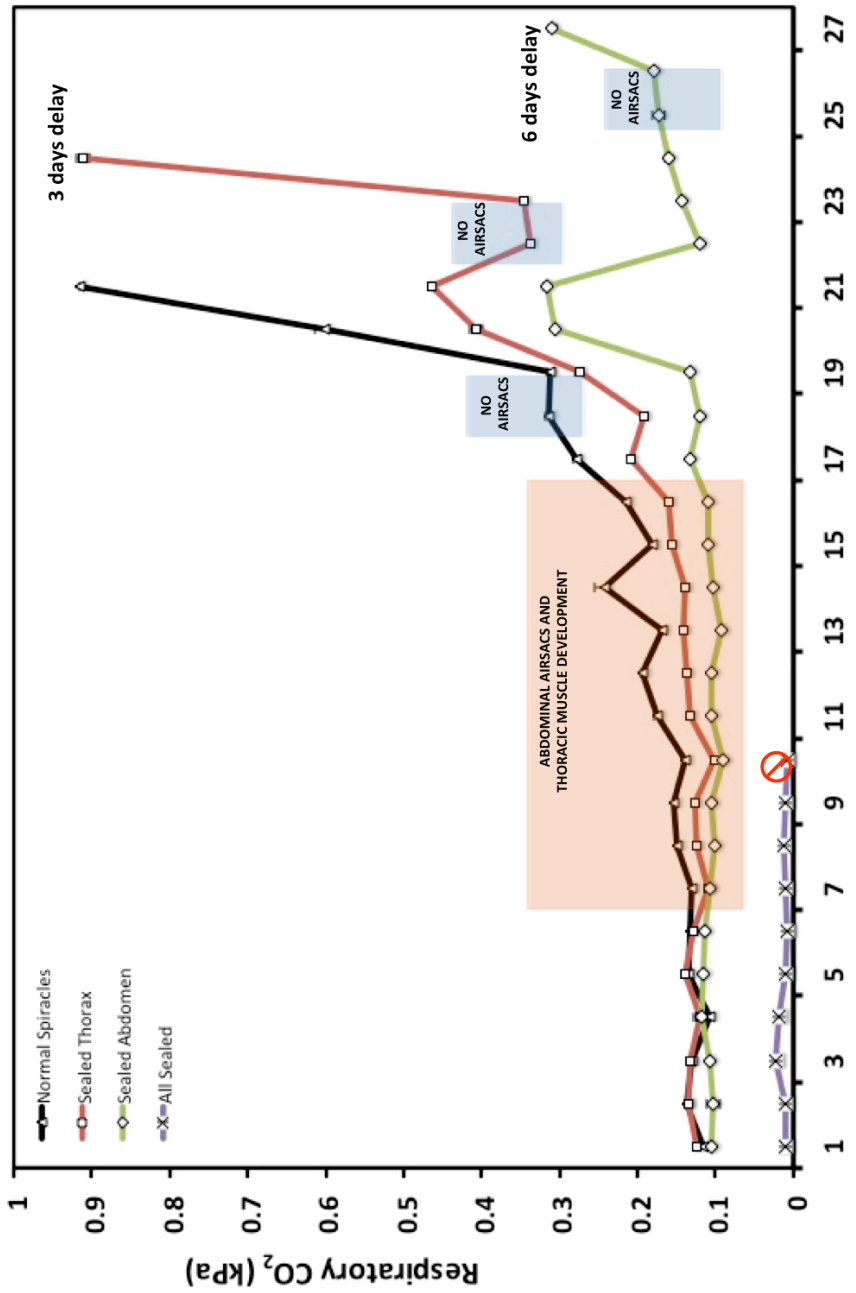


Figure 2.15: Metabolic profile of respiration during pupal metamorphosis in normal pupa and pupae with occluded spiracles.

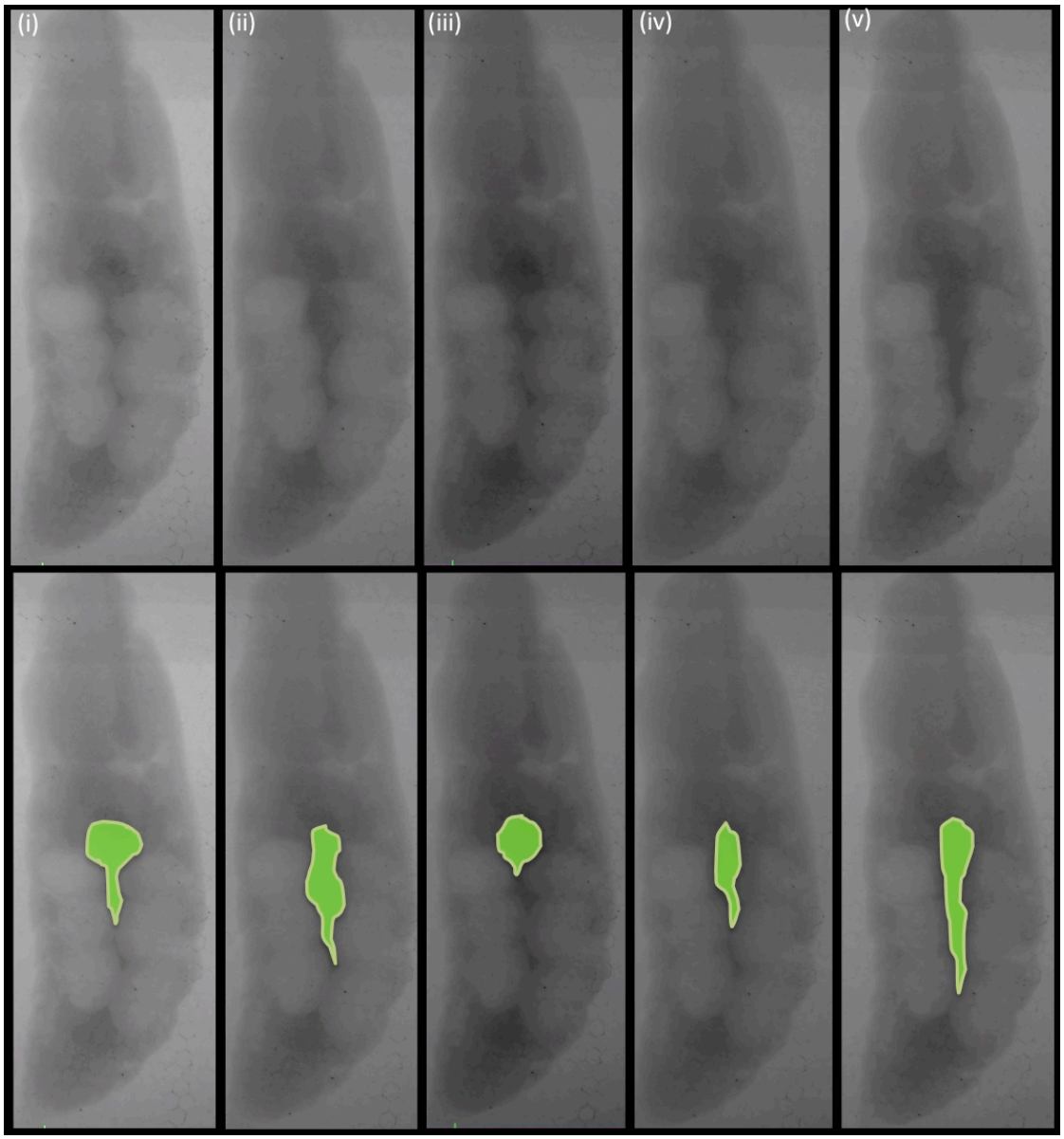


Figure 2.16: Pump-like movement of the foregut observed through fluorescence Micro-CT scans in *Manduca* pupa (i-iv < 45secs). The pulsatile movement of the foregut (green area) translates on to the adjoining airsacs and results in pulse-like distortion on the airsacs.

REFERENCES

Bell, R. A., Joachim, F.G., (1976). "*Techniques for rearing laboratory colonies of tobacco hornworm and pink bollworms*". Annals of Entomological Society of America 69, 365-373.

Ghabrial, A., Luschnig, S., Metzstein, M. M., Krasnow, M. A. (2003). "*Branching morphogenesis of the Drosophila tracheal system*". Annual Review of Cell and Developmental Biology, Volume 19, 623-647.

Horowitz, A., Simons, M. (2008). "*Branching Morphogenesis*". Circulation Research, Volume 103, 784-795.

Manning, G. and Krasnow, M.A. (1993). "*Chapter 11: Development of the Drosophila tracheal system".* In: The development of *Drosophila melanogaster*, (Eds.) Bate and Martinez, Cold Spring Harbor Laboratory Press, Volume 1, 609-685.

Ojeda-Avila, T., H.A. Woods, R.A. Raguso. (2003). "*Effects of dietary variation on growth, composition, and maturation of Manduca sexta*". Journal of Insect Physiology: 49: 293-306.

Sato, M., Kornberg, T.B. (2002). "*FGF is an essential mitogen and chemoattractant for the airsacs of the Drosophila tracheal system*". Developmental Cell, Volume 3, 195-207.

Schwartz, L.M., Truman, J.W. (1983). "*Homrmonal control rates of metamorphic development in the tobacco hornworm Manduca sexta*". Developmental Biology: 99: 103-114.

Wasserthal, L. T. (1996). "*Interaction of circulation and tracheal ventilation in holometabolous insects.* Advances in Insect Physiology, Volume 26, 297-351.

Wasserthal, L.T. (2003a). "*Chapter 7: Respiratory system. In: Lepidoptera. Moths and Butterfies*". Handbook of Zoology. Kristensen, N. P., Volume 2, 189-203.

Wasserthal, L.T. (2003b). "*Chapter 8: Circulation and thermoregulation. In: Lepidoptera. Moths and Butterfies*". Handbook of Zoology. Kristensen, N. P., Volume 2, 205-228.

Wigglesworth, V. B. (1972). "*Chapter 9: Respiration. In: The Principles of Insect Physiology,*" 357-410. New York: E.P. Dutton and Co., inc.

CHAPTER 3

ASSESSMENT OF SURGICAL IMPLANTS IN THE MOTH, *MANDUCA SEXTA*, DURING INTERMEDIATE STAGES OF METAMORPHOSIS USING MICRO COMPUTED TOMOGRAPHY

3.1 ABSTRACT

As proof of concept towards making biobots, we implanted active PCB probes into the flight muscles of a moth at an optimal pupal developmental stage. The growth of tissue around the probes was viewed *in vivo* using MicroCT images. Through this imaging system we were also able to determine the exact positioning of the MEMS probes in the flight muscle and the effect of their presence in adults after eclosion and also after normal flight. By combining our surgical procedure with *in vivo* MicroCT images we demonstrate that we can determine the exact location of probes in the tissue of interest, design probes of feasible dimensions that can serve as a platform for a multitude of electro mechanical systems and create a solid tissue-implant interface leading to the development of modified insects which can then be used to study insect migration and dispersion patterns and various other applications such as remote sensing, via telemetry.

3.2 INTRODUCTION

Over the past four decades, many attempts have been made to develop small (cm scale) autonomous flying machines (micro aerial vehicles; MAVs). These attempts have had limited success (Ellington 1999) because the power sources needed for

powering flight have not had sufficient energy and power density for sustained flight with man-made actuators. However, nature provides insects, which have evolved highly efficient muscle actuators and flight control systems, and can carry payloads that are equal to or higher than their own mass because they posses larger cross-sectional area of flight muscles with respect to their muscle volume (Kuwana 1999, Vogel 2001). Hence insects can serve as an attractive model for constructing biological robots, popularly known as "*biobots*", in which an intact biological system is incorporated into the design of a microsystem (Wooton 2000; Cary 1996; Louden 1995). Yet, one of the challenges in constructing a biobot has been the permanent attachment of micro-components on to adult insects. For instance, several research groups have successfully adhesively bonded telemetry electronics onto adult insects to track the insects' movements and migratory paths (Riley 1998; Riley 2005), and attached miniature "backpacks" for environmental monitoring, wireless communication, or biobotic manipulation of behavior (Cary 1996; Mohseni 2001; Kuwana 1999; Kutsch 1993). However, the adhesive bonds can be temporary and unreliable. The devices can fall off due to shock, body motions and subsequently induced inertial stress and strain forces, when the insect is in challenging situations such as confined spaces, foraging, or avoiding collisions during free flight. Attaching a payload to an active adult is difficult due to resistance offered by the insect against undesired fixing or restraints required during immobilization. Also, adult insects have wax/oil coated smooth cuticles or are usually, as in the case of Lepidopterans, covered with shed-able scales that add to the complexity of using adhesives to attach

microsystems. Finally, adult insect attachments lead to reduced flying agility, presumably because they perceive the load as foreign load.

Insects survive extreme surgery as shown by parabiosis experiments on silkmoths and other insects (Williams 1946). But surgical intervention in the adult stage of an insect poses another host of technical challenges. Surgically inserted foreign materials such as electronic probes or wires are always invariably more rigid than surrounding tissue. Presence of such objects in dynamic and constantly shifting tissues will cause the soft tissue to shear hence reduce the effectiveness of the inserted electronic device. Also, damaged tissues introduce experimental variability.

To solve the aforementioned problems associated with device attachment and subsequent machine-tissue interface during the adult stage, we have taken advantage of the pupal metamorphic stage of life cycle in a holometabolous insect, the tobacco hornworm moth, *Manduca sexta* (Lepidoptera: Sphingidae) (Figure 3.1), to implant and permanently tether devices into this immature stage. Insects grow by periodically molting to change their external skeleton and to accommodate a larger body volume for the next instar. The replacement of the exoskeleton that takes place during a molt presents a window for the repair of wounds that are caused by the implantation of external devices. With the goal of producing "biobots" that can be harnessed as micro-air vehicles (MAVs) with integrated MEMS sensors and actuators, we have developed a metamorphosis-based surgical method. This method, Early Metamorphosis Insertion Technology (EMIT: Patent CRF#D-3872-03), involves surgical implantation of the micro cargo platforms during early pupation. EMIT ensures a secure hold of the implant due to the metamorphic development of the surrounding adult tissue. We have

already demonstrated that implantation of such MEMS devices during metamorphosis does not adversely effect the insect's survival (Paul et al. 2006). Because the implants are placed in the early metamorphic stages, not only do the wounds heal, but also new tissue is formed around the inserted object. This enables the perception of the insert to be more natural after the adult insect ecloses from the pupal exoskeleton. Initial balloon-assisted flight control experiments in these hybrid sentinels have shown that the implants can readily and reliably modulate the insect's innate flight capability (Bozkurt 2009).

But the question of probability of target tissue-device interface and subsequent consequences due to the presence of a foreign body with such invasive methods always remains. Also, it is important to isolate target areas, realize potential available space on and inside these small-scale organisms and avoid regions of the body where presence of a foreign rigid substance could pose a barrier for normal development. The fate of such implantations can be evaluated by postmortem histological methods. But in order to generate viable sentinels an *in vivo* visualization of these target sites is more favorable. Diagnostic radioentomology is a fast emerging field (Greco 2012, Westneat 2003, Lee 2009) in which Micro-CT, X-ray synchrotron and MRI are some of the imaging technologies that are now becoming desirable methods to visualize these elusive small-scale organisms in 3D and in a non-invasive way.

In order to address our question we have used Micro-CT imaging to visualize the fate of our implanted devices in the *Manduca sexta* pupae. By using this technology, we not only simultaneously looked inside multiple live specimens with implants, but also tracked the insect's normal tissue development with and without implants. For this we

performed 4D Micro-CT scans where the same specimens were scanned, *in vivo* everyday, throughout their metamorphic period. The Micro-CT scans generated a 3D stack of images that could be sliced virtually to evaluate the fate of the target tissue and the implants. The scans on these sentinels were resumed once they emerged as adults so that we could see if the probes and tissue had undergone any displacement or damage during eclosion and also after free flight.

Our results demonstrate that Micro-CT imaging can allow us to create large number of viable hybrid insects with implanted MEMS devices. These sentinels when active, upon adult emergence, can serve as hybrid-flying vehicles and can be adapted for a variety of applications, such as to monitor insect migration and dispersion, and for environmental sensing via telemetry.

3.3 METHODS AND MATERIALS

A colony of the tobacco hornworm, *Manduca sexta* (Lepidoptera: Sphingidae), was maintained at Boyce Thompson Institute, Ithaca NY on artificial diet (Bell 1976, Ojeda 2003) at 26^0C, at 80% relative humidity, exposed to a 12:12h light: dark photoperiod. How many animals were used, genders, ages, etc?

The dimension of the implant was determined by measuring the cross section of the pupal thoracic cavity and the distance between the two primary bundles of thoracic indirect flight muscles, the dorso-ventral (*dv*) and the dorso-lateral (*dl*) muscles (Figure 3.2a). The design of the probe consisted of four prongs that targeted the main bundles of the *dv* and *dl* muscles.

The polyamide, PCB, flexible muscle probes (Figure 3.2b) were fabricated (Hughes Circuits, Inc., San Marcos, CA, USA) on a thick Kapton polyamide substrate (AP7156, DuPont^{TM}) and laminated with 17µm (0.5oz/ft^2) thick copper on one side only. Polyamide was the choice for the probe scaffold due to its pliable yet structurally sound, biocompatible and inert properties. A deposited copper layer served as the electrical conduit and was coated with liquid photo imageable (LPI) for insulation except at the excitation/ recording sites (Bozkurt 2009). These recording pads had electro-less nickel and immersion gold (ENIG) deposited on them for biocompatibility. These probes were stored in sterile packages until they were ready for implantation.

The pupae were anesthetized on ice (4^{O}C) for 10 minutes for the EMIT surgeries. In order to attach the four-prong thin-film polyamide muscle probe, four mesothoracic incisions in the overlying cuticle were created using a hypodermic needle (22G, Exel International, Fisher Scientific). The controls for this experiment comprised of two sets of pupae that were only anaesthetized on ice and served as the normal marker for adult emergence. After the surgical implantation, the pupae were then housed individually in a dark chamber to reduce the chances of contamination or infection. Upon emergence the adults were transferred to a large 2 X 2 X 2 m net tent, which provided suitable perches for proper expansion of the wings and also allowed free flight and normal nectar feeding.

The Micro-CT scans were performed using our in-house a GE CT-120 (GE Healthcare, Ontario CA). The custom protocol used for the *in vivo* scans of the pupae

were 2 minutes long with 360 projections (2 frame signal averaging) with a resulting resolution of 50μm. The live pupae were scanned (Figure 3.3) in groups of 5 specimens every day, without any contrast agents, from the 1st day of insertion of the probe at Day 4 of pupation until adult emergence. Two sets of control insects were used: One was exposed to the exact same amount of radiation as the experimental animals in order to determine the effect of radiation exposure on adult eclosion and the other set was kept unexposed to the X-ray completely to determine normal development and eclosion. The adult moths after emergence with implants were scanned twice: once using the standard 2 minutes protocol and also the 60 seconds fluorescence mode in which we could observe the implanted probe in real time. The images were processed using OsiriX imaging software to primarily visualize three major tissue types: thoracic flight muscles, the respiratory system comprising of tracheal network and abdominal airsacs and the digestive system.

3.4 RESULTS

The time series Micro-CT images taken throughout the metamorphic period of normal unaltered pupae enabled us to visualize the developmental time line of the flight muscles and the respiratory system. The thoracic muscles, primarily comprising of two groups of flight muscle bundles, *dv* and *dl*, were undistinguishable during the early pupal stages (Figure 3.4a). These muscles were more defined and compartmentalized from the rest of the developing adult tissue after Day 7 of pupation (Figure 3.4b). The whole body scans of the *Manduca* pupa revealed the components and gradual development of the respiratory system. The respiratory system comprised of tracheal

tubes that gradually ramified throughout the body during the metamorphic period. The other major structure observed were balloon-like airsacs in the abdominal cavity that also increased in volume over this developmental time line. These airsacs expanded to cover the entire abdominal cavity right up to the final day, i.e., Day 19, before adult eclosion (Figure 3.5a). There after these airsacs were observed to collapse reducing the sum total air volume inside the pupa (Figure 3.5b) and was followed by the emergence an adult moth soon after. These preliminary time series scans were used to determine the appropriate time frame and available sites for insertion of probe in the pupa. These numerous stacks of Micro-CT images enabled us to slice the pupae virtually in order to determine the available space in the pupae and the distance to the target sites/tissues so that implants of appropriate dimensions could be fabricated. The fabricated PCB muscle probes were inserted during Day 4 of pupation. Day 4 was chosen as the period for insertion of these probes because at this stage the pupae had already hardened its pupal exoskeleton but had not yet formed the final flight muscle bundles or had regenerated an extensive tracheal network at the target area. These factors ensured structural stability for the inserted probe, reduced mortality of the pupa and also avoided shearing of target tissue due to probe implantation. Another set of time-series Micro-CT images elucidated the fate of incorporating these implants in the pupae. Multiple pupae were scanned at a time (Figure 3.6b) that allowed us to select and save pupae that grew with correct positioning of the implants. The pupae with incorrect positioning of the probes could be rejected immediately that otherwise may have directly increased the probability of failure in generating a viable experimental moth. We were able to visualize the gradual tissue growth around the implanted

muscle probes (Figure 3.6a, c,d). Pupae, with the implants, eclosed at the same time as the unaltered controls. This indicates that the surgical procedure did not hamper the pupal development or its survival rates (Paul 2006).

