RESPIRATORY PHYSIOLOGY AND THE INITIATION OF MOLTING
IN THE TOBACCO HORNWORM, MANDUCA SEXTA

by

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Will Wilson

Dissertation submitted in partial fulfillment
of the requirements for the degree of Doctor of Philosophy
in the Department of Biology
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ABSTRACT

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Abstract

The regulation of body size is one of the fundamental unsolved problems in developmental biology. The control of body size requires a mechanism that assesses size and stops growth when a characteristic size is reached. In *Manduca sexta* the endocrine events that lead to a molt start when the larva reaches a critical weight. Molting is initiated by a size-sensing mechanism, but the nature of this mechanism has remained elusive. Here I show that this size sensing mechanism emerges from the changing relationship between the increasing body mass and the fixed tracheal system in each instar. As body mass increases, the demand for oxygen also increases, but the fixed tracheal system does not allow a corresponding increase in oxygen supply. The constraint on oxygen supply is relieved by the molt.

Using quantitative measurements of the tracheal system for several instars, I make theoretical predictions about the maximum rate of oxygen delivery that is permitted for each instar, and compare those to measured respiration rates. These metabolic data also allow me to propose a new mechanism that could account for the hypoallometric scaling of metabolic rate with respect to body mass.

Low oxygen tension causes larvae to molt at a smaller body size, consistent with the hypothesis that under normal growth conditions, body size is regulated by a mechanism that senses oxygen limitation. Nevertheless, larvae transferred to hypoxia in mid-instar are not induce to molt immediately; instead, their molt is delayed. This suggests that under certain stressful growth conditions, larvae may use an alternative mechanism to initiate molting. Indeed, starved or ligated larvae may never attain the critical weight, but are eventually able to molt. These larvae must use a size-independent mechanism, the “leaky prothoracic glands” (first proposed in the 1970s), that stimulates sufficient ecdysone secretion to trigger a molt. I examine the different potential sources of ecdysone-stimulating factors and present hypothetical mechanism for size-independent molting, which I then formalize with a mathematical model.
Dedication

To Papa, who taught me to think clearly;
To Mom, who fed and clothed me.
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1. Introduction

The cascade of signals that leads to the molt (or metamorphosis at the end of the final instar) is set in motion when the larva attains the “critical weight” (Nijhout and Williams, 1974b). Because larvae initiate metamorphosis based on a size-sensing signal rather than a timing signal, this suggested that there must be a physiological mechanism for size-sensing. Although the critical weight is easily measured at a phenomenological level, the physiological mechanism of size-sensing in Manduca has remained elusive.

In this introductory chapter, I review what is understood of the neuro-endocrine regulation of molting and metamorphosis in Manduca sexta, and then propose the hypoxia hypothesis, a physiological mechanism for size-sensing that could initiate the molting cascade. This hypothesis is based on the premise that the fixed size of larval tracheal systems within an instar imposes a ceiling on oxygen delivery, but oxygen demand continues to increase as body size increases. Therefore, there is a size in each instar at which oxygen supply is no longer adequate, and this could be the signal to initiate molting to the next instar, where the constraint on oxygen supply is relieved. The subsequent chapters are dedicated to the experimental testing of the predictions that follow from this hypothesis.

1.1 Why do caterpillars molt?

Why not have just one larval instar? The traditional explanation for why insects molt is that the sclerotized cuticle is a physical constraint on growth and the larva would need to shed this "box" in order to grow into a bigger one. This constraint does not apply to many insect species, however: "Most of the body surface of holometabolous larvae is unsclerotized and it would seem, therefore, that such animals ought to be able to do away with larval molting altogether" (Nijhout, 1981; Nijhout, 1998), especially given the costs associated with molting.

Molting is energetically expensive, as the larva must shed and replace its cuticle. Moreover, the molting (or pharate) stages are the most physiologically constrained and vulnerable times
of the growth trajectory: larvae are unable to feed, to move to escape predators, or to escape heat/cold. It is therefore the time at which physiological stress is the highest and also the time at which selection might be strongest.

Thus, another reason to be particularly interested in the regulation and process of molting is that the growth periods might be relatively easy to get through compared to the molting stages. It therefore seems reasonable to assume that much of the developmental physiology and biology of the larval stages will be dictated by the constraints and evolutionary pressures faced during the head capsule slipped (molting) stages.

1.2 The neuro-endocrine regulation of molting

Molting and metamorphosis are regulated by a neuroendocrine axis that involves the brain and the prothoracic glands (Nijhout, 1998). Specific neurons in the brain secrete the prothoracicotropic hormone (PTTH), which in turn activates the prothoracic gland to secrete ecdysone (Figure 1). Ecdysone is the hormone that triggers ecdysis of the old cuticle.

The functioning of the brain-prothoracic gland axis depends on many factors: the brain must be competent to send the PTTH signal; the brain must then make the developmental decision to initiate the pulse of PTTH; the prothoracic gland must be able to sense the PTTH signal, and be competent to respond by secreting ecdysone. All of these conditions (both the competence to respond and the developmental decision to initiate signals) are fulfilled only at specific times during the growth period or during each instar.

Attainment of the critical weight is thought to be the first developmental decision point, which leads to the pulse of PTTH. Thus the search for the signal that initiates molting and metamorphosis has focused on the physiological mechanisms that determine the critical weight.
1.3 **Molting and metamorphosis: what’s different about the metamorphic molts?**

The same cascade functions to trigger all ecdysone events, whether they are larval molts, exposure of the dorsal vessel and entry into the wandering stage, pupation, or adult development. However, the hormonal milieu in which the ecdysone events occur influences the type of event that will happen. When juvenile hormone (JH) is high, ecdysone stimulates a larval-larval molt; when juvenile hormone disappears from the hemolymph, ecdysone stimulates the larva to purge it gut contents and initiate wandering; another pulse of ecdysone then stimulates the transition to the pupal stage; in the pupal stage, ecdysone initiates development of the adult moth (Nijhout, 1998; Nijhout and Williams, 1974a).

In the final instar of *Manduca*, a drop in JH is required to allow PTTH secretion (indeed, an exogenous increase in JH delays PTTH secretion, gut purge and the onset of metamorphosis). However high levels of JH are the normal state in earlier instars, and in those stages JH does not inhibit PTTH secretion nor does it delay the molt.
The processes of pupal commitment for different tissues do not happen all at once in lepidopterans (Kremen and Nijhout, 1989; Kremen and Nijhout, 1998) or in *Drosophila*, but they do appear to be synchronous in the flour beetle, *Tribolium castaneum* (K. Preuss, pers.)
Heterochronic shifts have occurred between the processes of pupal commitment and the ecdysone events in different species. An important consequence of this observation is that instars are not homologous units and cannot be compared across species.