The newly eclosed adult moths were scanned using the same Micro CT protocol that was used for the pupal scans. The generated images (Figure 3.7a) show that the probe did not move or dislodge during the process of eclosion and that the target tissue was not damaged during this distortive, highly dynamic and strenuous phase. We were able to visualize the pulsating foregut and an *in vivo* internal view of the adult moths from the image stacks generated by the fluro-mode scans (Figure 3.7b).

The scans of the adult moths after a period of free flight indicated tissue damage in one of the primary flight muscles around the probes (Figure 3.8). The *dv* muscles appeared to be undamaged after free flight but the *dl* muscles showed considerable amount of damage around the inserted probe (Figure 3.8c).

3.5 DISCUSSION

During Lepidopteran metamorphosis, the entire larval musculature in the thoracic compartment is degenerated. The fibers of these digested muscles act as the template for the adult flight muscles where the developing myoblasts migrate to these fiber scaffolds to accumulate into muscle bundles (Rind 1983, Chapman 2012). The flight muscles are created *de novo* in order to support a physiologically different life stage. Results from this study indicate the efficacy of using Micro-CT imaging technology to observe the dynamics of tissue development, *in vivo*, in a cm scale insect during metamorphosis without the use of any contrast agents. The time series scans enabled

us to follow a single metamorphosing *Manduca* pupa and visualize, for the first time, the time line and intricacies of muscle tissue formation, compartmentalization and the ramification of tracheal system during this life stage. We also demonstrate for the first time that fluorescence CT scanning mode can be used to follow real time changes in the internal conditions of the moth.

Our novel EMIT surgical procedure explores the possibilities of harnessing nature's super fliers, in combination with appropriate mm-scale tissue actuators and sensors, to remotely study the natural environment around us. In our goal towards making such insect hybrid systems with implantable MEMS devices, Micro-CT imaging has proven itself as an invaluable diagnostic tool to determine the exact positioning of the probes in the *Manduca* flight muscle and the effect of their presence in adult moths after eclosion and also after normal flight. Information accumulated from these scans gives us an insight to further develop the design of such actuators so that we can minimize the amount of tissue damage due to the presence of these structurally rigid probes. The technological challenge in creating flexible implants is to make them pliable enough, while maintaining its structural integrity, so that it is comparable to the soft target tissue (Rogers 2010) and yet be easily implantable. Orientation of these implants with respect to the fiber orientation of muscle tissue is also very important as we observed, in our CT images, shearing of the *dl* muscles after free flight due to the positioning of the probe perpendicular to the orientation of the muscle bundle.

Nature provides us with unfathomable biodiversity among insects, but due to their elusive nature we still lack a proper understanding and adequate information on the internal morphology and developmental physiology for a majority of these specimens.

Using *in vivo* MicroCT imaging as a diagnostic tool, we prove that we can determine the exact location of probes in the tissue of interest, design probes of feasible dimensions that can serve as a platform for a multitude of electro mechanical systems and create a solid tissue-implant interface leading to the development of modified insects. These sentinels once assembled can then be used to study insect migration and dispersion patterns and various other applications, via telemetry.

3.6 ACKNOWLEDGEMENT

We thank the members of the *Sonic*MEMS group: Alper Bozkurt, Abhishek Ramkumar, Siva Pulla and Janet Shen for fabricating the muscle probes, Mark Riccio for his assistance with the Micro-CT scans and Boyce Thompson Institute Insect facility for the maintenance of the *Manduca sexta* colony. This work was supported by DARPA-MTO for Hybrid Insect MEMS program. The facilities used for this research include the Cornell Imaging center, Weill Hall: Ithaca Campus, *Sonic*MEMS laboratory, Boyce Thompson Institute and the Duffield Research laboratory at Cornell University.

3.7 FIGURES

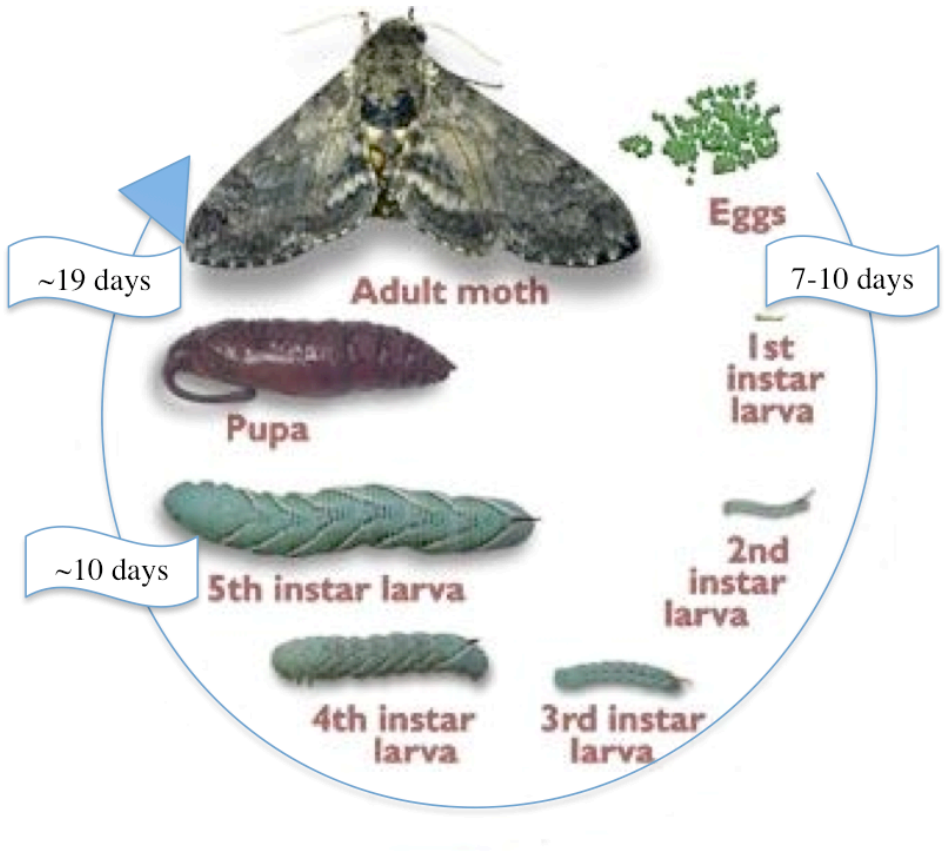


Figure 3.1: Life cycle of *Manduca sexta.* A pupa metamorphoses into an adult in approximately 19 days.

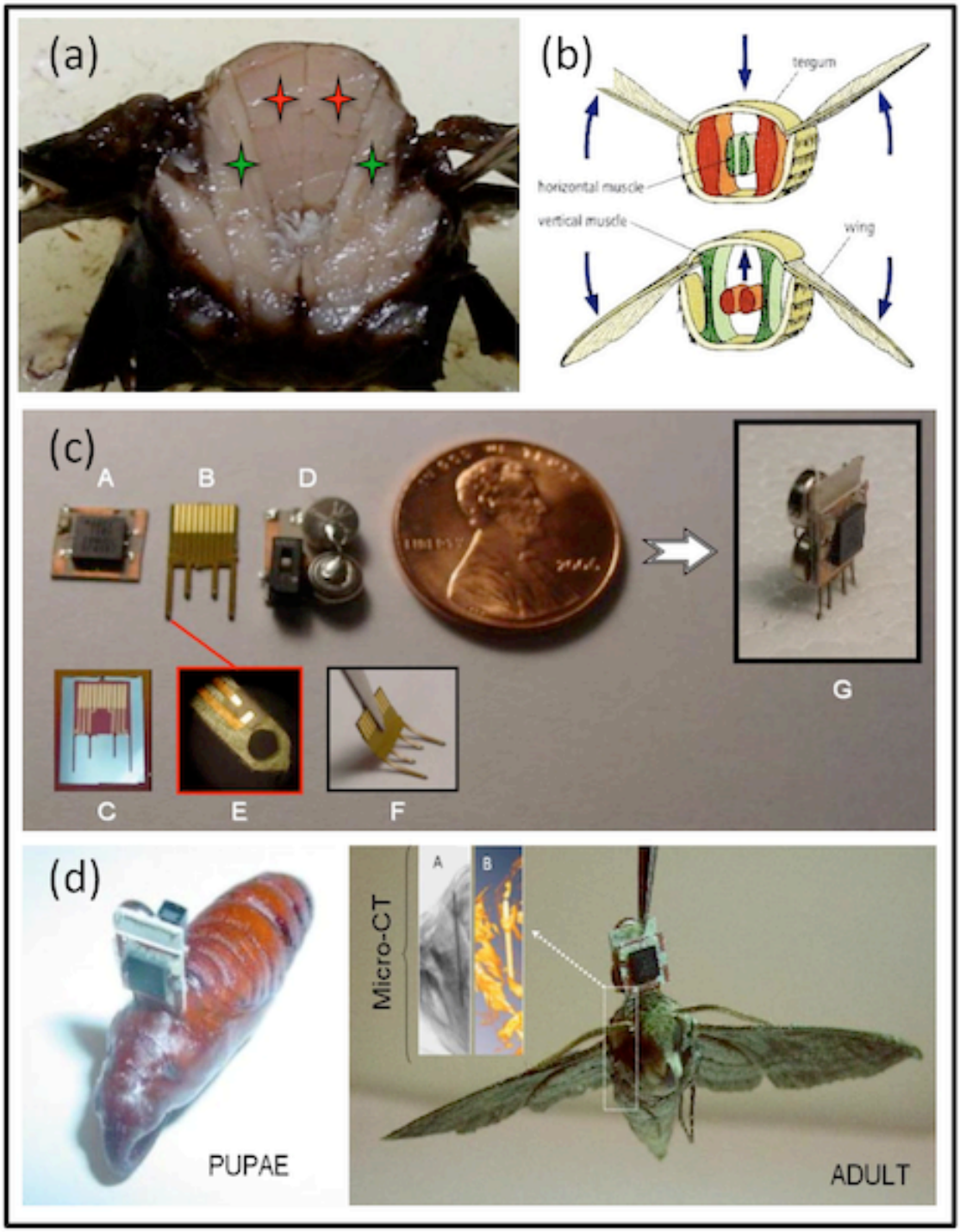


Figure 3.2: Probe design for flight muscle actuation. (a & b) cross-section of adult *Manduca* thorax showing flight muscles, dorso-longitudinal down-stroke muscle (DL)

and dorso-ventral up-stroke muscle (DV) and target locations (DL: red & DV: green) for electrical stimulation, (c) flexible polyamide probes for muscle stimulation where probe tips target the specific muscle sites and (d) pupa with implanted muscle probe emerges into an adult with strong adhesion at probe-tissue interface.

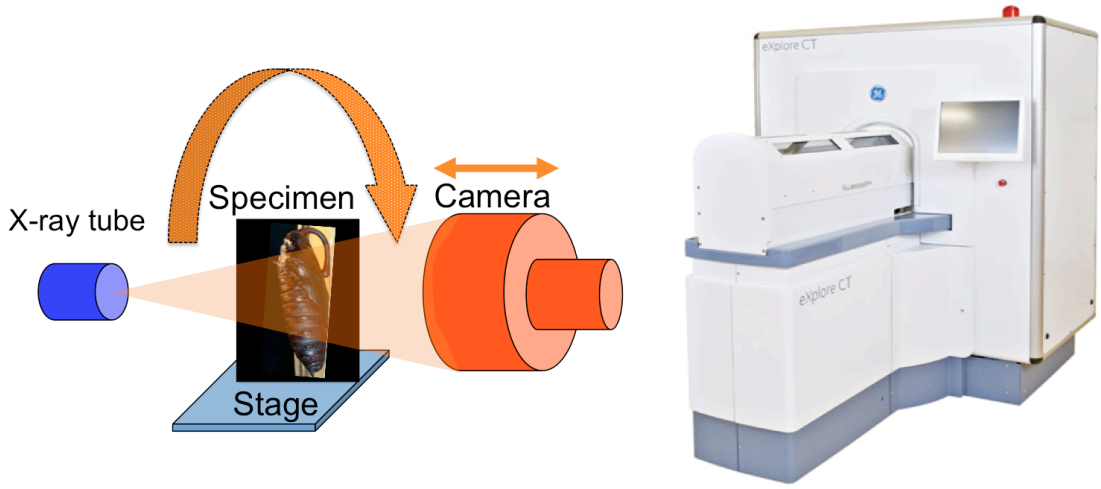
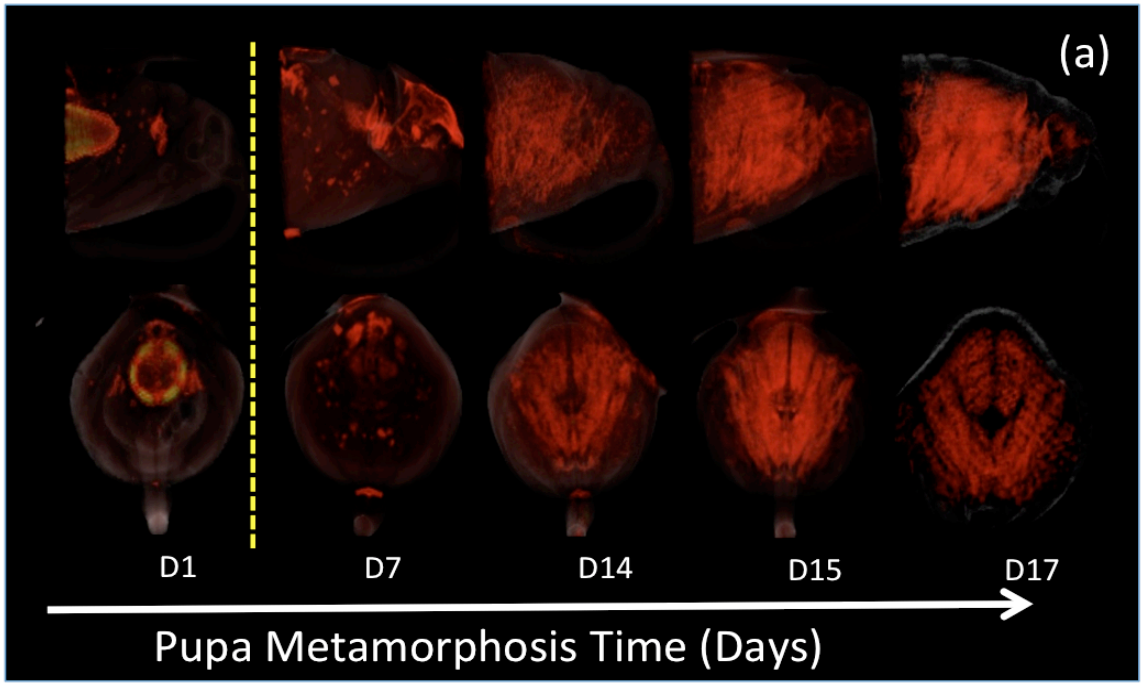


Figure 3.3: GE Micro-CT uses x-ray power of 50-120keV to generate peak spatial resolution of 25µm. The specimen is placed on a stage that is positioned in between the x-ray tube and the 360^{O} rotating camera.


(a)
D1
D7
D14
D15
D17
Pupa Metamorphosis Time (Days)

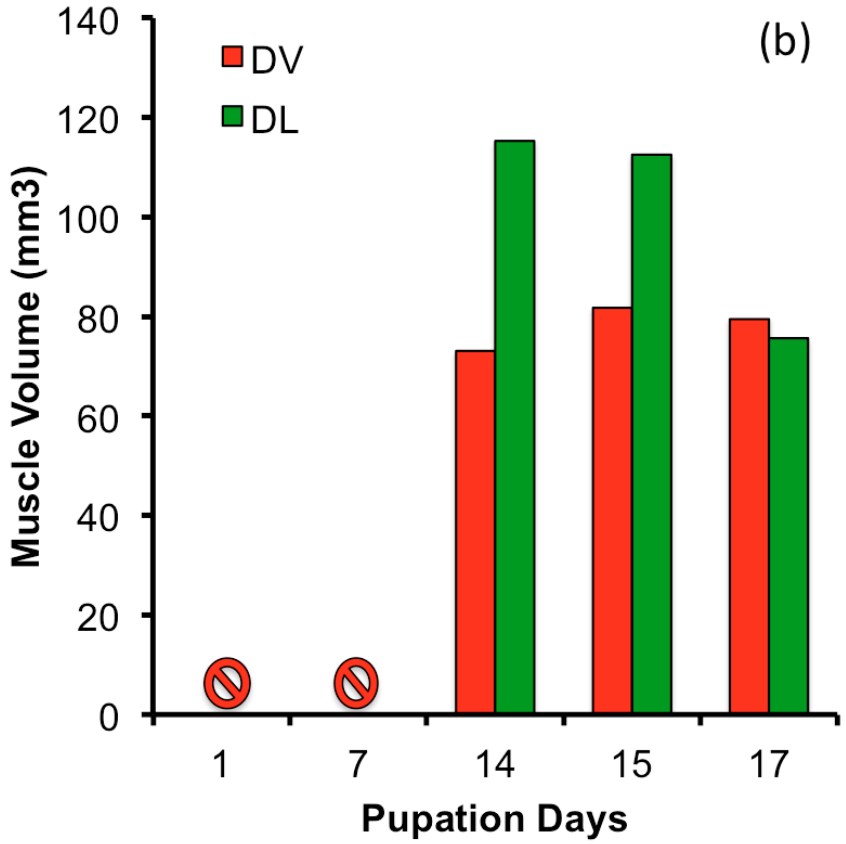
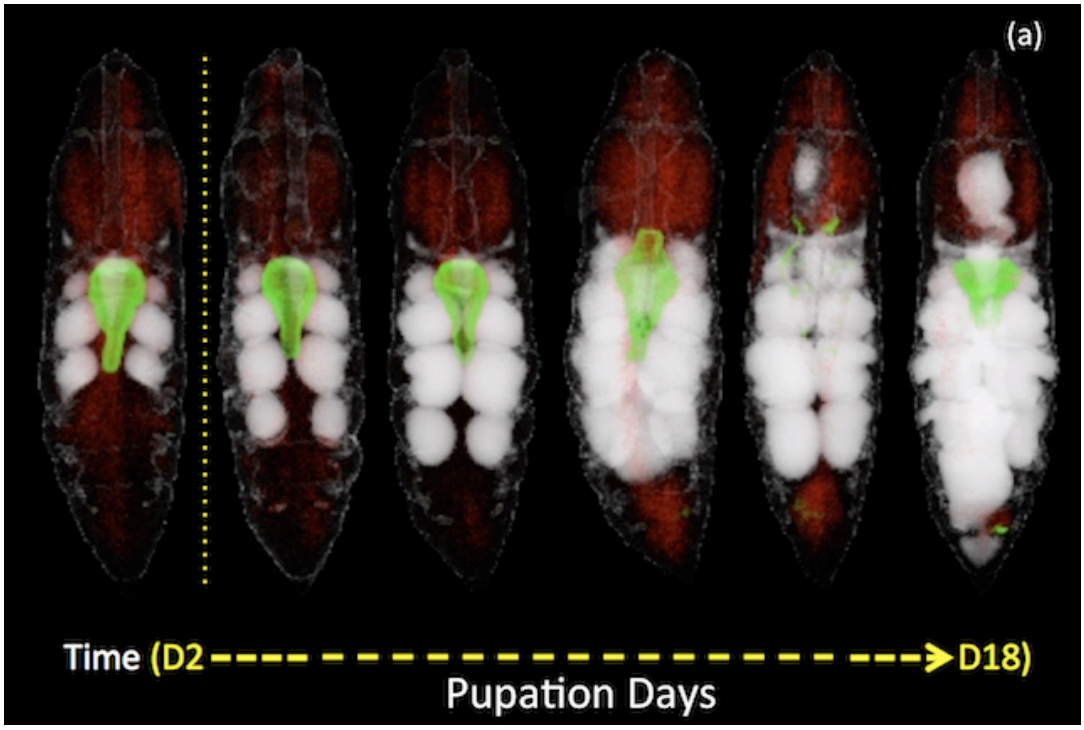

(b)
DV
DL
Muscle Volume (mm3)
140
120
100
80
60
40
20
0
1
7
14
15
17
Pupation Days

Figure 3.4: Development of thoracic upstroke (DV) and downstroke (DL) flight muscles during pupal metamorphosis. (a) *In vivo* micro-CT scans of *Manduca* pupa shows the appearance of the flight muscles at Day12 and clear tissue compartmentalization into DV and DL muscles at Day 14 and (b) DL muscles aggregate earlier than DV muscles and become more compact when the pupa is close to pupal eclosion.