In some species (Manduca, Precis) pupal commitment is not simultaneous for different tissues: it begins late in the fourth instar and continues through the beginning of the fifth instar. In the final instar, the mechanism that initiates the transition to wandering is inhibited by JH, because molting when some but not all tissues are pupally committed would result in an unviable larval-pupal intermediate. Thus, there is an additional layer of regulation that controls ecdysone secretion in the final instar.

In the work that follows, I first attempt to understand how larval-larval molts are triggered, and then examine how this basic mechanism has been modified or overlaid with additional regulation in the final instar of Manduca.

1.4 Critical weight

Nijhout and Williams (1974) showed that in the final instar of Manduca, the decision to molt is triggered by attainment of the critical weight, the weight at which starvation no longer delays the timing of exposure of the dorsal vessel and gut purge, the first sign of the transition to the pupal stage. The critical weight is a phenomenological measurement that suggests that the physiological mechanism that initiates molting is a size-sensing (rather than timing) mechanism.
Figure 4: Larvae initiate the molting process when they attain the critical weight (from Nijhout and Williams 1974)
The critical weight is the weight at which the cascade of signals leading to the molt are set in motion. Thus, starvation before critical weight delays the larva from attaining the critical weight and molting; in contrast, starvation after the cascade has been initiated does not alter the timing of the molt.

Attainment of critical weight is immediately followed by the Interval to Cessation of Growth (ICG), which can be divided into three phases:

1) The brain becomes competent to secrete PTTH. The critical weight precedes secretion of PTTH by about 24 hours in the fifth instar (Nijhout and Williams, 1974b), and possibly less time in the earlier instars.
2) Once the brain is competent, it secretes PTTH immediately, if a photoperiodic gate is open; otherwise it waits until the opening of the next photoperiodic gate. The photoperiodic gating of PTTH secretion occurs at all molts (Truman, 1972). The photoperiodic gating is probably an adaptive mechanism: larvae are particularly vulnerable to predators during the molt because they cannot move; it is advantageous to molt when predators are least likely to find them.

3) The prothoracic gland, if competent to respond, is activated by the PTTH signal and secretes an ecdysone pulse that triggers ecdysis. A timetable of physiological events after PTTH secretion has been established (Truman, 1972; Truman and Riddiford, 1974). In normal growth conditions, the PG is competent to respond, and ecdysone secretion is triggered by PTTH. In poor growth conditions ecdysone secretion is delayed but not prevented. It is unclear whether the delay is due to a delay in the PTTH signal, a delay in the attainment of the prothoracic gland’s competence to secrete ecdysone in response to PTTH, or an absence of PTTH secretion altogether.

Neck-ligation separates the brain, which secretes PTTH, from the prothoracic gland, which secretes ecdysone. Neck-ligated larvae are able to eventually molt, which suggests that the PGs alone will eventually secrete enough ecdysone to trigger a molt. Thus, PTTH is necessary for the correct timing of ecdysone secretion, but ecdysone secretion will eventually occur in absence of PTTH.

The first phase is the least well-understood: it is not known what triggers the brain to secrete PTTH. Nijhout and Williams (1974) showed that in the fifth instar of Manduca, the drop in JH renders the brain competent to secrete PTTH, and that until the JH titer is low enough, PTTH secretion is inhibited. The time delay required for the brain to become competent to secrete PTTH is the delay required for clearance of JH from the hemolymph.

In contrast to the final instar, in the earlier instars PTTH secretion normally occurs in the presence of JH. During larval-larval molts JH clearly does not inhibit PTTH secretion (Nijhout, 1998). Therefore, it is unlikely that a change in JH is involved in triggering larval-
larval molts. The larval-larval molts nevertheless exhibit the critical weight phenomenon. This suggests that the critical weight is not regulated by JH, but that perhaps changes in JH signaling are overlaid on the critical weight in the final instar in preparation for metamorphosis.

At least two questions present themselves:

(1) Does the drop in JH cause the critical weight in the final instar? If the drop in JH triggers molting in the fifth instar but not in earlier instars, what can account for the fact that JH inhibits the molting signal only in the final instar? What mechanism, necessarily different, would account for the molts and the critical weight phenomenon in earlier instars (larval-larval molts)?

(2) If CW in the final instar is caused by the drop in JH, how is the JH titer regulated as a function of size? In other words, what is the role of the drop in juvenile hormone in the size-sensing mechanism?

The primary evidence that the drop in JH titer causes the critical weight is that addition of exogenous JH early in the fifth instar delays gut purge; the treated larva will continue growing beyond its normal size of gut purge (Nijhout et al., 2006). When the larva has attained a very large size, the cuticle does allow further stretching, and the larva stops growing. It may remain at the same size for several days before initiating the gut purge.

The drop in JH is necessary for initiation of metamorphosis, but its role in regulation of molting and size-sensing remains unclear. The drop in JH, while necessary for the larval-pupal transition (competence and commitment of tissues to the pupal state), is not sufficient for the correct timing of the molt to the pupal stage. The drop in JH is permissive for initiation of metamorphosis.
1.5 *When the cascade breaks*

One way to study the function of the brain and prothoracic gland in the molting process is to disrupt the cascade of hormonal signals in a controlled way. Several factors are known to interrupt the function of the brain and prothoracic gland in triggering the molt. Neck-ligation physically isolates the brain and prevents PTTH from stimulating the prothoracic gland. Starvation prevents the larva from attaining the critical weight and it must therefore molt by an alternative mechanism. Malnutrition can, in some cases, also prevent the molting cascade, although it is not clear if malnutrition prevents the brain from secreting the PTTH signal or whether it renders the prothoracic gland incompetent to respond. Experiments (Safranek and Williams, 1984) have shown that the brain usually retains its competence to secrete PTTH which suggests that the problem probably lies in the competence of the PG to respond.

Larvae that are neck-ligated eventually molt although they never attain the critical weight; they must therefore be using an alternative molting mechanism that does not require the brain. When the normal functioning of the brain-prothoracic gland axis is interrupted, larvae usually molt, albeit with significant delay. The instar durations are usually variable, as are the final sizes. Because the brain is not involved in triggering these molts, the alternative molting mechanism is thought to be caused by the autonomous activity of the prothoracic gland, or by stimulation of the prothoracic gland by a factor secreted in the abdomen.
1.6 Leaky prothoracic glands

*Manduca* larvae that are starved or neck-ligated before the critical weight eventually transition to wandering, in spite of never attaining the required size. A similar delay in the transition to pupariation is observed in *Drosophila* larvae in which the PTTH neurons have been ablated.