(a)
Time (D2
D18)
Pupation Days

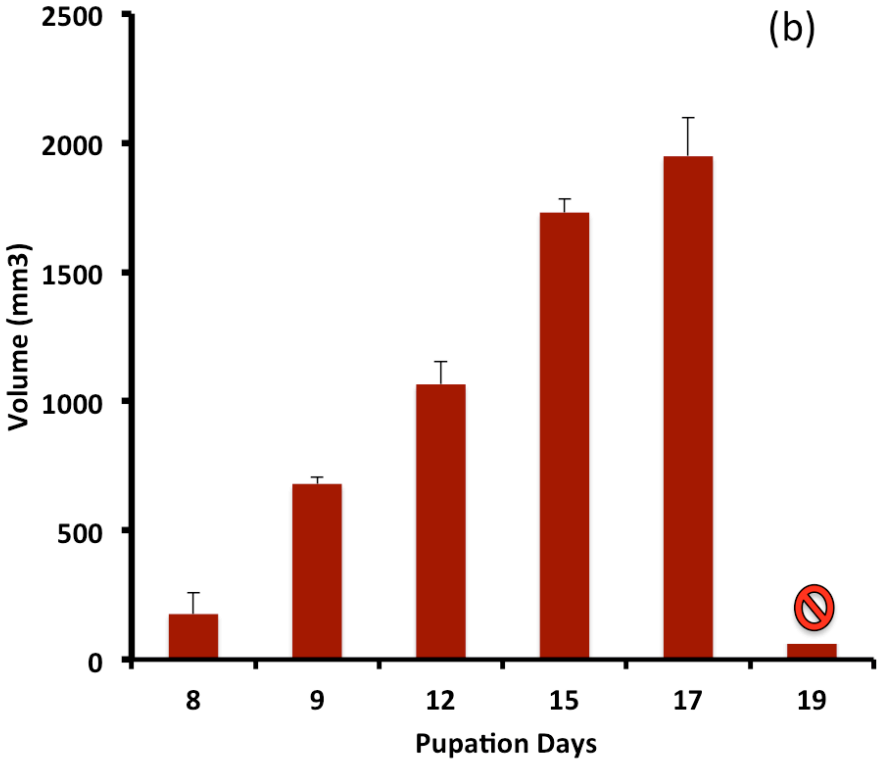
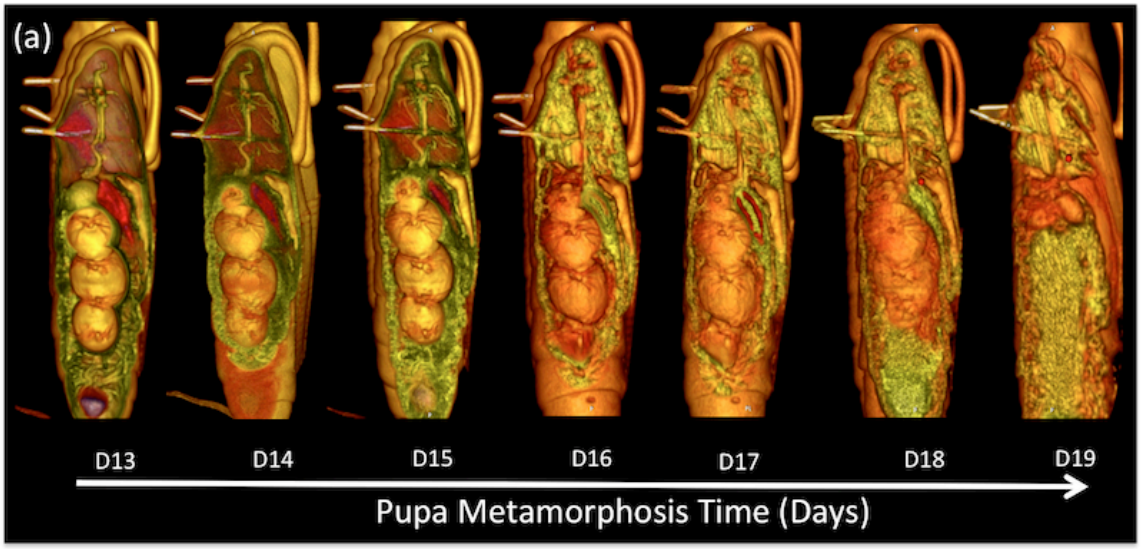

(b)
2500
2000
1500
1000
500
0
Volume (mm3)
8
9
12
15
17
19
Pupation Days

Figure 3.5: Development of the tracheal system, specifically airsacs, during pupal metamorphosis. (a) An *in vivo* scan of the pupal abdomen shows that the airsacs, a insect respiratory organ, continually develops during the metamorphic period and fills up the entire abdominal cavity and this results in an increase in the volume of the tracheal system (b) until Day 18 which is then followed by a sharp decrease in volume as the airsacs collapse a few hours before final adult emergence from the pupal shell.



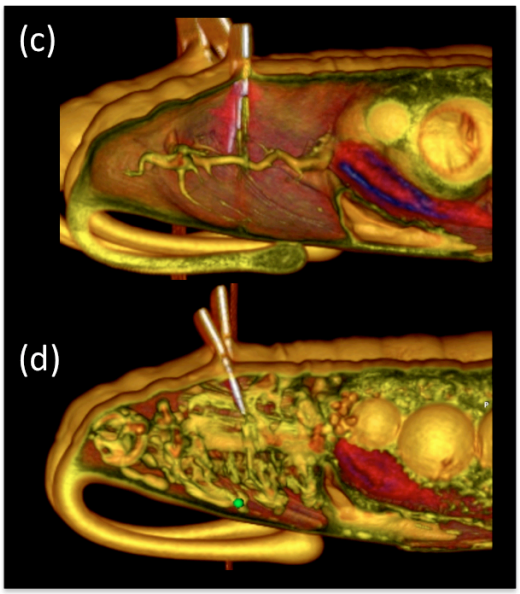


Figure 3.6: *In vivo* series scan of pupae (a) with PCB probe implanted at Day 4 of pupation where micro CT imaging enables us to determine and select multiple pupae (b) with correct placement and tissue integration on to the thoracic probes through time. (c) Shows an early stage pupa and (d) is the same pupa at a later developmental stage.

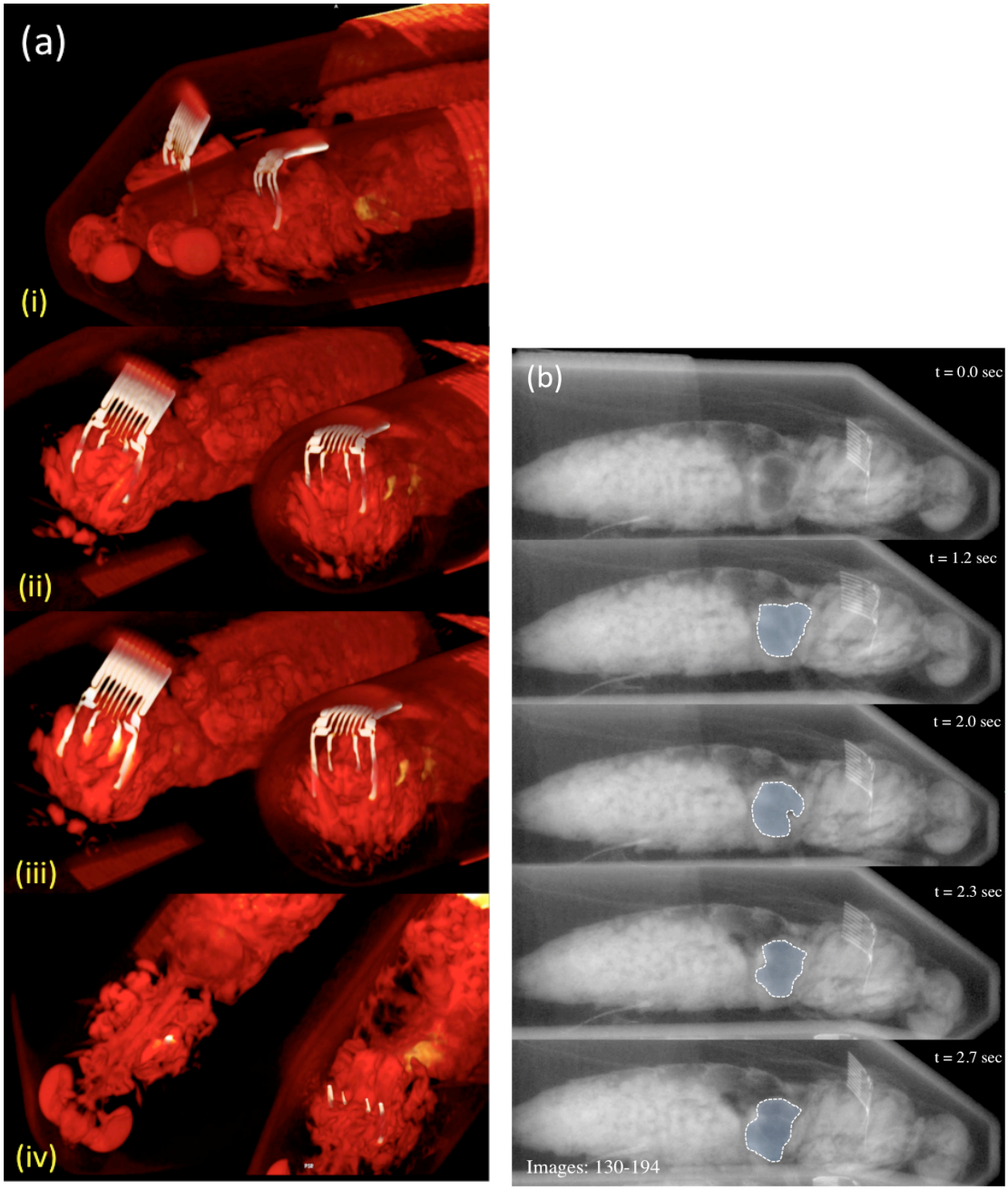


Figure 3.7: Successful emergence of an adult *Manduca sexta* moth after implantation of PCB probe at early pupal metamorphic stage where (a) slicing through the image stacks (i- iv) shows the correct placement of the active probe in the DV and DL

thoracic flight muscles and (b) shows a series of images of the adult moth with strong foregut (shaded) contractions.

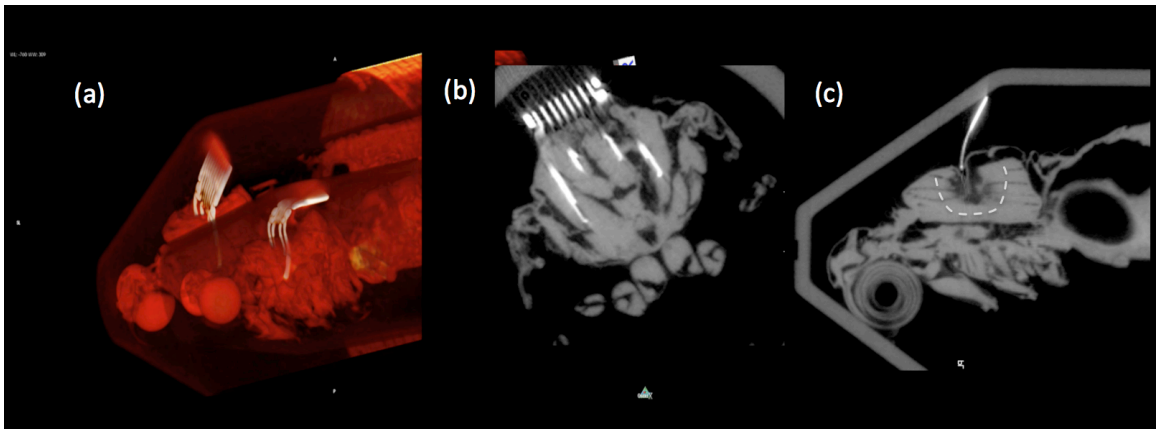


Figure 3.8: Micro-CT images of (a) fully functional newly eclosed adults with thoracic muscle implants inserted at Day4 of pupation, (b) virtual cross section of the thorax shows the accurate placement and stability of the active probes even after 3 days of adult emergence and (c) a longitudinal section of the scan of the same adults shown in panel (a) shows DL muscle damage (dashed line) after free flight in the vicinity of the probes.

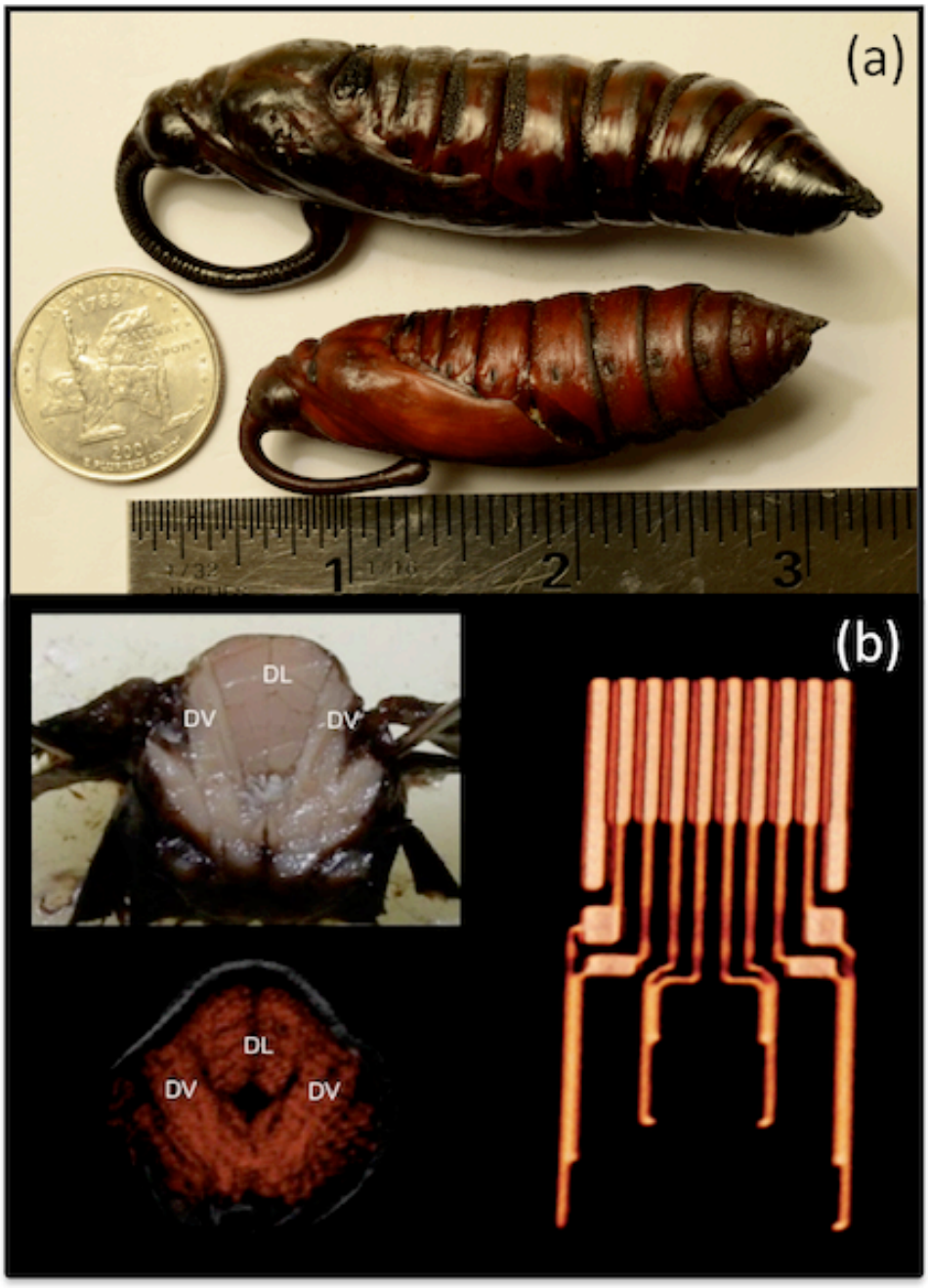


Figure 3.9: Moth pupae of varying sizes and across multiple species (a) can be scanned to determine the spatial dimensions of the target muscles via *in vivo* CT scans

(b) which in turn will aid us to design probes of specific dimensions for a custom fit, implantation and tissue adherence in order to generate active sentinels for research.

REFERENCES

Bozkurt, A, Gilmour, R.F., Sinha, A, Stern, D., Lal. A. (2009). "*Insect Machine Interface Based Neuro Cybernetics*". IEEE Transactions on Biomedical Engineering, 56:6, pp. 1727-33.

Cary, S.B., Moore, T.E., Conklin, T.A., Sukardi, F., and Kotiditschek, D.E. (1996). "*Insect Biorobotics: Electro-neural control of cockroach walking*". Abstract IEEE International Conference Robotics and Automation, Workshop WT3, Bio-Mechanics: pp. 42-54.

Chapman, R.F. (2012). "*The insects: Structure and function*". Cambridge University Press; ISBN: 978-0521113892.

Ellington, C.P., (1999). "*The novel aerodynamics of insect flight: applications to micro-air vehicles*". The Journal of Experimental Biology. Vol. 202: pp. 3439-348.

Greco, M.K., Tong, J., Soleimani, M., Bell, D., Schäfer, M.O. (2012). "*Imaging live bee brains using minimally-invasive Diagnostic Radioentomology*". Journal of Insect Science, Vol: 12 (89), pp. 1-7.

Kutsch, W., Schwarz, G., Fischer, H., and Kautz, H. (1993). "*Wireless transmission of muscle potentials during free flight of locust*". The Journal of Experimental Biology 185: pp. 367-373.

Kuwana, Y., Ando, N., Kanzaki, R., and Shimoyama, I. (1999). "*A radio telemetry system for muscle potential recordings from freely flying insects*". Proceedings of IEEEBEMS/EMBS conf., Atlanta, GA, Oct.1999: 846.

Lee, W., Socha, J.J (2009). "*Direct visualization of the hemolymph flow in the heart of a grasshopper: Schistocera americana*". BMC Physiology 9 (1), pp. 2- 12.

Louden, C. (1995). "*Insect morphology above the molecular level: biomechanics*". Annual Entomological Society of America 88: 1-4.

Menzel, R., Brembs, B., Giurfa, M. (2007). "*Cognition in Invertebrates",* in *Evolution of nervous systems,* J.H. Kaas, Ed. Oxford, England: Academic Press, Vol.II, Chapter 126, pp. 403-422.

Mohseni, P., Nagarajan, K. (2001). "*An ultralight biotelemetry backpack for recording EMG signals in Moths*". IEEE Transactions on Biomedical Engineering 48(6): pp. 734-737.

Paul, A., Bozkurt, A., Ewer. J., Blossey. B., Lal. A. (2006). "*Surgically Implanted Micro-Platforms in Manduca-Sexta"*, Solid State Sensor and Actuator Workshop, Hilton Head Island, June 2006, pp. 209-211.

Riley, J.R. (2005). "*The flight paths of honeybees recruited by waggle dance*". Nature 435(12 May 2005): pp. 205-207.

Riley, J.R., **Valeur, P., Smith, A.D., Reynolds, D.R., Poppy, G.M.** (1998). "*Harmonic radar as a means of tracking the pheromone finding and pheromone - following flight in male moths*". Journal of Insect Behavior 11(2): pp.287-296.

Rind, C.F. (1983). "*The organization of flight motorneurons in the moth, Manduca sexta*". Journal of Experimental Biology 102, pp. 239-251.

Rogers, J.A. (2010). "*Materials and mechanics for stretchable electronics*

". Science 327 (5973): pp. 1603-1607.

Vogel, S. (2001). "*Prime mover: A natural history of muscle",* New York, NY: W. W. Norton, ISBN: 0-393-02126-2.

Westneat, M.W., Betz, O., Blob, R.W., Fezza, K., Cooper, W.J., Lee, W. (2003). "*Tracheal respiration in insects visualized with synchrotron X-ray imaging*", Science, Vol: 299, pp. 558-559.

Williams, C.M. (1946). "*Physiology of insect diapause: the role of the brain in the production and termination of pupal dormancy in the giant silkworm Platysemia cecropia*". Biological Bulletin 90: 234- 243.

Wooton, R. (2000). "*From insects to microvehicles",* Nature, 403, pp. 144-145.

CHAPTER 4

EDUCATION CHAPTER 1:

DEVELOPING SCIENTIFIC INQUIRY AND MAKING CONNECTIONS IN BIOLOGY THROUGH LESSONS IN RESPIROMETRY AND HOMEOSTASIS

4.1 OVERVIEW

The goal of this curriculum was to introduce 10th grade Regents Biology students to the development of methods of bioinquiry, where the students would use the scientific method to integrate conceptual ideas in biology via experimentation and make connections among those ideas to form a working philosophy and in-depth understanding of the living world. This goal was achieved by examining the effect of changing environmental stimuli, such as temperature and humidity, on respiratory rate and how organisms compensate to maintain homeostasis on being exposed to such extrinsic challenges. I used the 5E learning cycle model (Bybee et al 1986, Bybee et al 2006): (a) engagement, (b) exploration, (c) explanation, (d) elaboration and (e) evaluation to organize my teaching curriculum, where the students used an invertebrate model, *Manduca sexta* larvae and pupae, for the study.

As an engagement activity, the students were first taught the concepts of respirometry in Module 1. During this activity, a real world example of changing environment, global warming, was brought to the students' attention so that they could correlate how various changes associated with global warming might affect invertebrate life cycle and eventually our entire ecosystem.

During the exploration activity, the students designed their experiment to quantify larval and pupal respiratory rates at three different ambient temperatures. The students also were exposed to two different observational tools for this bioinquiry lesson, i.e., two types of classroom respirometers, in order to gather the quantitative data and general qualitative data through observation.

The explanation section involved students analyzing the pooled classroom data to explain their understanding of respirometry and homeostasis, using the data from aforementioned tools and comparing the pros and cons of each method.

The elaboration section involved introducing the concept of homeostasis in Module 2. This module allowed the students to apply the concept of respirometry and homeostasis in context. By designing an experiment to determine the effect of temperature on larval development and pupal metamorphosis, they were able to build on their understanding of a dynamic environment and its subsequent effects on life forms. Their experience with experimental design during Module1 helped them further refine their experimentation skills. The students used standard methods of observation by recording the change in weight and size of larvae and pupae during the experimental period of three weeks. They were also given an internal view of the development of these life stages through MicroCT images, which they used in their final reports.

The students were evaluated using oral and written classroom quizzes for pre- and post assessment and a final report on their experiment. The final report was evaluated using a rubric that was specifically designed to grade the students based on their performance during the 5E learning cycle.

4.2 CLASSROOM IMPLEMENTATION

4.2.1 *Introduction and Background*

Cellular respiration is the controlled burning of food to release energy. This catabolic reaction can only occur at the cellular level when food is broken down by digestion into simpler elements such as sugars, amino acids and fatty acids. An organism functions seamlessly when its organs maintains a relatively constant chemical and physical condition, a homeostatic state, of its internal environment. Factors such as temperature, humidity, pH and photoperiod affect respiration in an organism. However, the natural tendency of an organism is to metabolically compensate for these changes in respiration in order to return to its stable state, where successful compensation leads to survival and failure to do so results in illness/disease or death.

The goal of this curriculum was to expose students to concepts of respiration, the effect of environmental factors on respiratory rate and the mechanisms by which organisms compensate to maintain homeostasis in a changing environment.

4.2.2 *Material and Methods*

4.2.2.1 Experimental Animals

Manduca sexta (Lepidoptera: Sphingidae) 2nd instar larvae were acquired from the Manduca Laboratory at Boyce Thompson Institute (Ithaca, NY 14853). These larvae were reared on artificial diet (Larval Diet, Carolina Biologicals Supply, Burlington, NC Larval Diet) at 26^{0}C, at 80% relative humidity, and a 12:12h light:dark

photoperiod. Healthy, similar size and weight larvae at 4th and 3rd instar were selected for the experiments on: (a) respirometry and (b) homeostasis.

4.2.2.2 Module 1: Respirometry

The respiratory system of insects is comprised of an extensive network of tracheal tubes (Figure 4.1). These tracheal tubes are conduits of gas exchange, where respiratory gases are delivered directly to tissues via convection and are exchanged from tissues via diffusion. Respiratory rates in insects vary based not only on the insect's life stage and metabolic activity, but also on the environmental conditions in which the organisms thrive (Contreras & Bradley 2010, Harrison 2012). Our experiment involved measuring respiratory rates of 4th instar *Manduca sexta* larvae and pupae at three different temperature treatments of 17^{O}C, 27^{O}C and 37^{O}C.

Respiratory rate is measured via respirometers. In this module we used two different kinds of respirometers, a glass tube manometer and a CO_2 probe. The glass tube respirometers were built in-house using a 25ml glass test tube, a size 6-rubber stopper, a 1ml disposable serological pipette (SigmaAldrich, St. Louis, MO) and glass marbles for weight (Figure 4.2). The idea was adapted from an educational website (Glass Tube Manometer). This respirometer measured the reduction in total air volume in the enclosed tube by consumption of O_2 and chemical absorption of CO_2 by KOH present in the manometer. The other respirometer used was a Vernier CO_2 gas probe (Vernier, Beaverton, OR), where the respired CO_2 was measured by the CO_2 sensor. The students were divided into groups of four, where each group was responsible for one temperature treatment.

4.2.2.3 Module 2: Homeostasis

This experiment involved observation of the effect of temperature on the developmental time of larval and pupal stages of metamorphosis in *Manduca sexta*. The 4rd instar larvae and pupae were subjected to similar temperature treatments as in the respirometry experiment, i.e., at 17^0C, 27^0C and 37^0C. The larvae were housed in 9x12" plastic chambers and were given access to food, ad libitum. The larval chambers were kept in (a) refrigerator set at 17^0C, (b) on the classroom laboratory bench for 27^0C and (c) in a chick incubator set at 37^0C for the three long-term temperature treatments. The data entry involved recording (a) the change in weight and length of larvae for two weeks, (b) the change in weight of pupae (primarily hygric loss of weight), (c) the time taken for the completion of these holometabolic life stages, and (d) the qualitative analysis of the internal metamorphic changes using MicroCT imaging and quantification of respiratory rates using a stop flow respirometer in the developing pupae during the experimental time-line of 3 weeks.

4.2.3 *Data Analysis*

The data gathered from all the groups for both modules were pooled together for data analysis, discussion and final reports. For the respiration module, the data from the Vernier measuring exhaled CO_2 and the glass tube manometer measuring consumed O_2 were used calculate the respiratory rates of the 4th instar larvae at temperatures of 17^0C, 27^0C and 37^0C. This data was also used to compare and contrast between the quality of data collected from the two types of respirometers. For the homeostasis module, the physical conditions, i.e., length, weight, morbidity and

mortality of the larvae and the pupae along with the time taken for development of each stage under the three temperature treatments, were recorded. The qualitative data for mortality was incorporated into the graph generated for the developmental time of each life stage at the different temperature treatments. The MicroCT data were used to visualize and qualitatively analyze the changes in the internal structure of the developing pupae. All the data were graphed using the NCES graphing website (NCES Graph).

4.2.4 *Outcomes*

4.2.4.1 *Module 1, Respirometry:*

Our respirometry experiments using the two classroom respirometers showed similar trends in respiration when larvae were exposed to three different temperatures. The glass tube manometer gave a measure of the amount of oxygen consumed in ml during the 5 minutes experimental time (Figure 4.3, whereas the Vernier gas probe detected the amount of carbon dioxide in PPM (Figure 4.4) released during the same experimental period. Respiratory rate including increased movement in larvae was the highest under hot treatment of 37^{O}C and lowest under cold conditions of 17^{O}C when compared to normal temperature of 27^{0}C (Figures 4.3, 4.4).

4.2.4.2 *Module 2, Homeostasis:*

Data for this module were gathered over a period of two weeks, as the students followed the insect's development through three larval instars and one pupal metamorphosis in parallel. The external morphology of the larvae, i.e., weight and

length, during this period of voracious feeding was expected and observed to be dramatic under normal conditions. Larvae at 17^{O}C showed the least amount of development and their growth remained stunted at the 3rd instar for the entire experimental period. Under the hot condition of 37^{0}C, the larvae grew faster compared to the larvae at 27^{0}C (Figure 4.5 & 4.6). Even though the larvae under 37^{0}C grew faster, 100% mortality was observed after observation Day 8.

The immediate morphological effect of temperature on pupal metamorphosis was not as apparent as was in the case of *Manduca* larvae. The weight of the pupae decreased during metamorphosis, primarily due to hygric loss from normal respiration (Harrison 2012, Levy & Schneiderman 1966a). The rate of respiration (Figure 4.7) and loss of weight (Figure 4.8) were higher in the pupae under 37^{0}C treatment when compared to pupae under 27^{0}C and 17^{0}C. Completion of metamorphosis and adult emergence under regular conditions was 19 days. This eclosion date was delayed under 17^{0}C (Figure 4.8) when compared to 27^{0}C.

Because the internal development of the organism could not be interpreted by general observation, the pupae were scanned on Day 3, 10 and 19 using a Micro-CT. The *in vivo* CT sections revealed the time line and intensity of adult tissue development in the pupae (Figure 4.9 & 4.10). The developmental of the tracheal network and the thoracic flight muscles during the first half of pupal metamorphosis was delayed at 17^{0}C and enhanced in the pupae under 37^{0}C when compared to 27^{0}C. Pupae at 37^{0}C had 0% emergence of adult moths, i.e., 100% pupal mortality, which corresponded with disintegration of thoracic muscle tissue and collapse of its tracheal system during the latter half of the metamorphic period.

4.2.5 *Interpretations*

During the respiration module, the students not only explored the basic concepts of respiration, but also had hands on and real life experience on how metabolism was regulated by extrinsic environmental factors. During this module, the students also learnt about the multiple types of respirometers, their limitations, their capacity and accuracy and the errors associated with each during data collection. During this module, discussion about endotherms and ectotherms served as a segue to the homeostasis module.

Most of the student groups had observed a delay in developmental time in the larvae at $17^{O}C$ and failure to molt into pupa and high mortality at $37^{O}C$. These observations led the students to design a scientific query to understand what occurs when a stable system is disrupted by changes in external environment. The main aim was to examine the long-term consequences of environmental changes on an organism, rather than the acute changes occurring over a snapshot of a few minutes. Failure in larvae to molt into a pupa, as well as stunted growth and high mortality, were directly correlated to changes in the treatment temperature. Based on their understanding of respiration, the students could conclude that the cause of high mortality in elevated temperature was high hygric loss and denaturing of essential proteins. Conversely, exposure to low temperatures stunted growth, as the organism could not reach its appropriate body temperature to carry out its metabolic activities.

4.2.6 *Assessment*

4.2.6.1 *Pre- and Post-Assessment*

Pre-assessment worksheets (see Supporting Material) were distributed at the beginning of each module, where the students were asked to describe their preliminary understanding of the subject. These quick response sheets were used to estimate the initial level of understanding on the subject and to concentrate on areas that lacked adequate understanding. Post-assessment worksheets primarily were comprised of the scientific report that the students were required to submit at the end of each module. These work reports, along with classroom participation, were graded on a rubric.

4.2.6.2 *Rubric*

The following rubric was used to assess students during each part of the activity. The term "expectations" refers to the content, process and attitudinal goals for a given activity. Evidence for understanding could be in the form of oral as well as written communication, both with the teacher and with other students.

The specifics of the rubric are listed in the table below.

1 = exceeds expectations

2 = meets expectations consistently

3 = meets expectations occasionally

4 = does not meet expectations

	Engage	Explore	Explain	Expand/Synthesis
1	Shows high level of interest in materials; asks relevant questions that demonstrate interest in the topic; helps other group members to	Takes over 15 measurements; measures features other than those suggested in the handout; clearly presents data and	Provides thorough and detailed answers to worksheet questions clearly	Is actively involved in summarizing the observations from the project; brings in real life examples and/or

	craft hypotheses to better explain the subject's underlying theory	creates clear graphs and/or tables; engages with partners in conducting the experiments	demonstrating comprehension of the material	applications to demonstrate understanding of the subject
2	Participates in filling out the objectives of the laboratory and contributes towards constructing the experimental design	Shows interest in the laboratory; takes at least 10 measurements; enters findings on the laboratory worksheet and conducts the experiment with group members	Participates in the discussion of the results	Participates in summarizing the project and discusses the observations
3	Participates in the group discussion with little interest; little effort addressing the objectives of the assignment	Demonstrates little interest in the experiment; minimal laboratory group participation; measurements (at least 5) and calculations may be written down, but with little interest	Participation during discussion is poor and demonstrates very little interest	Answers are simple direct observations
4	Does not participate in laboratory group activities and shows little interest in filling out lab objectives	Shows no interest in the experiment; enters little or no information in the work sheet and does not assist in group work	Does not participate during discussion of results	Does not summarize any experimental observations or results

4.3 ACKNOWLEDGEMENT

The development of this curriculum was supported through the Cornell BME NSF GK-12 program: DGE 0841291.

4.4 FIGURES

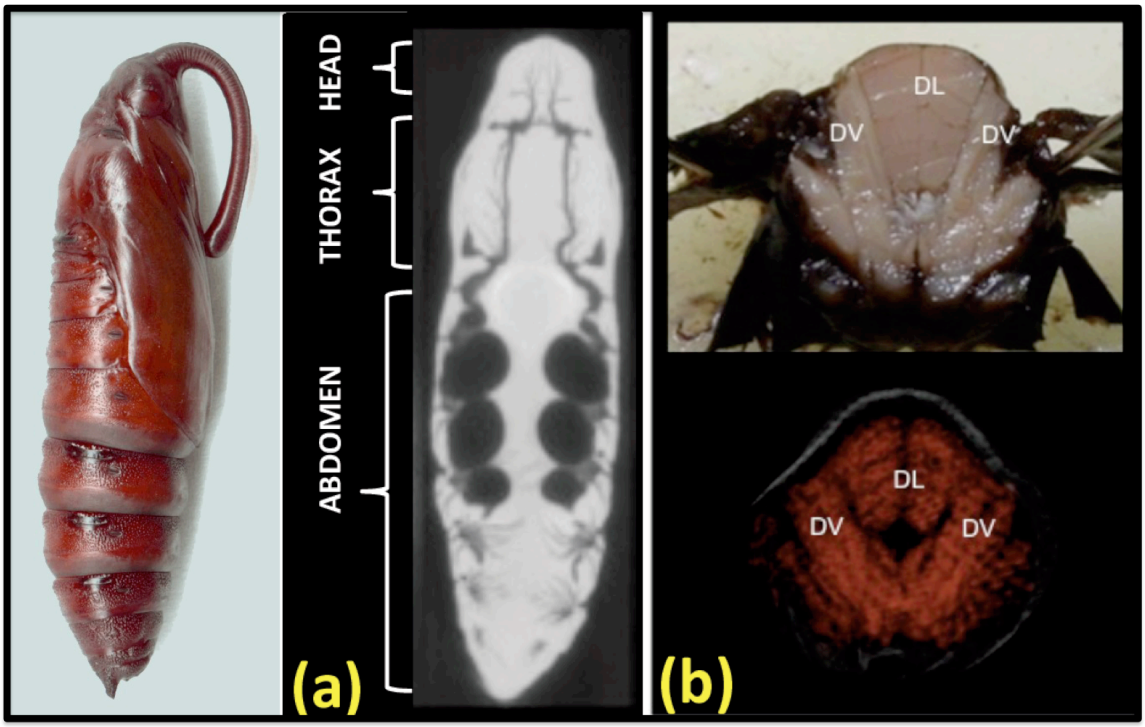


Figure 4.1: (a) Micro-CT scan (R) showing the respiratory system and compartmentalization of tracheal system in the head, thorax and abdomen in a *Manduca sexta* pupa (L). (b) Top - photograph showing the cross section view of the dorso-ventral (DV) and dorso-longitudinal (DL) flight muscles in the thorax; Bottom - micro-CT image of the same muscles.

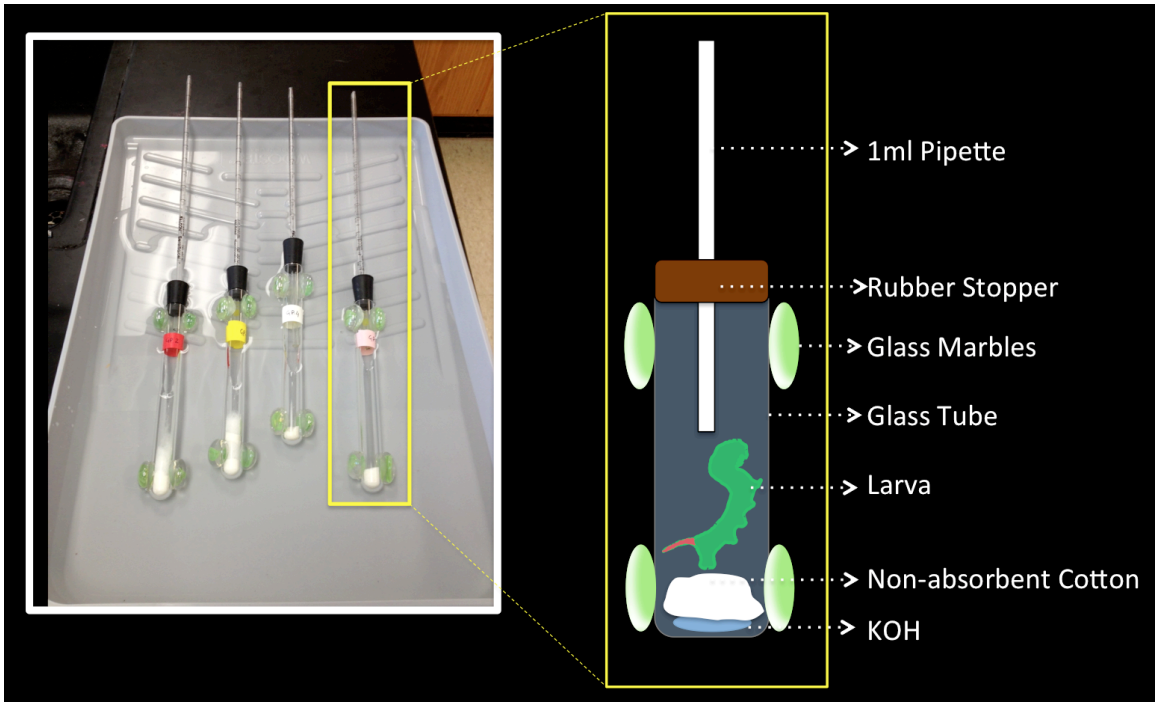


Figure 4.2: This in-house glass tube manometer was constructed using a glass tube, rubber stopper, 1ml disposable pipette and glass marbles glued on with hot glue for weight. 0.25ml of KOH was dispensed at the bottom of the manometer to absorb CO_2 and covered with non-absorbent cotton in order to protect the *Manduca* larva. This assembled manometer was immersed in paint trays filled with water of varying temperature during the respirometry experiments.

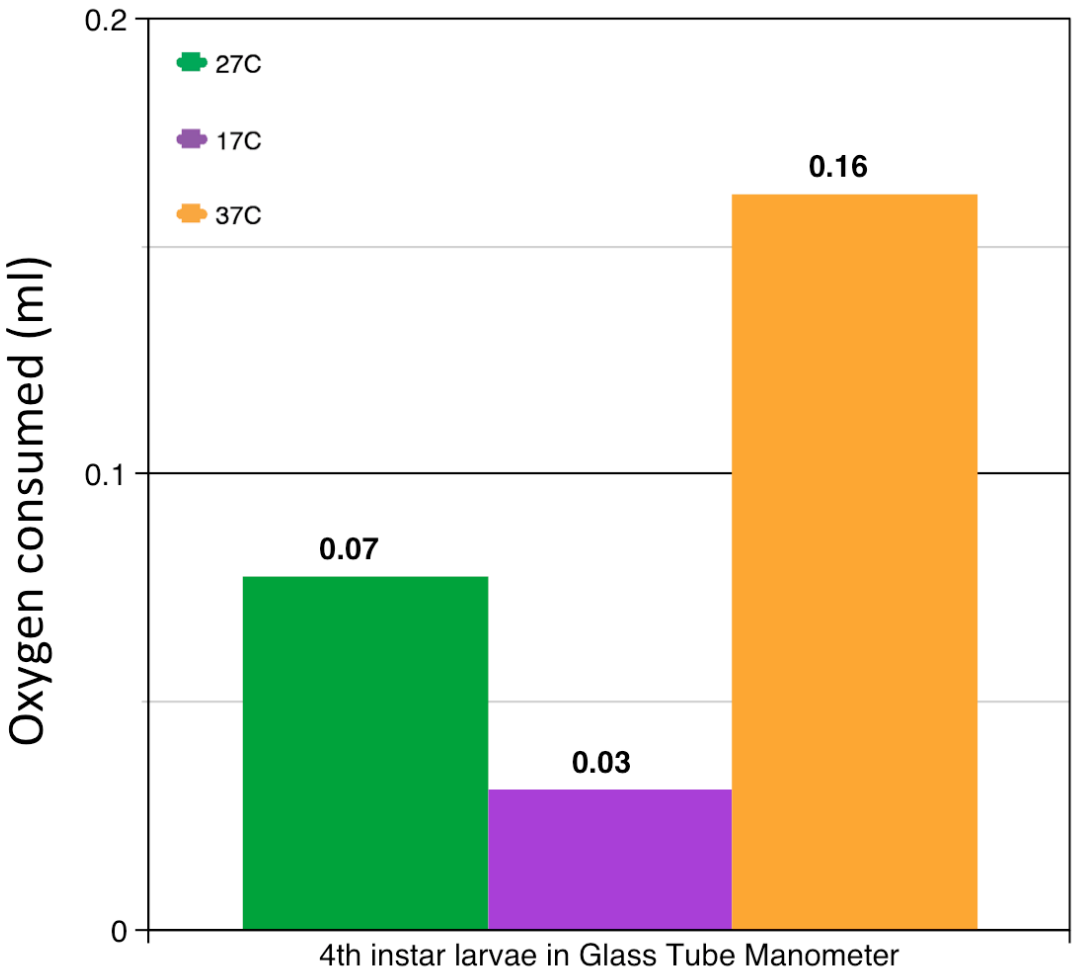


Figure 4.3: The respiratory oxygen consumed (ml) in the *Manduca* 4th instar larvae, was measured using an in-house glass tube manometer. The respiratory rate at hot temperature of 37^{O}C was almost double compared to normal room temperature of 27^{O}C. The respiratory rate at 17^{O}C was the lowest of all three temperature treatments.