The question that needs to be answered is why, in absence of PTTH, the ecdysone titer eventually rises enough to trigger a molt: why aren’t the larvae indefinitely stuck in one larval instar? Several explanations have been proposed to explain the delayed developmental transitions, the first of which is the “leaky prothoracic glands” hypothesis proposed by Truman (1972) and Nijhout (1976). The leaky prothoracic glands hypothesis is the idea that the autonomous activity of the prothoracic glands eventually causes enough accumulation of ecdysone in the hemolymph to trigger a molt.

Alternative possibilities to “leaky prothoracic gland” are (1) ecdysone is produced elsewhere in the body; (2) PTTH is produced in the ventral ganglia, and the phenomenon would be
more aptly described as leaky PTTH; (3) there are other ecdysteroidogenic factors such as bombyxin (lepidopteran insulin) and orcokinins (Yamanaka et al., 2011) which are found in the brain and ventral nerve cord; or (4) autonomous, nonlinear feedbacks of ecdysone on the prothoracic gland cause the PG to secrete a peak of ecdysone (Sakurai and Williams, 1989).

In considering the first possibility, it is important to note that when Manduca abdomens are isolated from the head and prothoracic gland by ligation, the abdomens never molt (Nijhout, 1976), which suggests that the abdomen is not a source of ecdysone. Nevertheless, the abdomen could be the source of a factor such as insulin (secreted by the fat body in the abdomen) that stimulates the prothoracic gland to secrete ecdysone.

The second possibility can be tested by using an antibody to stain for PTTH in the abdomen. The third possibility is known to exist, but it is unclear how strong the other ecdysteroidogenic factors are relative to PTTH. They are considered to be relatively weak compared to PTTH and insulin (Van de Velde et al., 2009).

Sakurai and Williams (1989) showed that high levels of ecdysone in the hemolymph inhibit the prothoracic glands from secreting more ecdysone. This provides a mechanism whereby the prothoracic glands shut down the production of ecdysone after secreting a large molting pulse. There is evidence in Drosophila (Varghese and Cohen, 2007) that ecdysone induces transcription of its own receptor in the PG and that it sensitizes the PG to its own signaling, thus leading to a positive feedback loop. This loop could cause the observed rise in ecdysone titers. The negative feedback induced by the high titers would then “shut off” this pulse.
1.7 Mechanisms that regulate ecdysone secretion from the prothoracic glands

Regulation of ecdysone secretion by the prothoracic gland has been extensively studied at the molecular level in *Drosophila* (Caldwell et al., 2005; Colombani et al., 2005; Layalle et al., 2008; Mirth et al., 2005; Shingleton, 2005; Walkiewicz and Stern, 2009; Walsh, 2009). These studies have shown that ecdysone secretion rate can be nutritionally modulated, and that activation of insulin signaling (PI3K) in the prothoracic gland accelerates ecdysone secretion whereas inhibition of insulin signaling decreases ecdysone secretion. For example, activation of insulin signaling in the prothoracic gland causes faster secretion of ecdysone and precocious transition to the pupal stage. Inhibition of TOR in the prothoracic gland causes an extension of larval development (by suppression of ecdysone secretion) without affecting larval growth rate, resulting in an overgrowth phenotype. Tuning the rate of ecdysone secretion (by manipulating insulin signaling in the prothoracic gland) can therefore modulate the timing of developmental events.

Nutritional modulation of the rate of ecdysone secretion affects larval growth rate and the timing of molts and commitment to pupation. The authors of these studies therefore
inferred from the change in final size that changes in the rate of ecdysone secretion must control the critical weight. However, I argue that because ecdysone secretion occurs several hours (sometimes days) after attainment of critical weight, ecdysone secretion cannot be involved in size-sensing and molt initiation; rather, it is a necessary piece of machinery in the cascade of events that executes the molting process, after it has been initiated.

Activation of insulin signaling in the prothoracic gland not only caused and precocious ecdysone release but also an enlarged prothoracic gland (Caldwell et al., 2005; Mirth et al., 2005; Walkiewicz and Stern, 2009). Mirth et al. (2005) proposed the following interpretation: the prothoracic gland's size relative to the body is the size-assessment mechanism, and larvae with enlarged prothoracic glands overestimate their body size and therefore initiate metamorphosis at a smaller size than the controls.

To follow up on this hypothesis, Colombani et al. (2005) used a genetic approach to modulate the basal levels of ecdysteroids in *Drosophila*. They manipulated the levels of PI3K in the prothoracic gland, and found that the prothoracic gland size is increased upon PI3K activation and decreased upon inhibition. Consistent with Caldwell et al. (2005) and Mirth et al. (2005), Colombani et al. (2005) also found that larvae whose prothoracic glands were stimulated by insulin signaling produced more ecdysone, whereas larvae in which insulin signaling was blocked in the prothoracic glands produced less ecdysone.

In addition, they found that increasing the size of the PG by activating Myc did not increase ecdysone secretion. Therefore, production of ecdysone is specifically modulated by insulin signaling in the PG, not by the size of the PG. Furthermore, Caldwell et al. (2005) found that overexpression of Raf in the prothoracic gland initiates premature metamorphosis without any change in size of the gland. These experiments suggest that the size of the prothoracic gland is not the relevant variable, but pointed to insulin or Raf signaling as the potential trigger for initiation of metamorphosis.

This lead to the idea that insulin could be the "metamorphosis initiating factor" (Koyama et al., 2008). To test this idea, Koyama et al. (2008) cultured early fifth instar wing discs with
ecdysone and showed that ecdysone increased expression of broad, a marker of pupal commitment. Neither wortmannin nor rapamycin (respective inhibitors of insulin and TOR signaling) had a suppressive effect on basal or 20E-induced broad expression (a marker of pupal commitment). Neither bombyxin nor bovine insulin increased basal levels of broad expression, which means that insulin and TOR do not induce pupal commitment. Although insulin and TOR signaling appear to modulate the rate of ecdysone secretion from the prothoracic gland, insulin and TOR do not control the "metamorphic switch".