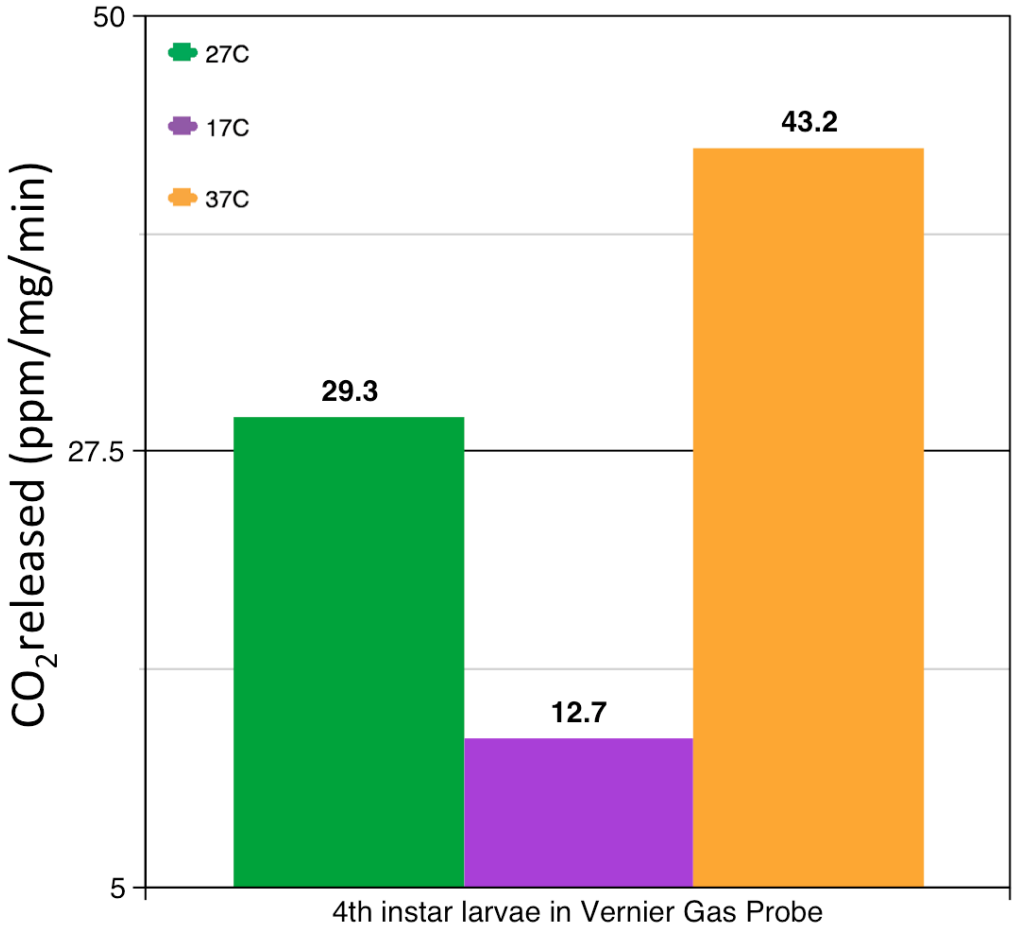


Figure 4.4: The mass specific rate of respiration in the *Manduca* 4th instar larvae, depicted by the carbon dioxide (ppm/mg/min) released, was measured using a Vernier CO_2 gas probe. The highest rate of larval respiration was recorded at 37^{O}C and the lowest at 17^{O}C.

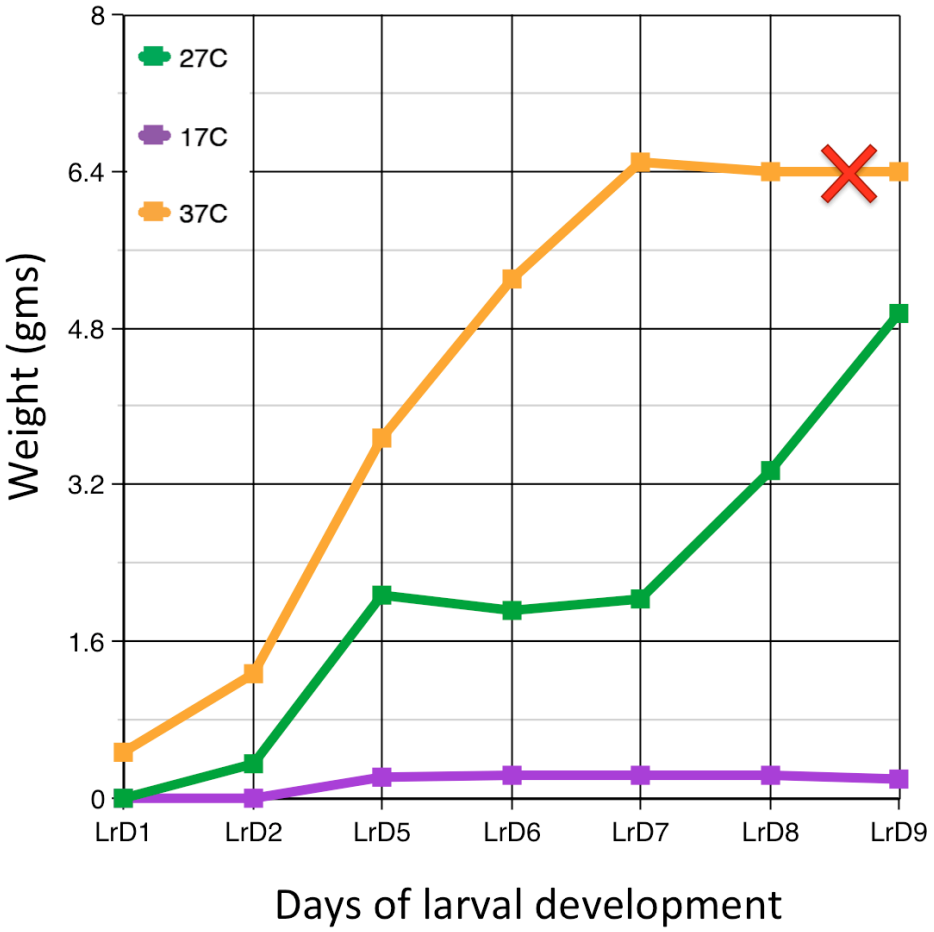


Figure 4.5: The larvae under hot treatment at 37°C gained weight faster compared to larvae at 27°C until Day 7. The change in weight of larvae at 17°C was negligible. Larvae that were alive after Day 7 at 37°C stopped feeding until 100% mortality was recorded at Day 9.

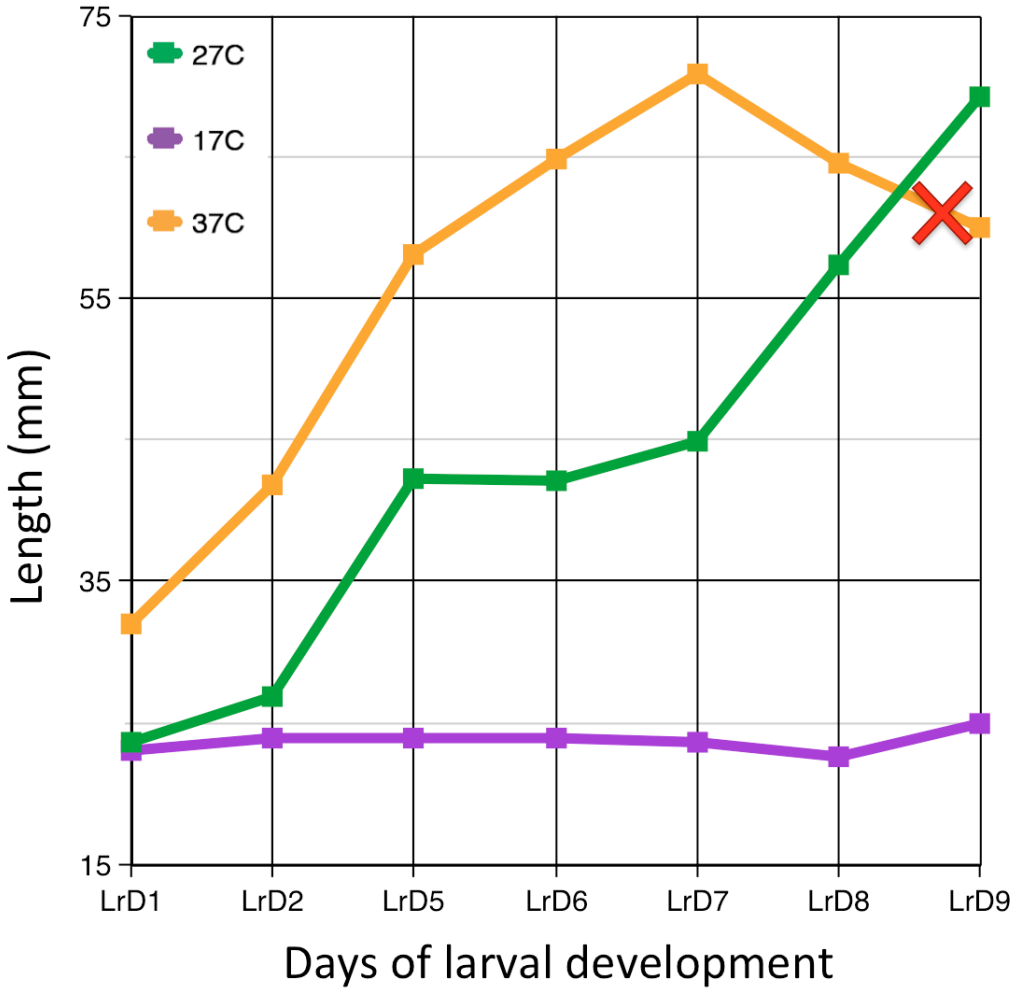


Figure 4.6: The larvae under hot treatment at 37°C showed rapid increase in length when compared to larvae at 27°C until Day 7. The growth rate was severely stunted under cold treatment of 17°C. The larvae under hot treatment started dying after Day 7 until 100% mortality was observed at Day 9.

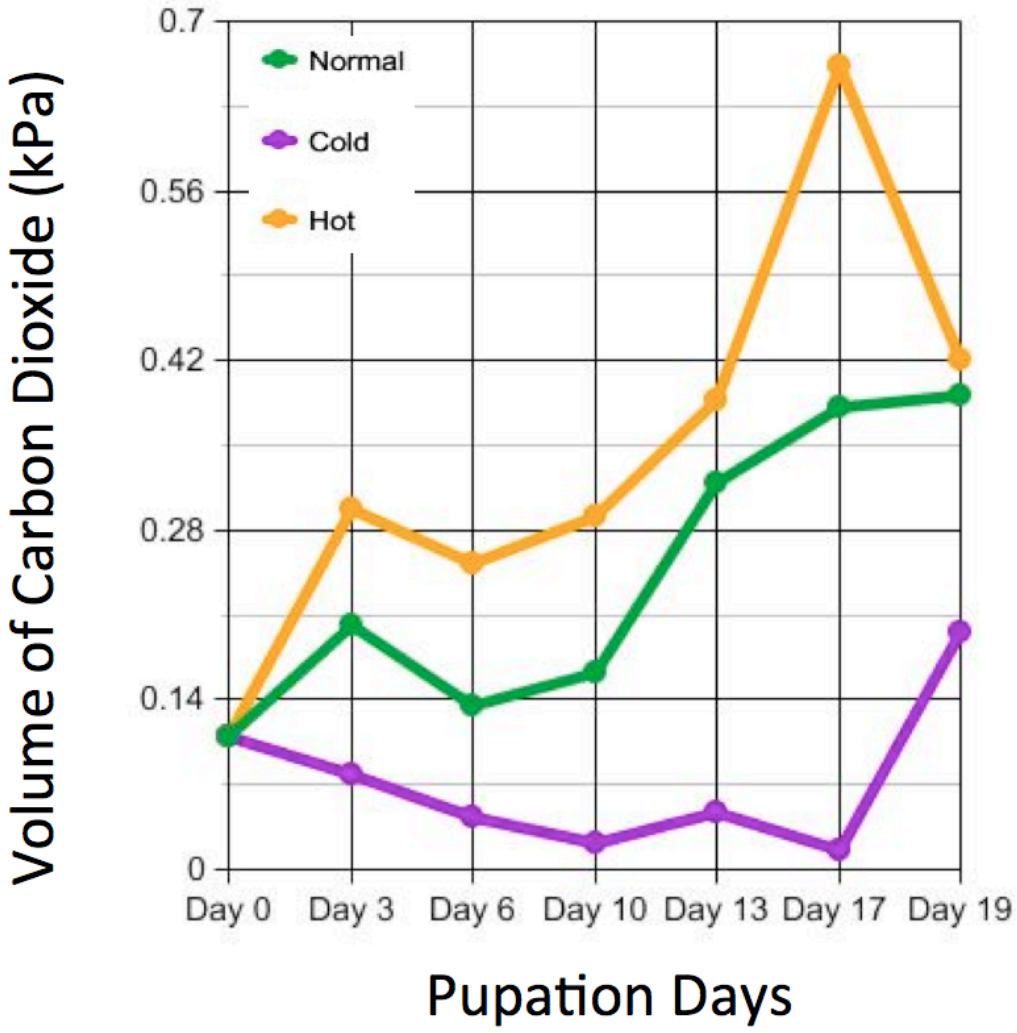


Figure 4.7: The rate of respiration during *Manduca* pupation, depicted by the rate carbon dioxide (kPa) released, was measured using a stop flow respirometer. The pupa at 37^0C respired at a higher rate (compared to normal conditions of 27^0C) until Day 17 of pupation, but failed to emerge as an adult and subsequently died at Day 19. At 17^0C the pupae respired at a lower rate compared to normal.

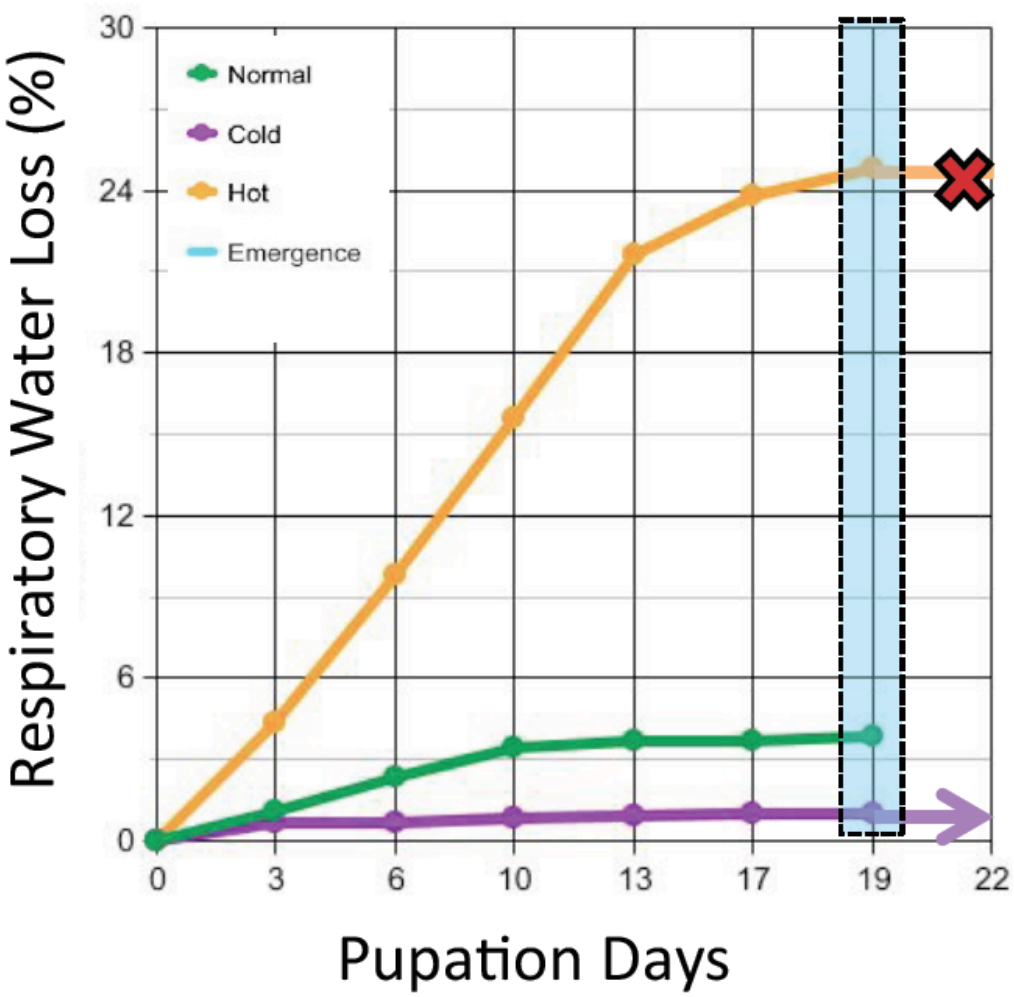


Figure 4.8: Percentage of water loss during pupation at three different temperature treatments. Day 0 is the first day when the 5th instar larva molts into a pupa. Pupae had the highest respiratory water loss under hot conditions at 37°C that proved to be lethal and resulted in 100% mortality. Under normal conditions at 27°C, the pupae emerged (blue box) on Day 19. The cold treatment at 17°C resulted in delayed development compared to the pupae at 27°C.

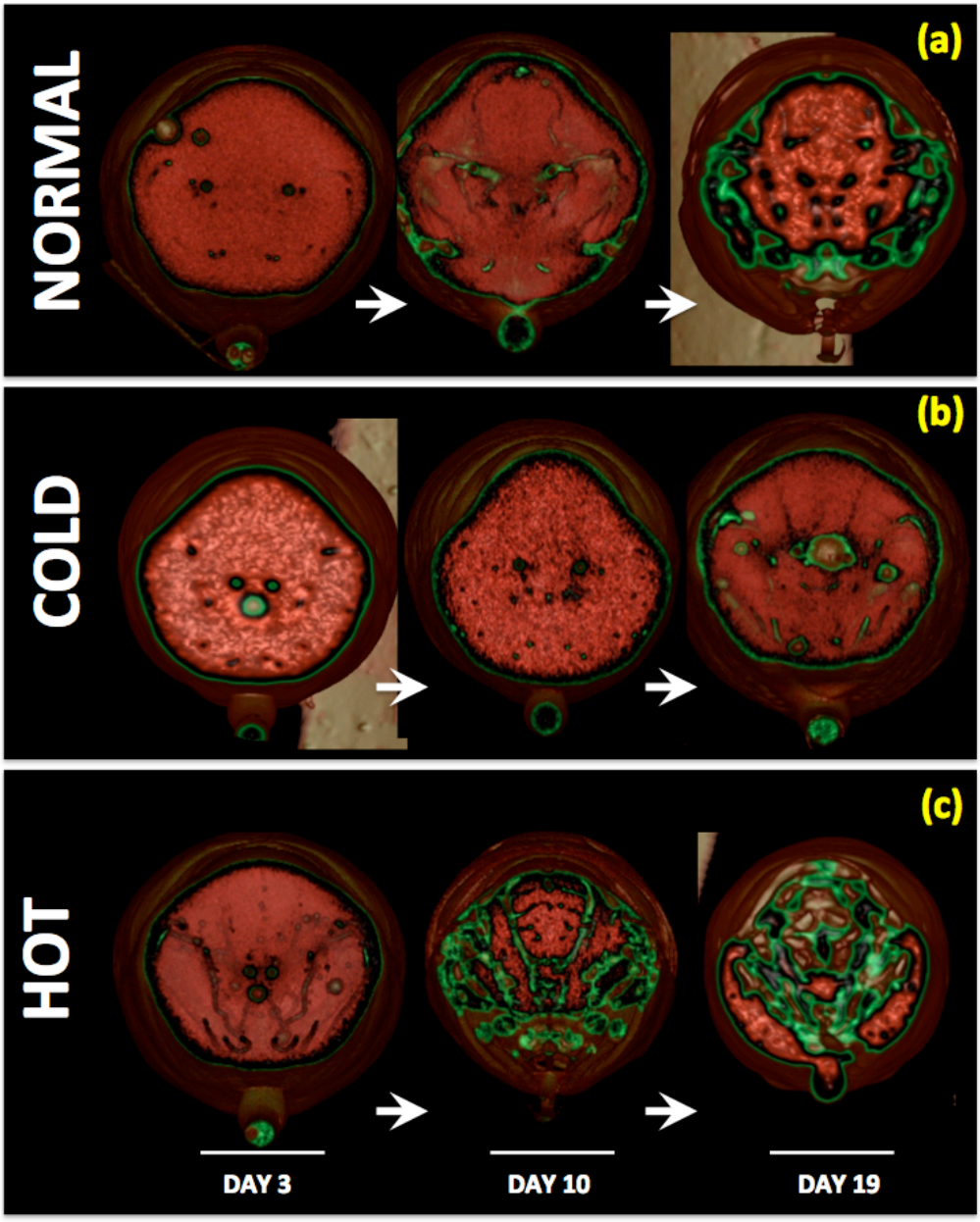


Figure 4.9: Micro-CT images of the cross section of a pupal thorax displaying the flight muscle compartment. (a) Shows the development of the flight muscles under normal temperature conditions of 27^{O}C, where the muscles start differentiating after Day10 of pupation. (b) Cold treatment at 17^{O}C shows delayed compartmentalization

of the flight muscles even at Day 19 and (c) the hot treatment at $37^{O}C$ shows that the muscle tissue differentiation starts earlier than normal (by Day 3), but the tissues start disintegrating as the pupa nears the eclosion date.

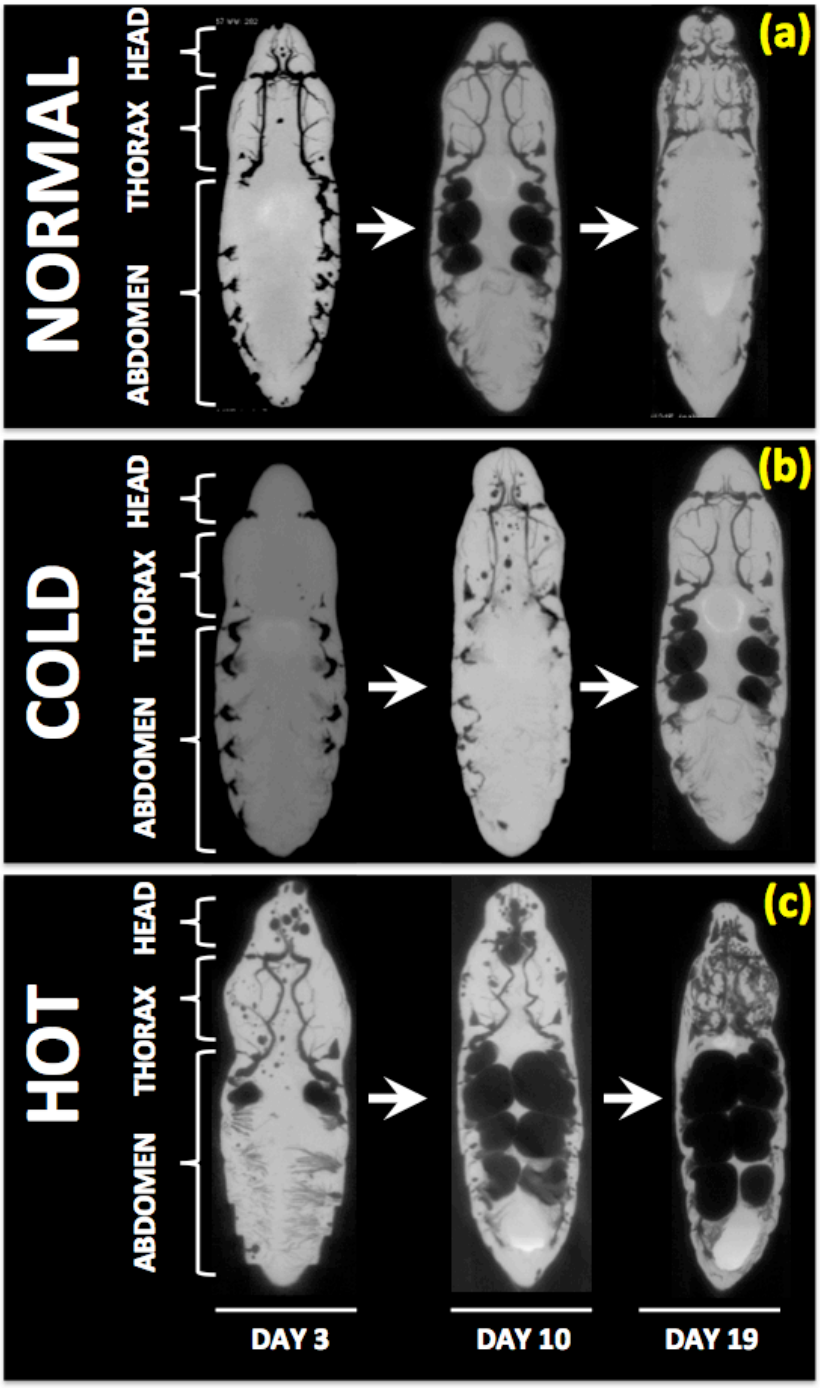


Figure 4.10: Development of the respiratory tracheal system in the *Manduca sexta* pupa when grown under different temperature conditions. (a) At normal temperature of 27^{O}C the pupa develops normally with increasing complexity in its tracheal system

and emerges into an adult at Day 19. The development is delayed when the pupa is raised under cold conditions of $17^{O}C$ and expedited when raised under hot conditions of $37^{O}C$.

REFERENCES

Bybee, R.W. et al. (1989). *Science and technology education for the elementary years: Frameworks for curriculum and instruction.* Washington, D.C.: The National Center for Improving Instruction.

Bybee et al. (2006) *The BSCS 5E Instructional Model: Origins and Effectiveness: A report prepared for the office of NIH.*

Contreras, H.L., Bradley, T.J. (2010): *Transitions in insect respiratory patterns are controlled by changes in metabolic rate*. Journal of Insect Physiology, Vol.56 (5), pp. 522-528.

Harrison, J.F., Woods, A.H., Roberts, S.P. (2012): Ecological and Environmental Physiology of Insects, Vol.3.

Levy, R.I., Schneiderman, H.A. (1966a): *Discontinuous respiration in insects. II. The direct measurement and significance of changes in tracheal gas composition during the respiratory cycle of the silkworm pupae*. Journal of Insect Physiology, 12 (1), pp. 83-104.

CHAPTER 5

EDUCATION CHAPTER 2:

LESSONS ON SCIENTIFIC INQUIRY AND METHOD BASED ON THE LARVAL FEEDING PREFERENCE AND LIFE CYCLE OF *MANDUCA SEXTA*

5.1 OVERVIEW

One of the biggest quests of our education system over the past two decades has been to teach children to be proficient in science. Being proficient in science involves developing the skill of acquiring knowledge in an evidence-based framework, where one generates one's own hypothesis on the subject matter and constantly extends, refines and revises their understanding with ever-evolving facts. Anyone proficient in science must have assimilated the art of scientific inquiry as a habit. According to National Academy of Science (NSE 1996), students K-8 should be able to:

1. know, use, and interpret scientific explanations of the natural world;
2. generate and evaluate scientific evidence and explanations;
3. understand the nature and development of scientific knowledge; and
4. participate productively in scientific practices and discourse.

Research in this area strongly indicates that we need to change our notions of what children, upon their initial entry to school, know and how they learn (Duschl 2007). In the past, science educators held the view that young children are concrete and simplistic thinkers, whereas present research shows otherwise (Duschl 2007). Children entering school already have a substantial knowledge of the natural world, much of

which is implicit and surprisingly sophisticated (Singer 2005). Children learn from available opportunities and are not restricted by their age or by the grade to which they belong. This rich but naïve understanding of the natural world, without appropriate and structured instruction of scientific concepts, may contradict scientific explanations and pose obstacles to the learning of science (Olsen 2000). It has been reported that previous methods of classroom teaching fall short at accomplishing this objective, as they focus on what students cannot do, rather than on what they can do.

Another topic of concern regards addressing the scientific method as a single list of steps that, when implemented systematically, generate reliable knowledge. The scientific method should be adopted by the student as a standard approach to a topic of interest or problem, an approach that involves an organized thought process. It is of utmost importance that when teaching science, teachers prepare a logically structured lesson plan where students get complete instructional support and opportunities for sustained engagement with the same set of ideas, based on a common theme over an extended period of time.

We developed the curriculum described below to teach a 6^{th} grade science class the systematic steps involved in rigorous scientific inquiry: PHEAC, i.e., problem identification, formulation of a hypothesis, experimental design, analysis of observations and derivation of a fact based conclusion, using an ecologically relevant example. The model organism used for this study was the moth, *Manduca sexta*, a known pest on tomato and tobacco crops. The curriculum involved generating lesson plans spanning over 4 weeks.

During the first week, the lesson plans aimed at introducing the model organism to the students. They learned its life history, habitat, ecological and agricultural impact, as well as its use in scientific research. This week was aimed at accruing knowledge on *Manduca* and developing a thorough understanding of its behavior and impact on people and the environment. During this time, students identified the problems or threats that this organism, at its larval stage, may pose. The second week's lessons instructed the students in how to formulate a hypothesis to address their problem, how to develop an experiment to test that hypothesis and how to identify dependent and independent variables in their experiment. All of these exercises were based around the central theme on *Manduca sexta* and its ecological impact. Finally, in weeks 3 and 4, the students conducted their experiment, pooled all their data for analysis to make an insightful interpretation of the data. Through observation and the subsequent description of a natural phenomenon, the life cycle and larval feeding preference of a moth *Manduca sexta*, the students came to discover and document patterns that led to a better understanding of how the environment around them functions.

5.2 LEARNING GOALS

For this exercise the students were expected to:

(1) Learn about the scientific method (PHEAC) via scientific inquiry, where they identify a potential ecological problem involving *Manduca sexta* larva as a pest and formulate a hypothesis on feeding behavior of the larvae.

(2) Design and execute an experiment using standard *Manduca* larval diet with and without food color and observe feeding preference of the larvae based on visual cues.

(3) Collect and pool class data, organize this data through graphs and tables, analyze it using simple statistical methods and interpret their quantitative results based on statistics and qualitative data through observation.

(4) Learn how to present scientific data by preparing a project report. Students may also use Powerpoint, Prezi or Xtranormal to present their understanding of the project to the class.

5.3 BACKGROUND INFORMATION, INITIAL OBSERVATIONS AND INVESTIGATION

The students at Boynton middle school, Grade 6, were introduced to the *Manduca sexta* moth, through a handout (Supporting Material 2) that discussed the natural history of the moth and they watched a movie on how the *Manduca* moth has been used in research (Manduca Flight Research Video). These activities established a background for the project, after which the students were informed, through a presentation, that naturally occurring *Manduca*, in their larval form, are known pests on tobacco and tomato crops. During this lecture, the students were introduced to concepts such as metamorphosis, organogenesis and the holometabolic life cycle of the moth. They also discovered in parallel that these moths have been used extensively in scientific research to study neural physiology (Riffell 2013) and flight dynamics (Dyhr 2012) and that they also have been used to create BioBorgs (Bozkurt 2009). A

terrarium to house a pair of male and female moths, their eggs and larvae, along with a tobacco plant was set up in the class. This display promoted daily interest among the students, as they would check on the status of the housed organisms as they entered the classroom.

Dealing with the live specimens gave the students a solid understanding of a larva's diet, its foliage foraging intensity and its economic impact on our agricultural crops. In addition, we showed the students a time-lapse video of the larvae feeding on a tomato fruit and tomato leaves, which demonstrated to the students that the larvae become voracious feeders during the third and the fourth instar. The students also observed that during this stage the larvae gained their maximum weight and body size.

Exposure to the live specimens prompted the students to ask if these insects were generalist feeders or whether their feeding was limited to tomato and tobacco plants. Some students were curious about the moth's habitat and whether the moths were found in upstate New York. Since we had these live creatures in the classroom, many students had formed an attachment to the larvae that they were taking care of and wondered if they were allowed to take home some larvae after our class was over.

In order to answer these questions, we posed a scenario in front of the class. With all the background information gathered about these moths, would it be advisable to release these eggs, larvae or adult moths into our environment? The class was asked to think of our habitat and the crops that are economically important, such as fruit trees - apples, grapes, peach and berries. They also were asked if their parents would agree to release these insects, especially if they had a vegetable garden.

5.4 HYPOTHESIS FORMULATION AND EXPERIMENTATION

Thinking through the questions posed to the students led them to investigate the potential of these insects becoming a common crop pest in the area. The class primarily wanted to investigate if the insects sought out their food via vision, so they designed a feeding preference assay with different colors of diet. The students were reminded of the mnemonic PHEAC and for an additional fun-filled activity they were shown a rap music video (Scientific Method Mr. Lee Song) that emphasized the steps of the scientific method.

With the problem to solve at hand, the next step was to develop a hypothesis – which we emphasized was very important in order to design a good scientific experiment. During the formulation of a hypothesis, the class came to understand the importance of having control variables as the baseline behavior. The diet variables were used to teach the class about dependent and independent variables (Supporting Material 2). Once these concepts were solidified, we set up the feeding preference assay, as described in detail in the Supporting Material 2.

5.5 ACTIVITY MATERIAL

- *Manduca sexta* 2nd instar larvae
- *Manduca sexta* larval diet (Carolina Biological Supply)
- Food color (Green, Blue and Red)
- Larval nursery kit (glass vial, sponge stopper, plastic stick, plastic cap)
- Glass tube rack
- Plastic tray

- Weight balance
- Measuring tape
- Examination gloves
- Hand sanitizer

5.6 TEACHER PREPARATION

The larval diet for the feeding assay was prepared a day ahead. The food dye was mixed when the diet was still hot and in a liquid state. For this experiment, 0.5ml of food dye was added to 300ml of diet, which generated twelve cups of diet (25ml) of each color (Figure 5.1). The diet cups were stored in the refrigerator, covered in a plastic bag, when the agar had cooled down to room temperature and had completely set. The diet cups could be stored up to 2 weeks. The *Manduca* eggs, once hatched, were maintained on a small piece of larval diet until the larvae reached their second instar stage in approximately seven days time. Details on identifying *Manduca sexta* larvae can be found at the Manduca project website. Second instar larvae of approximately similar size and weight were selected and kept separately. These larvae were ready to be used next day in the feeding preference assay. The handout (Supplemental 5.3) was distributed to the students at least one day before the experiment started.

The students were divided into groups of at least two. The three experimental colors and the colorless control diet in the assembled larval nursery (Figure 5.2) were distributed. Each laboratory bench had the second instar larvae set aside to be sorted

out and distributed to the groups once everyone was ready to begin the experiment. The temperature and humidity of the room were also recorded.

5.7 GROUP WORK AND RESULTS

After each group was assigned their choice of diet colors, they measured the weight of their diet cups, the weight and the length of the larva. They repeated their observations every alternate day for 10 days. During this time, the groups collaborated and shared their observations with each other (Harwood 2004). These discussions allowed the students to explore their results and the differing possibilities of unexpected results. The experiment concluded when the larvae went into the final larval molt. Once the experiment was completed, all the groups pooled their data into one datasheet and used this information for data analysis. The class was provided with an evaluation rubric that would help them to prepare a complete project report (Table 5.1).

After performing simple statistics on the collected data, results showed that the *Manduca* larvae showed almost equal preference for the blue and the green diet and very low preference for the red or colorless diet. Furthermore, the class observed cases of mortality in the red diet treatment group.

5.8 WRAP-UP DISCUSSION

The students prepared their respective reports, where they designed a cover page to illustrate their understanding of the *Manduca* project. Figure 5.3 (a & b) reflect their vision and inference on the question they had set forth to answer. Students understood the consequences if this animal was introduced in their environment. But

what initially caught them off guard was the preference for the blue diet color. Upon reflection and calculating the mean for their data with standard error, many addressed that there was no difference, statistically, between the preference for blue vs. green and that the color wavelength of blue and green was very similar and both may have been undistinguishable by the larvae. The reports also addressed the possibility of human error, where handling the larvae may have caused a change in behavior or may have introduced bacteria (or germs) making the larvae sick, and hence, unable to eat and grow.

Upon further introspection, many students concluded that vision may not be the guiding stimuli for feeding preference in *Manduca* larva and that perhaps feeding preference was determined either by olfaction or by taste. To further investigate the potential role of olfaction, the students were encouraged to examine the anatomy of the larvae under a magnifying lens and to determine whether the larvae had well-developed eyes for better vision, versus antennae for olfaction or developed mouthparts for taste. Since the students were unable to detect an antenna on the head capsule of the larva, they suspected that the larvae might have taste buds that enabled them to select their food. To elaborate on this insight, future experiments were proposed that involved formulating a new hypothesis to test whether the larvae chose their food by vision or by taste.

5.9 CONCLUSIONS

Overall, the purpose of this exercise was to familiarize 6th grade students with the steps of scientific inquiry and method and to provide them with an understanding

of how scientific research is conducted. The lectures and the hands-on experiment of the exercise revolved around a central theme where none of the concepts of scientific inquiry was kept abstract. The class also learned that answers to a scientific question constantly evolve and that they may need to refine their experimental design until they have sufficient data to arrive at a strong conclusion.

The students appeared to have thoroughly enjoyed the experience of conducting a hands-on scientific experiment with live animals. At the same time, they made connections in their knowledge bank regarding how our actions impact our environment. They also came to understand that every aspect of life and learning should be questioned and analyzed based on the facts at hand and that any missing information should be filled in by research and personal investigation.

5.10 ACKNOWLEDGEMENT

The development of this curriculum was supported through the Cornell BME NSF GK-12 program: DGE 0841291.

5.11 FIGURES

Figure 5.1: Larval diet cups of assorted colors.

Figure 5.2: Larval nursery set up with diet cup, glass tube, plastic stick and a stopper sponge.

Figure 5.3 (a): Examples of the cover page of reports submitted by the students.

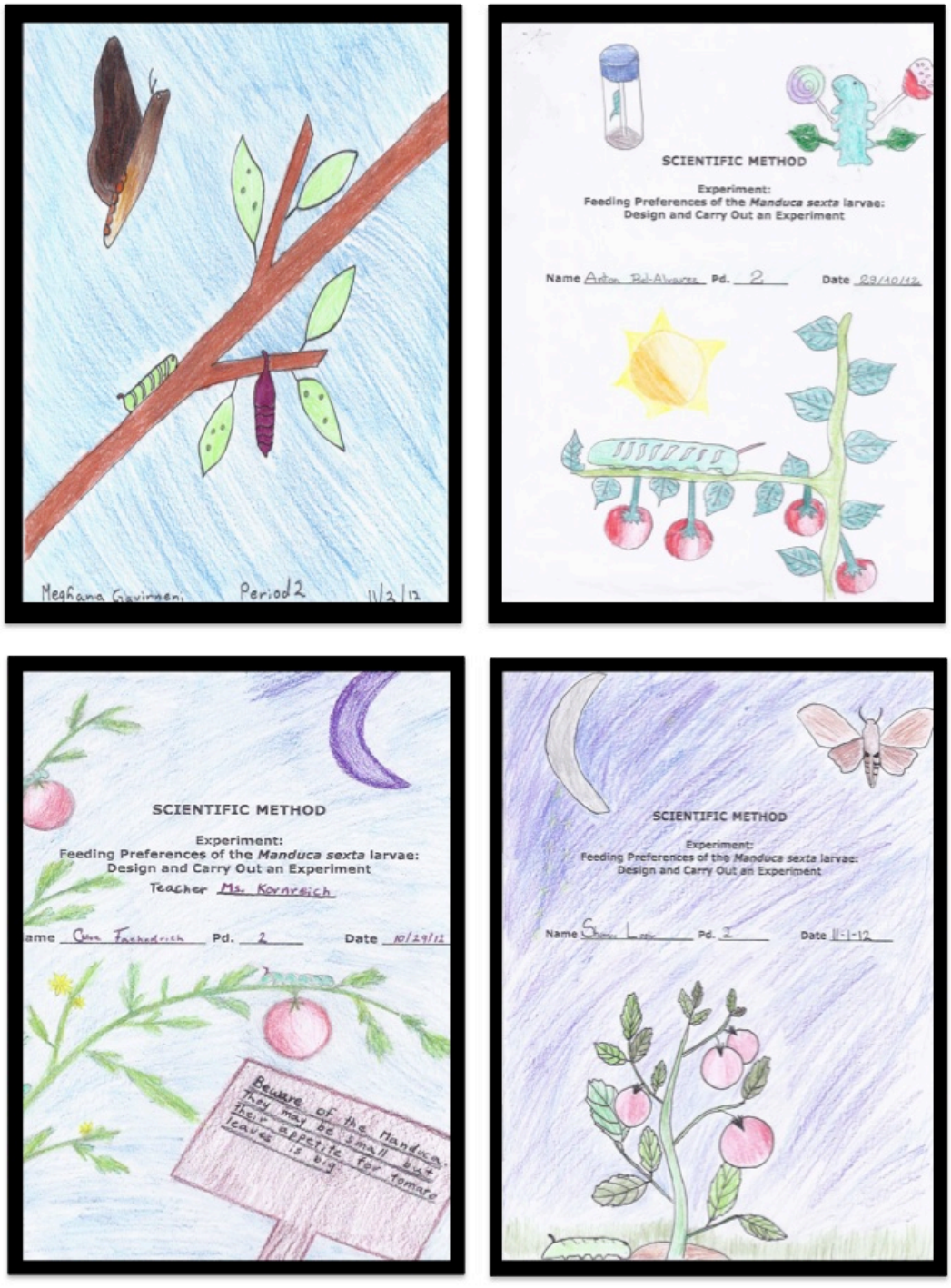


Figure 5.3 (b): Examples of the cover page of reports submitted by the students.

5.12 TABLES

Table 5.1: Rubric used for evaluating the laboratory report.

EXCELLENT! SHINES BRIGHTLY.	A JOB WELL DONE	MORE TIME AND/OR CARE WAS NEEDED?
1. Your cover page is extraordinary and includes all of the requested information. 2. The content of your report is relevant and interesting. 3. Your ideas are fully explained. 4. You expanded beyond the ideas from the guidelines. 5. You have well organized paragraphs. 6. There are no spelling or grammatical errors.	1. Your cover page is well represented and includes most of the requested information 2. Your ideas are sometimes supported and are generally informative. 3. You followed most of the guidelines. 4. Your paragraphs are generally well organized. 5. You have few spelling and grammatical errors.	1. Your cover page needs more planning and it is missing a lot of information. 2. Your ideas are limited and/or unrelated. 3. You did not follow the guidelines. 4. You have several spelling and grammatical errors.

REFERENCES

Bozkurt, A., Gilmour, R.F., Sinha. A., Stern, D., Lal, A. (2009). "*Insect Machine Interface Based Neuro Cybernetics*". IEEE Transactions on Biomedical Engineering, 56:6, pp. 1727-33.

Duschl, R.A., Schweingruger, H.A., Shouse, A.W. (2007). *Taking science to school: Learning and teaching science in grades K-8."* ISBN: 0-309-66069-6, National Academy Press, Washington, D.C.

Dyhr, J., Cowan, N.J., Colmenares, D.J., Morgansen, K.A., Daniel, T.L. (2012) "*Autostabilizing airframe articulation: animal inspired air vehicle control*". IEEE Proceedings of the Conference on Decisions and Controls (CDC).

Olsen, S., Loucks-Horsley, S. (2000). *Inquiry and the National Science Education Standards: A guide for teaching and learning."* ISBN: 0-309-51895-4, National Academy Press, Washington, D.C.

Harwood, W.S. (2004). *A new model for inquiry: Is the scientific method dead?* National Science Teachers Association (NTSA). Journal of College Science Teaching, Vol. 33, No. 7.

Singer, S.R., Hilton, M.L., Schweingruger, H.A. (2005). *America's Lab Report: Investigations in high school science",* ISBN: 0-309-65286-3, National Academy Press, Washington, D.C.

Riffell, J.A, Lei, H., Abrell, L., Hildebrand, J.G. (2013). "*Neural basis of a pollinator's buffet: olfactory specialization and learning in the Manduca sexta moth."* Science 339, pg:200-204.