Later studies (Gu et al., 2009; Walsh, 2009) showed that insulin signaling is not sufficient to stimulate ecdysone secretion from the prothoracic gland. Walsh (2009) studied the impact of nutrient deprivation on ecdysone secretion. She found that starvation on days 1 and 2 of the fifth instar of Manduca decreases the capacity of the prothoracic gland to secrete basal levels of ecdysone. The decrease in ecdysone secretion due to starvation was not rescued by insulin injection; furthermore, PTTH-stimulation of insulin-injected, starved prothoracic glands did not elicit ecdysone secretion. Therefore, she concluded that "insulin does not directly stimulate ecdysone secretion in absence of nutrients".

Gu et al. (2011) show that insulin/PI3K/Akt signaling in the PG is triggered by PTTH, and that wortmannin (inhibitor of PI3K) inhibits ecdysone secretion in a dose-dependent manner. They also note that PTTH triggers activation of Erk signaling, which is not triggered by insulin. Therefore, PTTH triggers both insulin and Erk, which are necessary for ecdysteroidogenesis. Blockage of insulin signaling partially blocks ecdysteroidogenesis. Insulin signaling in the prothoracic gland appears necessary for the PG to be competent to respond to the PTTH signal, but it is not sufficient to trigger ecdysone secretion.

Thus, for the larva to molt via the critical weight in normal conditions, three conditions need to be met: (1) the larva must grow to the critical weight and have a mechanism to sense it, (2) the brain needs to secrete PTTH after attainment of critical weight, and (3) the prothoracic gland must be competent to respond and secrete ecdysone. It is likely that inadequate levels of insulin signaling (due to starvation or knocking out the insulin receptor or PI3K in the PG) render the prothoracic gland incompetent to respond. In this sense,
adequate insulin signaling is necessary for the normal functioning of the critical weight mechanism, but it cannot be said to “trigger” or “control” the molt.

1.8 The time and place of the critical weight

The critical weight occurs approximately 24 hours before PTTH secretion in the fifth instar of *Manduca*, and therefore precedes the ecdysone event by more than a whole day. Ecdysone secretion from the prothoracic gland is part of the machinery that executes the critical weight signal, but comes into play only after the size-sensing mechanism has operated. Tinkering with signaling pathways in the prothoracic gland will have effects on the rate and timing of ecdysone secretion, but cannot be said to shift the critical weight or be the organ by which the critical weight is sensed, because the critical weight phenomenon happens before the prothoracic gland is implicated in the molting signaling cascade.

**Figure 8: Critical weight happens in the brain, before the prothoracic gland is implicated in molting**
Starvation and ligation experiments show that the brain is necessary for the normal timing of the molt. Starved larvae are delayed relative to fed controls until the critical weight, after which they are synchronous with controls. Nutrition is no longer necessary after the critical weight, which occurs 24 hours before PTTH secretion in the fifth instar of Manduca. Ligated larvae are delayed relative to fed controls and relative to starved larvae close to the critical weight, and do not become synchronous with controls until after PTTH secretion. Only after PTTH secretion is the brain no longer necessary for a normal time course to the molt. Because PTTH secretion occurs in the brain, it is likely that the size-sensing mechanism involves the brain.

1.9 Candidate triggers for PTTH secretion

Different-sized strains of Manduca have different critical weights in the fifth instar, and the critical weight is always proportional to the initial weight of the instar. I found similar results in the earlier instars; the critical weight is always ~4.8 times the initial weight of the instar. This suggests that the critical weight might be determined by structures set at the beginning of the instar.
The most obvious new properties that are set at the beginning of each instar are associated with the new external skeleton that is deposited at each molt. These include the size of the head capsule, the spiracles and the tracheal system. The soft exoskeleton of the body wall is not fixed in size but grows by intercalation throughout the instar, thus it is not a good candidate for limiting growth. Although the head capsule does not grow and could thus constrain growth by limiting food ingestion rate, studies have found that absorption in the gut, not ingestion rate, is the rate limiting step for growth in *M. sexta* (Woods and Kingsolver, 1999). Therefore, the head capsule does not provide an obvious constraint to growth and is an unlikely candidate for a size-sensing mechanism.

The spiracles and tracheal system deposited at each molt could constrain growth by limiting oxygen delivery. In insects, the tracheal system delivers air primarily by diffusion directly to every cell in the body. In the late stage of each larval instar, the respiration rates of *M. sexta* caterpillars come close to the maximum oxygen delivery rate allowed by the tracheal system of that instar. Greenlee and Harrison (2005) show that freshly molted *Manduca* larvae are able to maintain oxygen delivery to match metabolic need at low oxygen levels (5%), but
larvae nearing the molt could not maintain adequate gas exchange at only mildly hypoxic conditions (15%) (Greenlee and Harrison, 2005). Therefore, oxygen limitation could be the functional constraint to which molting is a solution.

Similar constraints have been observed in grasshoppers. Given that that tracheae could be limiting oxygen supply and that insects seem to be nearing the maximum delivery rate of oxygen, we hypothesized that critical weight in each instar could be the size at which oxygen delivery becomes compromised.

Studies in other insects, such as beetles, corroborate this hypothesis. Greenberg and Ar (1996) found that Tenebrio molitor (beetles) larvae reared in hypoxic conditions had a greater number of molts, and conversely that those reared in hyperoxic conditions had a reduced number of molts, relative to control (normoxic) larvae (Greenberg and Ar, 1996). Loudon (1988, 1989) also found that Tenebrio molitor larvae underwent additional molts when reared in hypoxic (10% O2) conditions (Loudon, 1988; Loudon, 1989). The larvae reared in hypoxic conditions attained a smaller final body size than controls in spite of additional molts, because their growth rates were reduced. This is consistent with the idea that oxygen limitation is the constraint to which molting is a solution, and hypoxic larvae run into this constraint at smaller sizes.

A large body of work shows that oxygen levels influence body size in insects (Dudley, 1998; Frazier et al., 2001; Greenberg and Ar, 1996; Harrison and Haddad, 2011; Kaiser et al., 2007;
Lane, 2002; Loudon, 1988; Loudon, 1989; Tenney, 1985). Hypoxia not only reduces body size in most species in which this has been studied (Harrison et al., 2006), but also constrains the evolution of increased body size by limiting the variation available to selection (Klok and Harrison, 2009; Peck and Maddrell, 2005). Very large and very active insects have dealt with the constraints of a tracheal system by evolving large internal air sacs and active mechanisms for ventilating their tracheal system, thus reducing the length of the path over which oxygen needs to diffuse to reach internal tissues (Chapman, 1998; Wigglesworth, 1930). It has been suggested that the evolution of gigantic insects in the Paleozoic was associated with a high level of atmospheric oxygen (Dudley, 1998; Harrison et al., 2006; Kaiser et al., 2007). Nevertheless, the physiological mechanisms by which oxygen levels influence body size are poorly understood.