National Science Education Standards (1996), National Academy Press,

Washington, D.C.

CHAPTER 6

CONCLUSION AND FUTURE DIRECTIONS

Insects without doubt are the most important heterotrophs in the terrestrial ecosystem. The importance of their presence not only revolves around them being active as pollinators, agents for nutrient transfer through ecosystem or as pests but also passively as aids to understand ourselves better. We have common ancestry with insects about 600 million years ago and almost half of our genes are orthologous to each other. This commonality not only helps us to provide antidotes by using insects as models for biological research but also poses challenges in designing insect-specific insecticides. Insects, like us, respond to environmental shifts by modulating their physiological responses. These responses usually have a faster turn around due to the short life span of the model insect and allows for more experimental replication resulting in robust conclusions. Diversity among insects allows for better comparative studies. Also, using insects as models for physiological studies has less ethical complications and is preferred by many scientists. The bottleneck in using insects to study physiological responses has been its size. But miniaturizing sensors-actuators and development of high-end imaging techniques have proven to be a big step towards overcoming these scaling problems.

Diagnostic radioentomology using phase contrast x-ray imaging, x-ray tomography and MRI is fast becoming a popular method to study insects of varying sizes and complexities. These imaging tools, specifically Micro-CT not only have the capacity to study morphology in 3D but, as shown by my research, can also be used to

investigate active physiological and developmental changes in an insect of cm-scale. Advancement of this imaging technology in combination with others such as PET and MRI can proceed to answer questions that many environmental physiologists have struggled to visualize *in vivo*. Questions such as morphological coupling and interaction between the respiratory and the circulatory systems of an insect, modulations in the same due to environmental, behavioral, metabolic or developmental changes may now be possible using this scale-appropriate technology. My research using Micro-CT imaging shows that metamorphic development of the thoracic flight muscles and the respiratory system in *Manduca sexta* pupa occurs in tandem. These findings, through 4D, time lapse and fluorescence CT imaging, also reveal the dynamic development of the respiratory organ, airsacs, in the pupal abdomen.

My experiments on spiracular occlusion reveal that spiracular conductance plays an important role in metamorphosis where partial or complete occlusion results in delayed developmental time. This result puts forth the function of this poorly studied respiratory organ in *Manduca* pupa. Further comparative research using CT imaging across Lepidopteran species and other insect families will reveal the presence and function of this crucial organ and may prove as an important tool to investigate the effect of environmental shifts in these organisms.

From the temperature treatment experiments on *Manduca* pupa we have seen that a change in environmental conditions *always* produces a change in its internal state. Respirometry experiments showed that instantaneous change in temperature led to passive buffering and behavioral changes in both *Manduca* larva and pupa. Long term

exposure to the same changes in stimuli resulted in delayed development, altered metabolic rates and even caused mortality. Future experiments using the same imaging and respirometric technology over extended periods of time and generations may reveal the developmental plasticity, specifically in metamorphic development of the respiratory system. This could be used to answer questions on adaptation and macroevolution in insects. These studies will not stay limited to answering the effect of environmental variation on the insect's physiological processes and systems in a single life stage but can easily extend over its complex and multiple life stages- four stages in a holometabolous and three in a hemimetabolous insect. Combining similar studies with genetic assays and study of the insect's transcriptome one can determine the genes that are responsible for normal development of the insect as well as changes in genetic expression when exposed to varying environmental and sensory stimuli.

In order to construct an efficient insect biobots my research used the findings on the time line of pupal metamorphosis, using Micro-CT, as the benchmark to implant MEMS probes for secure tissue integration. But to deploy a viable sentinel by overriding its innate behavioral response requires thorough knowledge of the insect's behavioral and physiological plasticity. It is still not clearly understood as to what behavioral mechanisms allow insects to buffer physiological stress and how this response varies at the intraspecies level? The approach towards solving this problem in the HIMEMS project has been to target and actuate the neural network rather than large muscles. But the nervous system of an insect is far more delicate and prone to damage than the robust musculature. The metamorphic development of the adult neural network could not be deduced using Micro-CT due to lower resolution

capability (25μm). Nano-CT with 2μm resolution offers a strong possibility to overcome this hurdle and may help visualize the changes in neural topography. This new information may provide us with a developmental time-line and present a window of opportunity to implant neural probes into the insect with minimum damage.

The GK-12 program introduced me to the avenue of teaching inquiry-based science in middle and high schools. This opportunity presented itself as the perfect stage to apply my research and understanding of the biological sciences in a novel way where student's curiosity, on topics relatable to day-today life, could be built and answered through critical observation and simple inexpensive experimentation. In order to do this, the program primarily aimed at building a solid foundation on teaching the students the methods of scientific inquiry. From my time spent with the students, I observed that examples of animal diversity compounded with the presence of live organism in the class and observing the dynamic changes in it created genuine enthusiasm among the students. This led them to research more, independently, on the biology of the subject. But in biology use of animal specimens leads to a lot more waste than acquiring new knowledge. So as a future direction towards building a strong educational tool, a virtual database comprising of 3D scans of a diverse collection of organisms is a must. This collection of virtual images will allow students to not only observe and study the diversity of organisms on this planet but will also allow then to virtually *dissect* them for detailed study without actually destroying a specimen. This effort would eventually contribute to preserving the biodiversity of this planet.

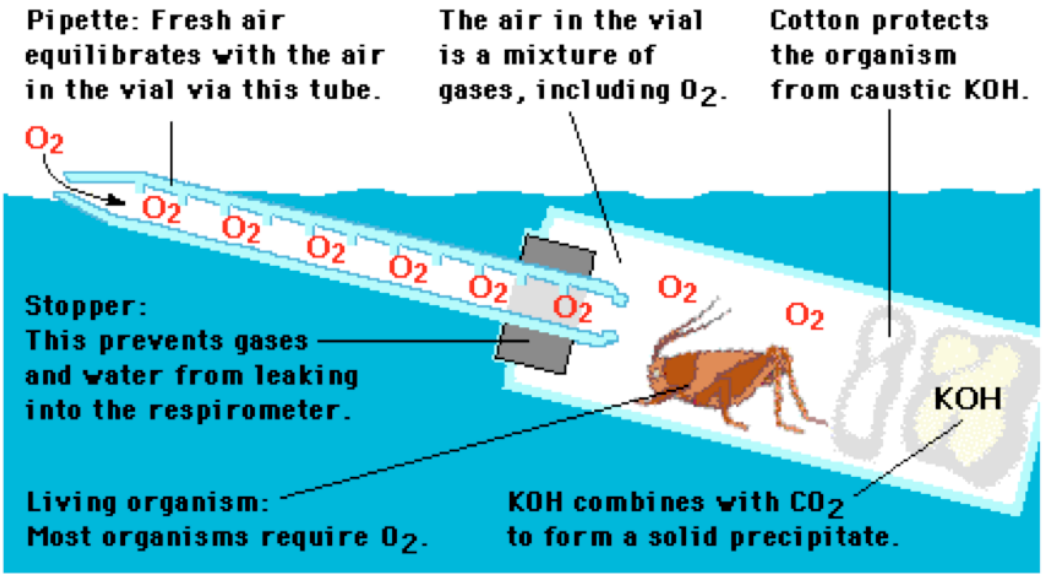
SUPPORTING MATERIAL

1. CHAPTER 4: RESPIRATION & HOMEOSTASIS

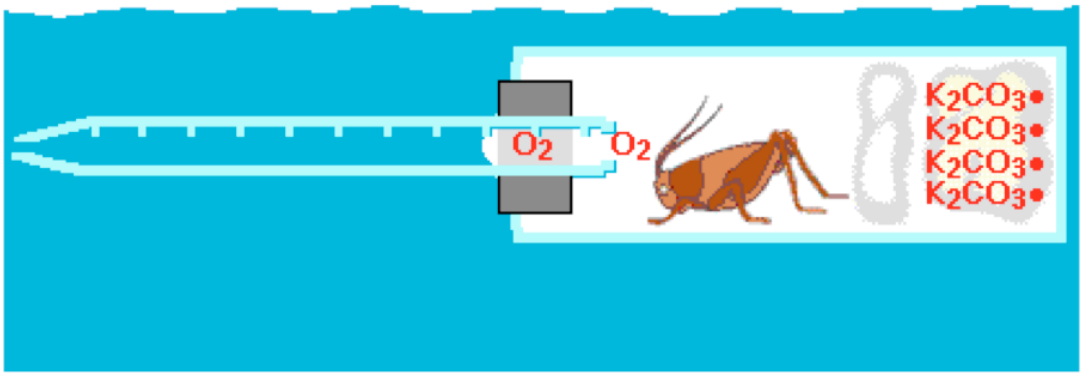
RESPIRATION LABORATORY HANDOUT

EXPERIMENTAL DESIGN:

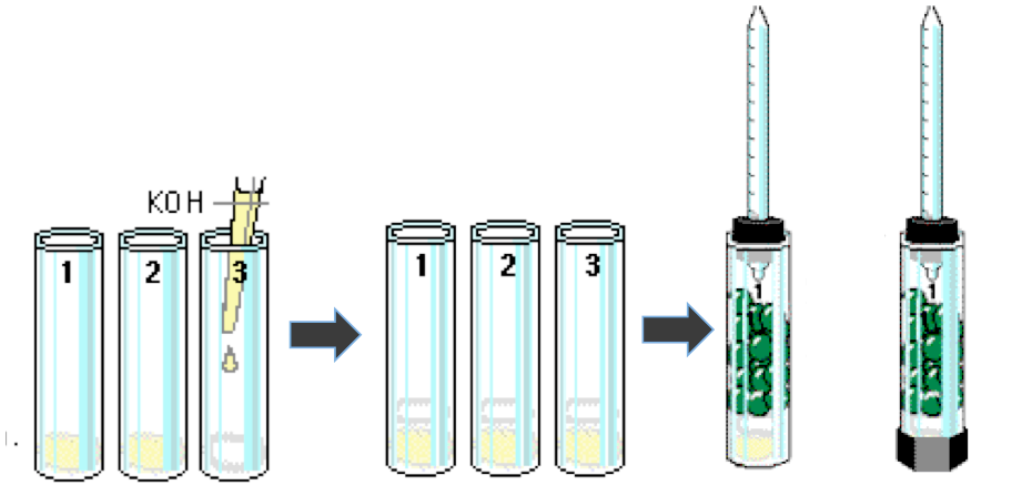
The illustration shows the basic feature of a respirometer. This unit measures changes in gas volume related to the consumption of oxygen.



When the tip of the respirometer is submerged, no addiitonal air can enter. As the oxygen is used up, the pressure of gases inside the respirometer decreases. This causes the water to enter the pipette. The CO_2 that is produced combines with the KOH to form a solid precipitate of K_2CO_3. The water that enters the pipette due to decrease in gas volume is directly proportional to the amount of O_2 consumed and thus allows us to measure the rate of respiration.



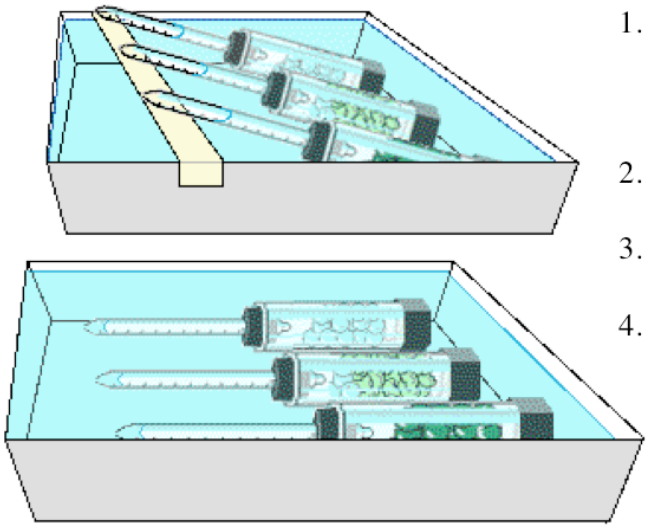
ASSEMBLING THE RESPIROMETER:



1. Soak absorbant cotton with KOH solution

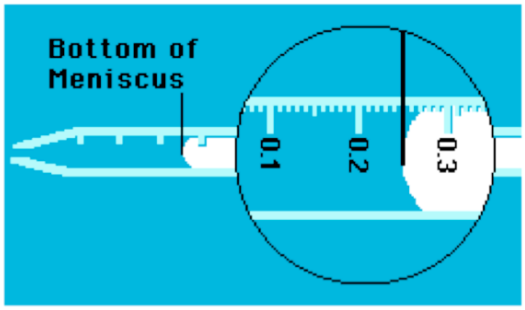
2. Cover with dry nonabsorbant cotton

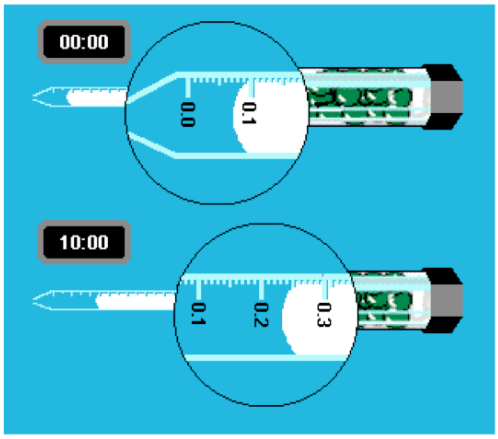
3. Place experimental organism in tube and seal with rubber stopper



1. Place the respirometers in water bath, resting on a sling for 5 minutes to equilibrate.
2. Lower tip of respirometer into bath.
3. Take initial reading on pipette.
4. Take 3 readings at 5 minutes interval.

The meniscus reading on the pipette gives us the amount of O_2 consumed. The unit on the pipette is in ml.





EXPERIMENTAL ORGANISMS:

(A) *Manduca sexta* larvae and germinating seeds at three different temperatures: (a) Normal room temperature (27^{o}C), (b) Hot temperature (37 ^{o}C) and (c) Cold temperature (17 ^{o}C).

Summary of lecture on respiration-

$$C_6H_{12}O_6 + 6\,O_2 \rightarrow 6\,CO_2 + 6\,H_2O + HEAT + 38\ ATP \quad \text{(Equation 1)}$$

$$\text{Rate of respiration} = \frac{\text{Change in the volume of gas}}{\text{Change in time}} \quad \text{(Equation 2)}$$

$$= \frac{\Delta y}{\Delta x} = \text{slope}$$

Question: How can rate of cellular respiration be measured? Circle the correct answer.

a) Measure the amount of glucose consumed

b) Measure the amount of oxygen consumed

c) Measure the amount of carbon dioxide produced

Calculate the rate of respiration for:

Larvae at:

NORMAL (27^{0}C)	HOT (37^{0}C)	COLD (17^{0}C)

Seeds at:

NORMAL (27^0C)	HOT (37^0C)	COLD (17^0C)

(1) In lab you were shown two methods to measure respiration in *Manduca* larvae. Describe the difference between the two methods (hint: what did each measure). Also describe why both the methods are correct ways to measure respiration? (3 points)

(2) Which treatment of temperature on larvae resulted in the highest respiratory rate? Why? (2 points)

(3) Which treatment of temperature on larvae resulted in the lowest respiratory rate? Why? (2 points)

(4) Is there a difference in the rate of respiration between larvae and germinating seeds? Explain your answer? (3 points)

(5) State your conclusion about respiration in *Manduca sexta* larvae and germinating seeds at different temperatures. (4 points)

HOMEOSTASIS

HOMEOSTASIS PREASSESMENT AND LECTURE

Homeostasis (Pre-assessment)

1. Why is it important for living things to maintain a relatively stable internal environment?

Living things need a relatively stable internal environment to maintain balance within the organism. When one organ system deviates from the "set point," other organ systems become affected and work less efficiently. When the set point is established, the body is balanced and in good health. Constant deviations from homeostasis are the causes of such diseases as diabetes and hypertension.

2. What are three ways that the human body's internal environment may become unstable?

Answers may vary based on the ability level of the students, but may include injury, temperature changes in the external environment and illness due to pathogens.

3. What happens to our body when we are exposed to temperature extremes? What do we do to control our internal environment?

When we are exposed to extreme heat, we sweat to cool off (we may take off a layer of clothing); when we are exposed to extreme cold, we shiver to generate body heat (along with adding layers of clothing).

4. What is homeostasis?

Biological balance within living things

5. Explain how insulin helps regulate blood sugar. What happens to individuals who cannot produce insulin?

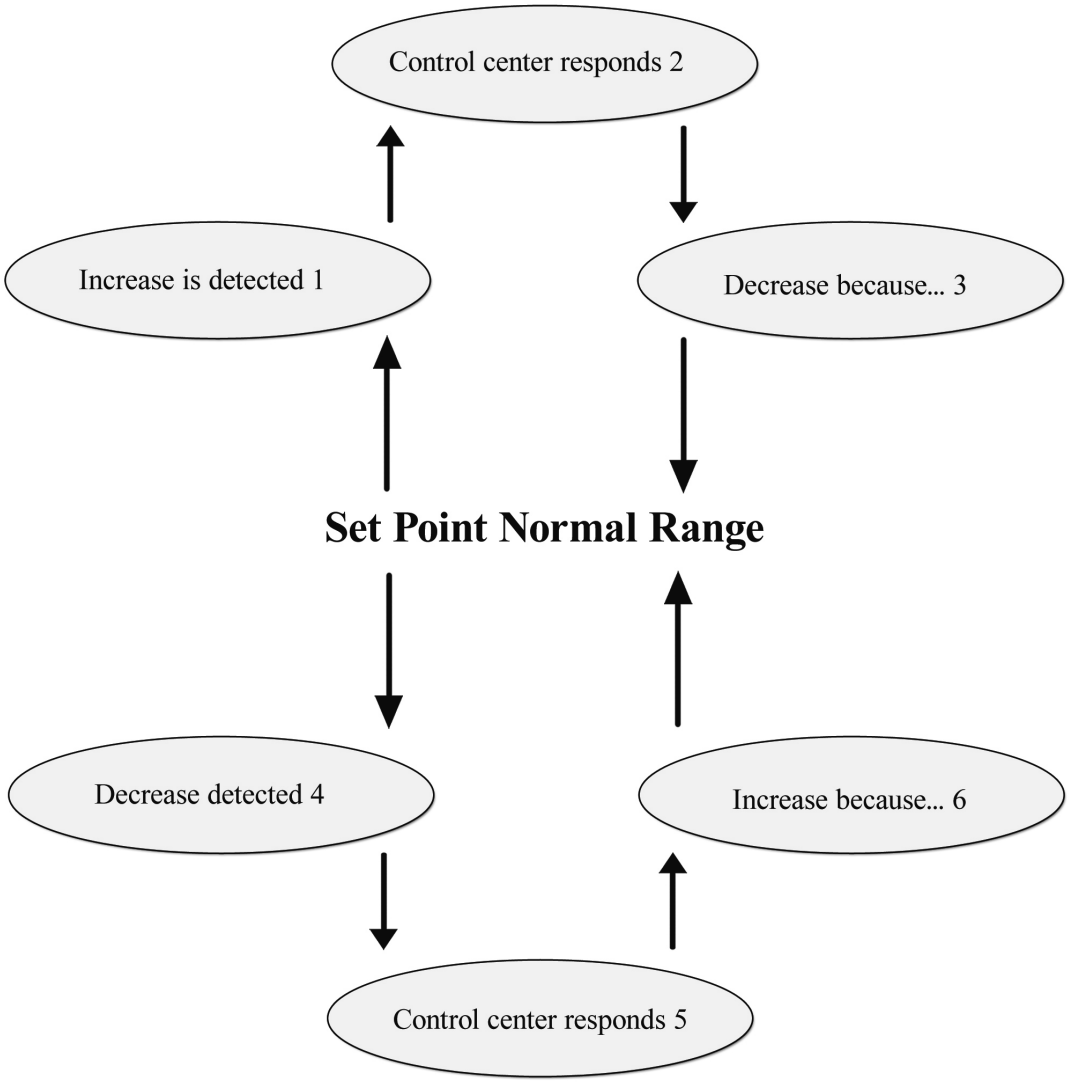
When you eat, the level of sugar in your blood rises. Insulin is a hormone that helps bring the sugar level down to the set point. If you cannot produce insulin, your body has no way of lowering the level of sugar in the blood and it becomes a health problem.

6. What is hypertension? What may cause it?

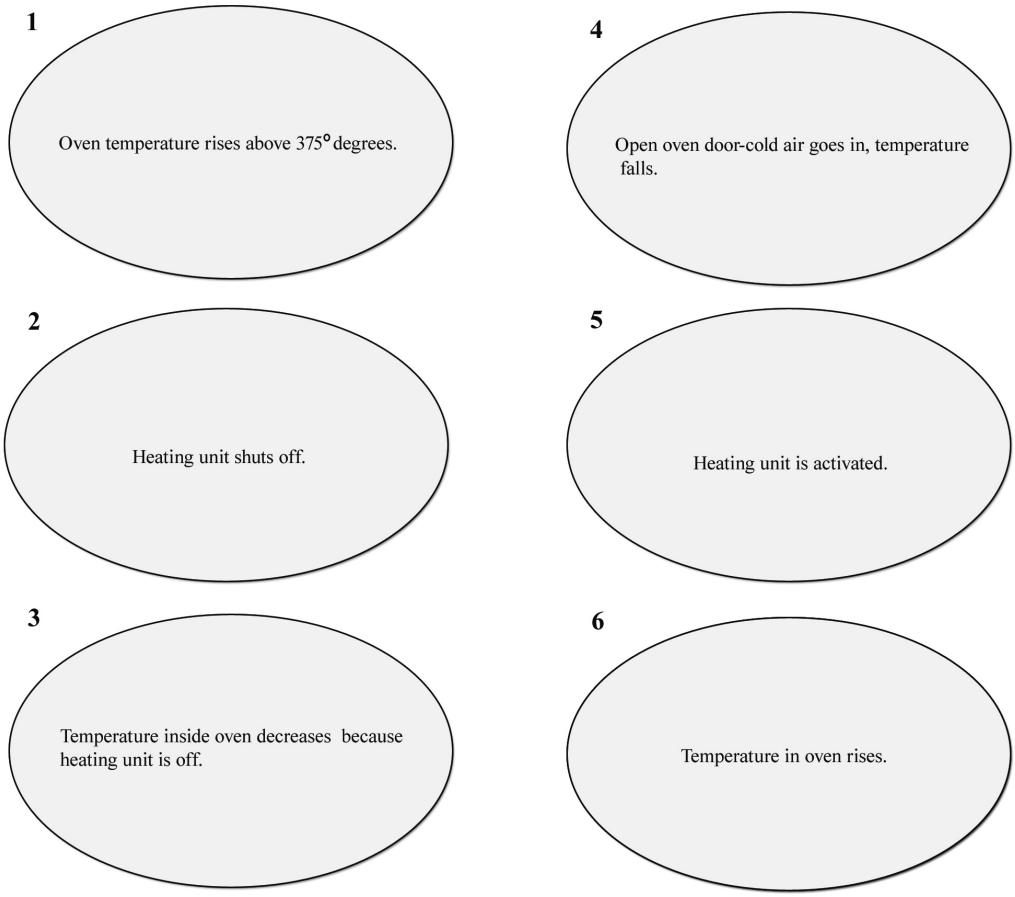
Hypertension is another name for high blood pressure. It may be caused by obesity, too much sodium in the diet, heredity (answers may vary).

EXAMPLE:

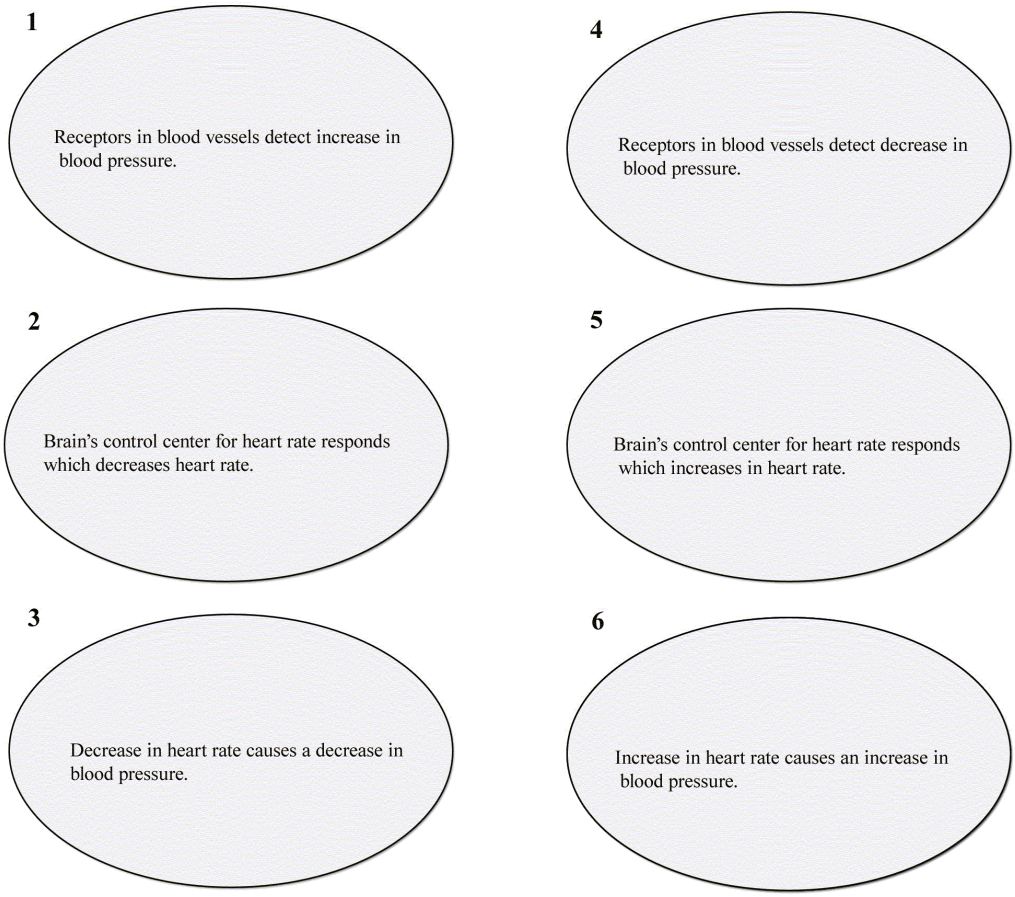
Negative feedback system



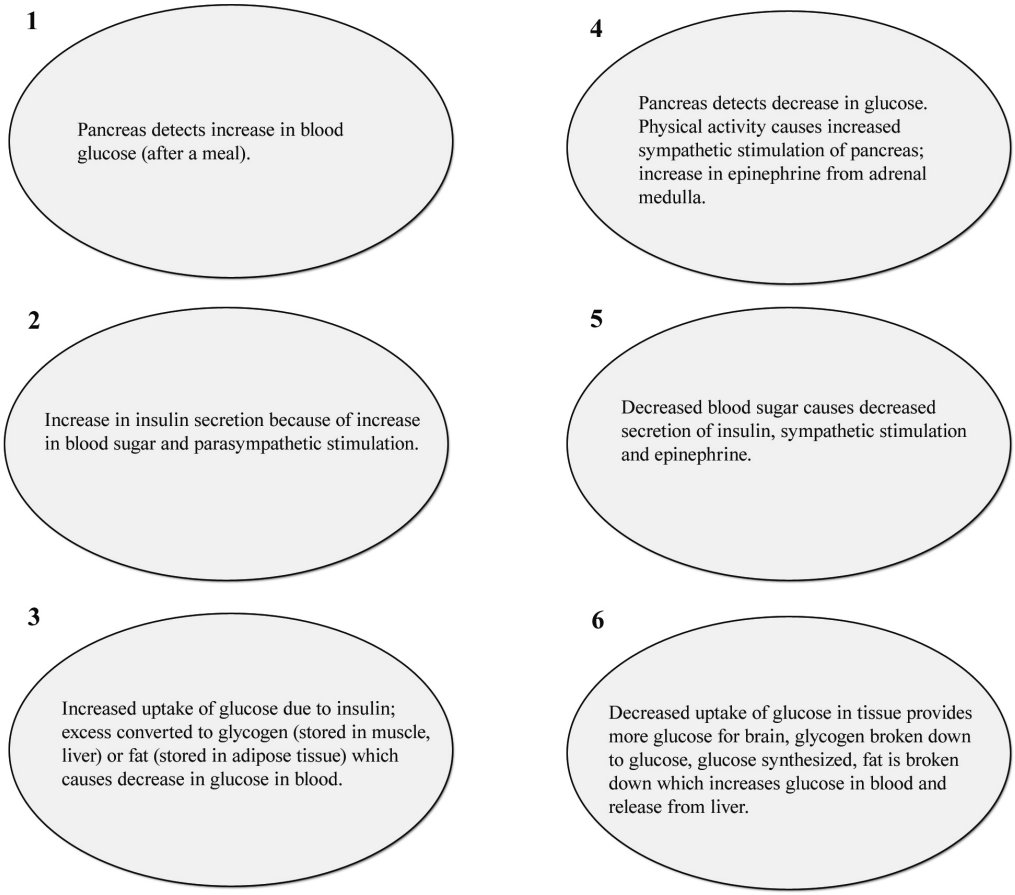
Regulation of oven temperature at 375° F



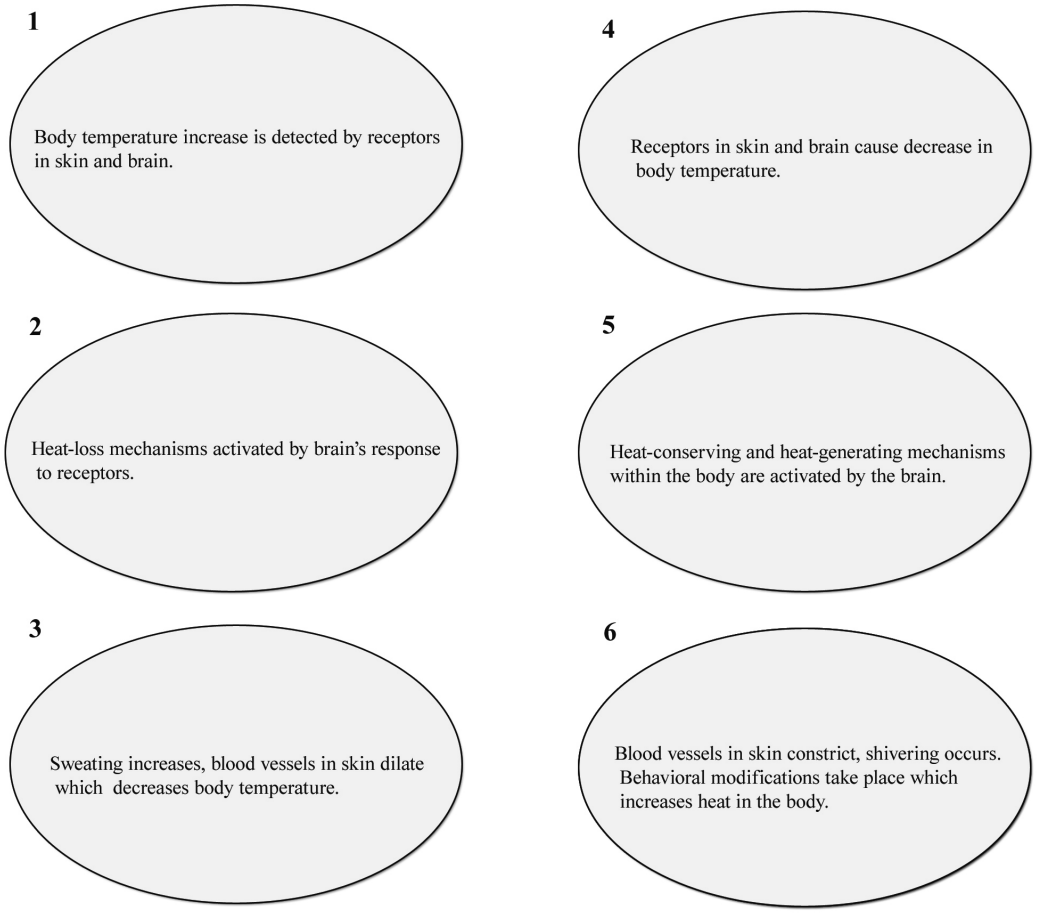
Blood Pressure Regulation



Blood Glucose Regulation



Body Temperature Regulation



2. CHAPTER 5: FEEDING PREFERENCE ASSAY FOR MANDUCA LARVA

LESSONS ON DEPENDANT AND INDEPENDENT VARIABLES:

PRELIMINARY INFORMATION AND PREASSESMENT

What is a *Manduca sexta?*

Directions: Use the following three websites at home, in study hall, at lunch, or after school to complete the activity about the *Manduca sexta* (activity is at the end of the reading). These sites are also linked on **Mrs. Kornreich's Blackboard page**. The websites have great pictures and videos of *Manduca sexta,* so be sure to check them out if you are able! If accessing the Internet is a problem, you may use the reading below the websites to complete the homework. Have fun and explore!

http://www.butterfliesandmoths.org/species/Manduca-sexta

http://entomology.unl.edu/k12/caterpillars/hornworm/hornwormpage.html

http://eol.org/pages/506145/overview

Introduction

Manduca sexta, referred to as the tobacco hornworm (caterpillar) or as the hawkmoth or sphinx moth (adult), is a common insect in a wide variety of habitats, such as tobacco fields and vegetable gardens.

Metamorphosis

The M. sexta goes through each of the following stages: egg, larva, pupa, and adult. Hornworm **eggs** are deposited mainly on the lower surface of leaves, but can also be found on the upper surface.

Larvae are cylindrical (shaped like a cylinder) and bear five pairs of legs up front in

addition to the legs near the back. One of the most striking features is the "horn" located on the last segment of the caterpillar.

The first stages of the larvae have very long horns compared to the fifth and final stage. Hornworms primarily feed on tobacco and tomato, but will occasionally feed on potato and pepper crops and other plants in the Solanaceae family, hence their name Manduca, which is the Latin word for "glutton". One or two hornworms can easily eat all the leaves on a tomato plant. Mature larvae drop to the soil and burrow, where they pupate, or change into their next stage of development. Pupae are brown in color and large.

Adults are large moths with long forewings. Their abdomen is marked with orange-yellow spots. They are strong fliers with a rapid wing beat and are sometimes mistaken for humming birds. Adults are generalist nectar feeders, meaning they feed on all types of flowers.

Distribution

The tobacco hornworm is more common in the southern United States, especially the Gulf Coast states. Its range extends northward to New York. It also ranges south through Mexico and the West Indies to Argentina.

Importance

Larvae are defoliators (they eat leaves) and can be considered pests in gardens and a menace to tobacco growers due to the high levels of eating.

Hornworms are an appealing organism to researchers because they are easily raised in the laboratory on artificial diets, have a short life cycle, and their large size allows for scientific investigations on their various systems.

Questions for Homework Directions: Use the resources provided to respond to the questions in the spaces below in complete sentences.

1) Write quantitative or qualitative attributes (remember our vocab?) to describe *Manduca sexta* in A) its caterpillar form and B) its moth form.

A) _____

B) _____

2) When *Manduca sexta* is a caterpillar what does it like to eat? When it is a moth what does it like to eat?

---

3) Can we find *Manduca sexta* in our own backyards? Why or why not?

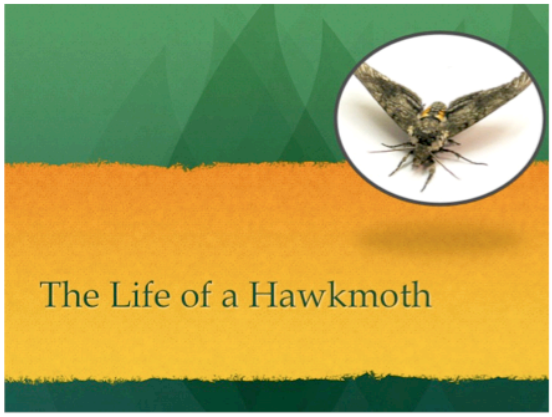
_____

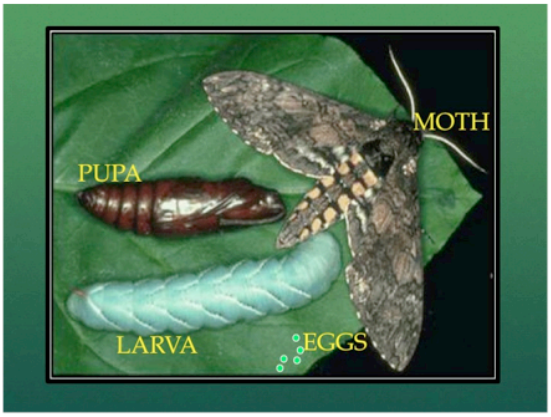
Readings obtained on 9/25/12 from:

http://entomology.unl.edu/k12/caterpillars/hornworm/hornwormpage.html

LECTURE SLIDES:

The lecture slides were left incomplete on purpose where the students are asked to fill out the title for each slide.





Job of a Pupa

Vocabulary!!!

METAMORPHOSIS

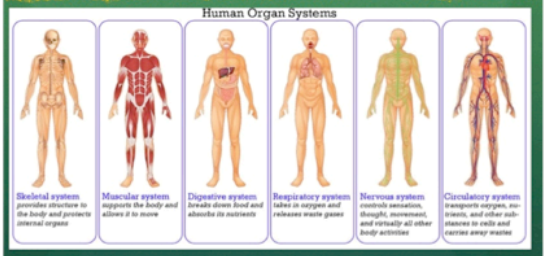
(Morph = change shape)

A biological process where the organism changes itself completely to accommodate a new lifestyle.

Example-

Larva into a Pupa and a Pupa into an Adult Moth.

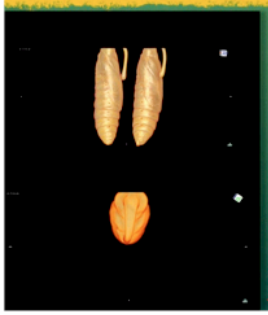
Organ Systems

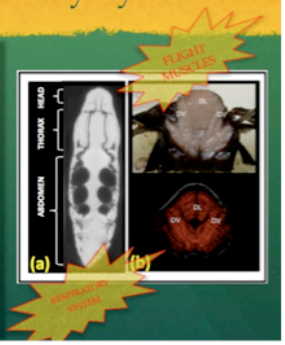


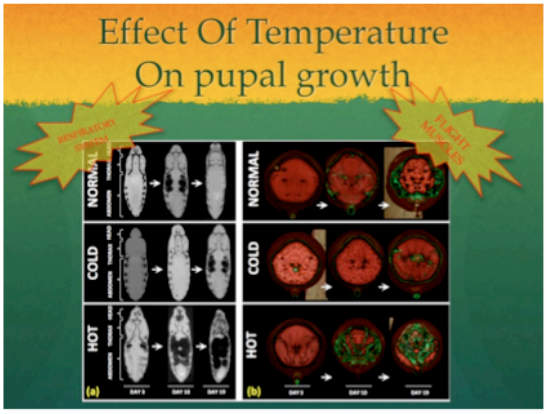
Organ systems in Moths?

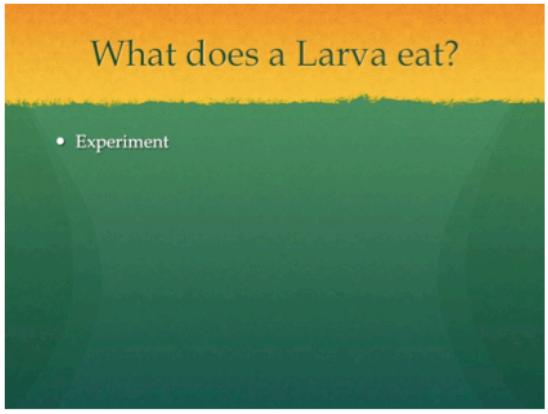
LARVA	PUPA	ADULT MOTH
Nervous system	Nervous system	Nervous system
Circulatory system		Circulatory system
Respiratory system	Respiratory system	Respiratory system
Digestive system	Digestive system	Digestive system
Excretory system		Excretory system
Muscular system	Muscular system	Muscular system
Reproductive system	Reproductive system	Reproductive system

Pupa Respiratory System









WORKBOOK:

Problem:

Manduca sexta are found to naturally exist year round in Hawaii and the southern states of our country. But due to very low temperatures during winters, these insects fail to survive in the northern parts of the US. *Manduca* larvae are known pest on tomato and tobacco crops. These larvae are leaf eaters and completely defoliate these agricultural crops. But can these leaf eaters also damage fruits and vegetables? Upstate NY produces a lot of fruit crops such as apples, peaches, blueberries and grapes. If *Manduca sexta* were to establish itself in our state, would the larvae pose as a threat to our crops? Would it be a threat to the green leaves or also on the colorful fruits and vegetables? Please design an experiment to test this hypothesis.

Objectives:

In this lab you will:

• design and carry out a biological experiment.

• make observations of *Manduca* larvae feeding behavior.

• accurately record observations and organize data.

• draw conclusions and make inferences based on data.

Materials:

• *Manduca sexta* larvae

• Weight scale

• *Manduca* diet

• Food color

• Apple leaf tea and tomato leaf tea

• Glass tube containers

• Plastic caps for diet

• Sponge stoppers

• Plastic stick

• Tube rack

Procedure:

In this lab, you will be asked to consider the feeding behavior of *Manduca* larvae and to also think of a question that you will then attempt to answer by measuring the quantity of food consumed by one larva. What are five characteristics of a good experiment?

1. _____

2. _____

3. _____

4. _____

5. _____

Your experimental design should include each of the five characteristics you listed. You should also think about how you will collect your data and how you will analyze

the data.

1. Formulate a question about the feeding behavior of *Manduca* larvae. For example, you might ask, "Do *Manduca* larvae have a color preference in the foods they eat?" Make sure your question is testable with the materials and the amount of time available. On the last page, using complete sentences, address the following:
    a) State the hypothesis. This is what you think will happen based on something you have observed or read.
    b) Identify the constants. These all the things you keep the same. They are also called the control variables.
    c) Identify the independent, or experimental, variable. This is the one thing you are testing. It is called the independent variable.
    d) How will the data be collected?
    e) How many replicates, or trials, will there be?
    f) Check with me to be sure your question and approach will be appropriate.

2. Decide what two food materials are necessary for your experiment and get them.
3. Collect your larval food choices.
4. Label your experimental setup and fill in your worksheet.
5. Weigh the food cap.
6. Put together the glass tube, diet cup, sponge and the plastic stick.
7. Put in your larvae in the glass tube setup.
8. Examine your experiment on Monday, Wednesday and Friday.
9. Record the change in the amount of food eaten by weighing the food caps.

10. Record change in the weight of larva.