1.10 Hypoxia hypothesis

At the beginning of the instar, the tracheal system is large enough to supply sufficient oxygen to the tissues. As the larva increases in size and its metabolic demands for oxygen increase, the oxygen delivery rate asymptotically approaches a ceiling determined by the dimensions of the tracheal system. In contrast, demand increases as body mass increases. Therefore, the ratio of supply to demand decreases as the larva progresses through the instar. The critical weight could be sensed when the ratio of oxygen supply to demand has decreased to a critical ratio. Oxygen limitation could be the physiological mechanism by which the larva senses its size and initiates molting.
Figure 11: Hypoxia hypothesis.

Figure 12: The hypoxia hypothesis, another visualization
In contrast to previous authors who argue that the critical weight is controlled by the prothoracic gland and the rate at which it secretes ecdysone, I hypothesize that:

1) oxygen supply is a functional constraint to which molting is a solution, and hypoxic larvae sense that constraint at a smaller size.
2) The size at which the oxygen delivery becomes insufficient to meet demand sets the critical weight of the larva and initiates the endocrine cascade that leads to the molt.

Two fundamental questions that need to be answered: (1) what is the trigger for the PTTH signal; (2) what determines the prothoracic gland’s competence to respond to PTTH and secrete ecdysone?

1.11 Recapitulation

The goal of this research is to find the signal to molt, a size-sensing mechanism in *Manduca*, that has the following characteristics:

1) is probably sensed in the brain,
2) is not nutritionally triggered, although it requires adequate nutrition to function properly,
3) sheds light on the functional relevance of larval-larval molts,
4) is a relational mechanism rather than an "absolute size" sensor; e.g. it should work based on the changing relationships between body parts or between the body and the environmental context in which it functions.
5) Must explain the observation that the critical weight is proportional to the initial weight of the instar.

The hypoxia hypothesis is consistent with all the conditions listed above. If the hypoxia hypothesis for critical weight is true, we should observe the following:
1.11.1 Tracheal morphology.

The hypothesis is based on the assumption that tracheae do not grow during the instar but increase in size discretely at each molt. Therefore, tracheae should be more densely distributed on freshly molted caterpillars, and become more sparsely distributed as the larva progresses through the instar. They should become densely distributed again after each molt.

1.11.2 Respirometry.

If the tracheae do not grow within instars but increase in size discretely at each molt, the tracheal dimensions (set at the beginning of the instar) impose a ceiling on the maximum oxygen consumption rate. The respiration rate should asymptotically approach this ceiling as the larva progresses through the instar. The ceiling constraint should be relieved at the molt.

1.11.3 Critical weights.

If the critical weight of each instar occurs at the size at which the ratio of supply to demand of oxygen has decreased by a critical factor, one should be able to manipulate the critical weight by altering ambient oxygen levels. In low oxygen levels larvae should attain the critical ratio of supply to demand at a smaller size, and therefore molt an effectively reduced critical weight.

These three predictions will be experimentally tested in the following chapters.
2. Tracheal Morphology and Growth Pattern

2.1 Introduction

The hypoxia hypothesis for molting hinges on the idea that tracheae do not grow within instars; they must increase in size discretely at each molt. This chapter aims to determine whether the pattern of tracheal development in Manduca is consistent with the hypoxia hypothesis by looking at how the tracheal morphology changes within and between instars.

Wigglesworth noted that "in view of the fact that the entire tracheal system is lined by an unbroken cuticular membrane, it is difficult to see how new air-filled tracheal tubes could be formed in the absence of a moult" (Wigglesworth, 1954). Wigglesworth observed that this was the case in Rhodnius, where the changes in the tracheal system during the instar are restricted to migration of tracheoles in the tissues depending on oxygen needs (Wigglesworth, 1954).

The sum of the areas of the cross-sections of the branches at any hierarchical level of the tracheal system is approximately equal to the cross-sectional area of the main tracheae (Krogh, 1941). This morphology is functionally important because "The rate of diffusion along a tube is proportional to its cross-section. If the total cross-section of the branches grew greater as they divided, the cross-section of the main trunk would still remain limiting. If the converse were true, the terminal branches would limit diffusion" (Locke, 1954). When the sums of cross-sectional areas are constant at all levels of branching, there is no branching level of the tracheal system that is rate-limiting relative to the others.

Tracheal growth in Manduca will be examined in this chapter in relation to growth of the body. The dorsal gut tracheae were chosen because they are the most easily visible and are consistently identifiable (therefore allowing comparisons from one individual to another). I also examined the tracheae supplying the brain, the organ that initiates the hormonal molting cascade.
I examined the thickness of homologous transverse tracheae. The tracheal mass of fifth instar larvae increases by an order of magnitude, approximately in step with the increase in total body mass (pers. comm., Bryan Helm, SICB 2011). This prompted me to investigate whether the apparent discrepancy between the increase in tracheal mass concurrent with little apparent growth in tracheal size could be explained by an increase in the thickness of the tracheae due to addition of endocuticle.

Finally, I investigated the tracheal volume to test whether the tracheae

2.2 Materials and Methods

2.2.1 Tracheal morphology

Tracheae were examined using two imaging methods. First, the larvae were anesthetized under CO2 and the tracheae were stained. To stain the tracheae, I filled them under vacuum with a cobalt solution, which was then precipitated as black cobalt sulfide. I dissected the larvae dorsally and observed whole body mounts at low magnification to compare the gross pattern of tracheation early and late in the instar. Low magnification images were taken with the Olympus DP17 camera attached an Olympus SZX16 stereo-microscope, using the Olympus Microsuite Biological Suite software for Imaging Applications (Olympus, Minneapolis, MN).

Second, I dissected tissue from the dorsal midline of the midgut with accompanying tracheae from unstained larvae and mounted them on a microscope slide. These preparations were photographed with a Hammamatsu Orca-ER C4742-95 digital camera (Sewickley, PA) attached to a Leica DMRBE microscope (Bannockburn, IL) compound microscope, and Simple PCI imaging software (Sewickley, PA). The tracheal area on the high magnification images was estimated using the threshold tool in Image J.

2.2.2 Tracheal sections.

To examine possible changes in tracheal thickness, I made sections of homologous transverse tracheae (connecting two spiracles) in early and late fifth instar larvae. I dissected
out the transverse tracheae of two early fifth instars (<2g) and two late fifth instars (>10g). Then I put the tracheae in progressively higher % ethanol solutions (from 50%, 70%, 95%, 100%, 100% + Orange G stain, 50% ethanol + 50% hemo-D, 100% hemo-D; 30 minutes for each solution). The stain colored the tracheae a bright orange color. The tracheae were left overnight in hemo-D on the mixing plate. Then the tracheae were put into paraffin (50% hemo-D, 50% paraffin, then 100% paraffin). The paraffin was kept in an oven at 60 degrees C. The tracheae were left in 100% paraffin in the oven overnight. The tracheae in paraffin were poured into a mold and cooled at room temperature.

The microtome was used to cut thin sections of the paraffin with the tracheae embedded in it. The thin sections were placed on microscope slides. The paraffin was removed using Hemo-D, and then I used Permount to mount a coverslip on the slide. These preparations were photographed with a Hammamatsu Orca-ER C4742-95 digital camera (Sewickley, PA) attached to a Leica DMRBE microscope (Bannockburn, IL) compound microscope, and Simple PCI imaging software (Sewickley, PA).

2.2.3 Tracheal volumes

Tracheal volumes were estimated using the water displacement method: Caterpillars of third (N=18), fourth (N=24) and fifth (N=23) instars were anesthetized under CO₂, weighed on a microbalance (0.0000 gram precision), and then submerged in soapy water. They were placed in a vacuum jar attached to a vacuum pump, and the pump was turned on for 15 minutes to suck the air out of the tracheae. Subsequently, the vacuum was released, and the tracheae were allowed to fill with water for 1-2 minutes. A vacuum was pulled a second time for 10 minutes and then released. The caterpillars were removed from the water and dried with paper towels, and then weighed. The difference in weight is the estimate of the tracheal volume (1 gram of water = 1 mL of volume).
2.3 Results

2.3.1 Gut tracheae

The tracheae of early fourth and fifth instar larvae are more densely distributed than those of the late fourth and fifth instars, which suggests that the tracheae spread out but do not grow as the rest of the body grows during each instar. The tracheae of the late fourth instar are more similar to the tracheae of the late fifth instar than to the early fifth, and there is a discontinuous change in the tracheal system at the molt from the fourth to the fifth instar. The images show that the tracheae of the early fourth and fifth instars are highly folded and curled whereas those of the late fourth and fifth are taut and much less densely distributed. The curled tracheae in the early part of the instar represent "built-in slack" to allow for growth.
Figure 13: Tracheae are densely distributed early and sparsely distributed late in each instar.

In the fifth instar, the caterpillar increases in size from 1.2 grams to about 10 grams, so mass increases almost 10-fold. Therefore, the linear dimension of the caterpillar should increase by $10^{1/3}$ or 2.15 times, and the area should increase by $10^{2/3}$ or ~4.6 times. If the tracheae do not grow, but the area of the gut epithelium has increased by 4.6 times, then the area of the tracheae on the late fifth instar should be $1/4.6$ times, or approximately 20-25%
of the area of the tracheae on the early fifth instar. For a given unit of area of gut epithelium, the area occupied by tracheae in a late fifth instar should be approximately 20-25% of that in an early fifth instar larva.

Table 1: Area of gut tissue occupied by tracheae as a function of age in instar.

<table>
<thead>
<tr>
<th></th>
<th>Average mass (g)</th>
<th>St. dev. mass</th>
<th>Average % occupied by tracheae</th>
<th>St. dev. % area occupied by tracheae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Late fourth (N=6)</td>
<td>1.20</td>
<td>0.19</td>
<td>9.70</td>
<td>0.96</td>
</tr>
<tr>
<td>Early fifth (N=6)</td>
<td>1.85</td>
<td>0.22</td>
<td>30.40</td>
<td>5.57</td>
</tr>
<tr>
<td>Late fifth (N=6)</td>
<td>10.70</td>
<td>1.00</td>
<td>12.39</td>
<td>0.93</td>
</tr>
</tbody>
</table>

The area occupied by tracheae in a late fifth instar is 40% of the area occupied by tracheae in an early fifth instar, which is more than predicted (20-25%) but still a significant decrease from the initial distribution of tracheae. Some error may be introduced by the fact that the gut wall is thicker in the late fifth than early fifth. Based on these observations, the supply per unit of tissue at the end of the instar is reduced to 40% of what it was at the beginning of the instar.

2.3.2 Brain tracheae

The brain increases slightly in size from the late fourth to the early fifth instar, but there is no difference in size between the brain in the early fifth instar and the late fifth instar. Even though body mass increases almost 10 fold, the brain remains the same size (2 brain lobes, about 1 mm wide, throughout the instar). The tracheae supplying the brain do not change noticeably.
Figure 14: Brain of late fourth (top), early fifth (middle), and late fifth (bottom) instars. Tracheae are stained black with cobalt sulfide.
2.3.3 Tracheal sections

Brian Helm (SICB, 2011) presented data suggesting that the mass of tracheae increases within the fifth instar of *Manduca*, suggesting growth of the tracheal system within the instar, and this seems incompatible with the hypothesis that tracheae do not grow within instars, but only increase in size at each molt. One possible explanation could be that within instars, tracheae increase in thickness but not in volume. The following histological sections of homologous transverse tracheae from an early and late fifth instar larva show that the walls of the tracheae from late fifth instar larvae are significantly thicker than those from early instar larvae.

![Figure 15: Location of spiracles and transverse tracheae.](image)

Dorsal midline of gut

Spiracle and associated tuft of tracheae

Transverse trachea
Figure 16: Thickness of the transverse tracheae increases during the instar
The increase in thickness of the tracheal walls could account for the fact that the mass of tracheae increases within each instar, but the volume does not.

2.3.4 Differences between the early instars and the final instar
In the fourth instar, the tracheal epithelium begins to detach a little after halfway through the instar, around attainment of critical weight. The pictures of the stained tracheae on whole mount specimens show the stained (dark) lumen of the tracheae, surrounded by a clear tube that is presumably the tracheal structure that will become functional in the subsequent instar. The images also show some tracheal tubes with a shiny white filling, which could represent either an air bubble (possible), or the tracheal epithelium detaching itself in preparation for the molt.
Figure 17: In the late fourth instar (0.8g), the old tracheae begin to detach in preparation for the molt.

Figure 18: In the late fifth instar, apolysis is not observed.
Apolysis of the tracheal lining happens relatively early in the fourth instar, around critical weight. In contrast, apolysis of the tracheal lining is not observed in the fifth instar even after critical weight. This difference is probably due to the fact that in the fifth instar, there are two pulses of ecdysone: the first pulse does not cause a molt but induces the wandering stage and pupal commitment of the epidermis, and the second pulse induces apolysis and the pupal molt.

### 2.3.5 Tracheal volume

To quantitatively determine if the tracheal system grows within instars, I measured the volume of the tracheal systems within and across instars.

**Table 2: Mean tracheal volumes in the third, fourth and fifth instars**

<table>
<thead>
<tr>
<th></th>
<th>Mean tracheal volume (mL)</th>
<th>St. dev.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Third (N=18)</td>
<td>0.0036</td>
<td>0.0032</td>
</tr>
<tr>
<td>Fourth (N=24)</td>
<td>0.0315</td>
<td>0.0221</td>
</tr>
<tr>
<td>Fifth (N=23)</td>
<td>0.4207</td>
<td>0.1731</td>
</tr>
</tbody>
</table>
Figure 19: Initial tracheal size scales isometrically with initial mass of instar

Figure 20: Tracheal volume does not increase within instars, but increases discontinuously at each molt.
The initial tracheal volume scales with 0.19 Mass$^{1.04}$, which corresponds to isometric scaling. The tracheal volume decreases slightly in the late part of the third and fourth instars, but not in the late part of the fifth instar. The decrease in tracheal volume in the late third and fourth instars is probably caused by apolysis of the tracheal lining in the second half of the third and fourth instars, which I observed in the dissections. Apolysis of the tracheal lining, in preparation for the molt, causes the tracheal volume to become functionally smaller. Lease et al. (2006) also find that tracheal volumes decrease as grasshoppers progress through an instar, and attribute this to compression of the tracheae by tissue mass.

2.4 Summary

These observations indicate that:
The tracheae thicken but do not increase in volume in keeping with the rest of the body during each instar;
The tracheae change discontinuously at the molt;
The curls in tracheae of early stage larvae may be “built-in slack” to accommodate growth.

The predicted area occupied by tracheae at the beginning and end of the fifth instar is quantitatively consistent with the hypothesis that tracheae do not grow during the instar. The detachment of the tracheal lining occurs during the second half of the instar in the early instars, but does not occur after critical weight in the final instar, because the larva must first go through the wandering stage before shedding its cuticle and tracheal system at the pupal molt.

There are several reasons for oxygen to become increasingly limited as the larva progresses through the instar:
1) As the body mass increases, demand for oxygen will also increase.
2) As body mass increases, it may compress the tracheae, constricting air flow.
3) If the tracheae stretch as the body grows, the distances from the spiracles to the tips of the spiracles may increase, and oxygen flow will be reduced by the inverse of distance (Fick's law of diffusion).
4) As the larva prepares for the molt, the old tracheae detach in preparation to be shed, which results in a functionally smaller tracheal system.

5) If the increase in body mass is mediated by cell size (Kato et al., 1987; Kato and Riddiford, 1987), the dimensions of larger cells could constrain the diffusion rates of oxygen into cells.

### 2.4.1 Looking ahead

1) How do tracheal dimensions (length, cross-sectional area, and volume) scale relative to body mass, across instars?

2) Given the type of morphology and growth discussed above, what signatures can be expected on the respiratory pattern? The fixed size of the tracheal system should impose a ceiling on the respiration rate.

3) What does the scaling of tracheal volume with body mass imply for the rate of oxygen diffusion from the spiracles to the tips of the tracheoles? How would the volume of the tracheal system have to scale with body mass in order to reset itself completely to the same oxygen supply safety margin at each subsequent instar? To answer these questions, it is necessary to calculate the rate of diffusion of oxygen into the tracheae based on Fick's law of diffusion.
3. Tracheal Dimensions and Maximum Theoretical Supply Rate of Oxygen

3.1 Introduction

The fixed size of the tracheal system within each instar imposes a ceiling on the maximum rate of oxygen that can be supplied to the cells by diffusion. In this chapter, I measure the scaling relationships of structures set at the beginning of the instar. Using quantitative measurements of the tracheal system and Fick’s law of diffusion, I derive the theoretical constraints on the oxygen supply rate imposed by the supply system within each instar.

The following scaling relationships are studied:
1) scaling of head capsule width with initial body mass of instar,
2) scaling of the length of tracheae with body mass,
3) scaling of spiracle cross-sectional area with body mass,
4) scaling of volume of the tracheal system with body mass at the beginning of each instar.

Head capsules are a structure like the tracheal system that is set at the beginning of each instar, and does not grow within the instar. Therefore, head capsules and tracheae might be expected to scale similarly, unless the functional importance of these structures dictates that they should scale differently. The functional importance of head capsule is to provide the structures that are able to withstand the forces exerted by the mandibles on food. The length of mandibles and the surface area of muscle attachment are probably the functionally relevant dimensions. The functional importance of the tracheal system is to provide a path for oxygen to diffuse to each cell in the body. The functionally relevant dimensions are the path length of diffusion (tracheal length) and the tracheal cross-sectional area.

Based on the estimated dimensions of the tracheal system and Fick's law of diffusion, I make a rough estimate of the maximum theoretical rate of diffusion of oxygen to the tracheoles determined. The theoretical calculation suggests that oxygen delivery rates might
present a constraint on final body size. The theoretical predictions will be evaluated against measured metabolic rates presented in the following chapter.

3.2 Materials and Methods

3.2.1 Head capsule widths

Caterpillars were photographed in dorsal view under an Olympus SZX16 stereo microscope and photographed with the Olympus DP17 camera. The width of the head capsule was measured using computer imaging software. Head capsule widths were measured at different stages within instars to compare within and between instar variation, and to ascertain that head capsules do not grow within instars.

3.2.2 Tracheal length

Caterpillars of third and fifth instars were placed in dorsal view under an Olympus SZX16 stereo microscope and photographed with the Olympus DP17 camera. The width of the caterpillars at the first abdominal spiracle was measured using using the Olympus Microsuite Biological Suite software for Imaging Applications (Olympus, Minneapolis, MN).

3.2.3 Spiracle cross-sectional areas

Caterpillars of second, third, fourth and fifth instars were anesthetized under CO$_2$, and then placed on their side under the Olympus SZX16 stereo microscope and photographed with the Olympus DP17 camera. Larvae at various stages in each instar were measured, in order to confirm that spiracle diameters do not change within instars but increase discretely in size between instars. The Olympus Microsuite Biological Suite software for Imaging Applications (Olympus, Minneapolis, MN) was used to highlight the area of the spiracle and calculate the area of the prothoracic, first abdominal, and tail spiracles (um$^2$).
3.3 Results and Discussion

3.3.1 Head capsule widths

Head capsule widths do not change within instars but do change across instars.

Table 3: Head capsule widths by instar.

<table>
<thead>
<tr>
<th></th>
<th>First</th>
<th>Second</th>
<th>Third</th>
<th>Fourth</th>
<th>Fifth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>852</td>
<td>1346</td>
<td>2132</td>
<td>3655</td>
<td>6100</td>
</tr>
<tr>
<td>head capsule width (um)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>St. dev.</td>
<td>21</td>
<td>49</td>
<td>96</td>
<td>123</td>
<td>132</td>
</tr>
<tr>
<td>N</td>
<td>35</td>
<td>28</td>
<td>46</td>
<td>29</td>
<td>10</td>
</tr>
</tbody>
</table>

Figure 21: Scaling of head capsule widths with initial mass of instar (Error bars representing the standard deviations are included, but are very small).
The head capsule width scales with Initial Mass$^{0.25}$. Under isometric scaling, we would expect a linear dimension to scale with volume$^{1/3}$ (or mass$^{1/3}$). Therefore the head capsule scales slightly hypoallometrically with the initial mass of instars.

### 3.3.2 Tracheal length

The tracheal length was estimated by approximating the caterpillar as a cylinder. Because the tracheae go from the spiracles to the dorsal and ventral midlines along the gut, the path from spiracle to tracheole was estimated as $1/4$ of the circumference of the cylinder. This estimate of tracheal length is a slight overestimate, because not all tracheae reach all the way to the midline.

![Schematic cross-section of a caterpillar](image)

*Figure 22: Schematic cross-section of a caterpillar, showing that the longest tracheae need to be approximately 1/4 of the circumference of the caterpillar.*
To estimate the circumference of the caterpillar, I measured its diameter at the level of the first abdominal spiracle. The diameter was used to calculate the circumference of the caterpillar.

![Figure 23: Scaling of tracheal length with body mass.](image)

The diameter of the caterpillar scales with Mass^{0.35}, which is very close to the 0.33 scaling exponent we would expect in the case of isometric growth.

### 3.3.3 Spiracle cross-sectional areas

Spiracles are functionally important because they are the point of entry of oxygen into the tracheal system. Because larvae breathe by diffusion, the dimensions of the spiracles determine the maximum oxygen flow rate.

Tracheole sprouting can direct oxygen flow to oxygen-deficient tissues, but the spiracle size determines the maximum rate of oxygen diffusion into the system as a whole. Therefore, oxygen supply can be redistributed during the instar, but can be increased only at the molt.
Because spiracle cross-sectional areas do not change within instars, only between instars, the way in which the tracheal size scales with mass at each molt will be an important determinant of maximum oxygen flow at each instar.

Table 4 shows the average spiracle cross-sectional areas (measured in micrometers$^2$) and standard deviations, for three different spiracles.

Table 4: Average cross-sectional areas (µm$^2$) of three types of spiracles for instars 2-5.

<table>
<thead>
<tr>
<th></th>
<th>Average PG spiracle</th>
<th>St. dev. PG</th>
<th>Average 1$^{st}$ abdominal spiracle</th>
<th>St. dev. Abd. Sp.</th>
<th>Average tail spiracle</th>
<th>St. dev. Tail</th>
</tr>
</thead>
<tbody>
<tr>
<td>Second</td>
<td>5545.45 (N=6)</td>
<td>908</td>
<td>1940 (N=6)</td>
<td>376</td>
<td>13978 (N=6)</td>
<td>1811</td>
</tr>
<tr>
<td>Third</td>
<td>18965 (N=6)</td>
<td>1887</td>
<td>11229 (N=6)</td>
<td>1817</td>
<td>36603 (N=6)</td>
<td>3464</td>
</tr>
<tr>
<td>Fourth</td>
<td>86406 (N=10)</td>
<td>14393</td>
<td>64866 (N=5)</td>
<td>14932</td>
<td>121524 (N=9)</td>
<td>23532</td>
</tr>
<tr>
<td>Fifth</td>
<td>471860 (N=6)</td>
<td>54833</td>
<td>395786 (N=6)</td>
<td>40413</td>
<td>594230 (N=9)</td>
<td>80378</td>
</tr>
</tbody>
</table>
Figure 24: Scaling of spiracle cross-sectional areas with body mass

The differences between the prothoracic, abdominal and tail spiracles are greatest in the second instar, and they become almost the same size by the fifth instar. The allometric coefficient varies depending on the spiracle; whether this indicates functional differences between spiracles is not clear.

Under isometric growth, a surface area scales with mass$^{2/3}$ (or volume$^{2/3}$). Therefore, if the spiracles grew isometrically with the body, the scaling exponent for spiracle cross-sectional area would be 0.67. The measured scaling exponents range from 0.73 to 1.03, so spiracle cross-sectional area scales hyperallometrically with mass.

The head capsule scales with mass$^{0.30}$ (Figure 20), which is approximately what we expect based on a null model of isometric growth. In contrast, the spiracle cross-sectional areas have larger exponents than the 0.67 exponent expected based on the null model of isometric growth.
If ingestion of food was rate-limiting for growth, we would expect the head capsule and mouthparts to scale hyperallometrically with body mass, because larger mouthparts would relieve the supposed constraint on ingestion rate. The fact that mouthparts scale isometrically whereas spiracle cross-sections scale hyperallometrically suggests that ingestion of food is not the rate-limiting constraint on growth, but oxygen delivery might be.

### 3.3.4 Estimated tracheal volumes

If the tracheal system has the same cross-sectional area at each level of branching (Krogh, 1941; Locke, 1954; Wigglesworth, 1954), then its volume could be predicted based on the shape of a cylinder whose length is the average path length of trachea, and whose diameter is the diameter of the spiracle. Assuming that the tracheal system can be approximated as a cylinder, I calculate its volume as the product of the cross-sectional area and the length. This estimate will be compared with measurements of tracheal volume to determine if it is possible to approximate the tracheal system as a cylinder.

The length of the tracheal system scales with $6115.8 \times \text{Mass}^{0.35}$ (length in $\mu$m, mass in g).

The cross-sectional area of the tracheal system scales with $105.52 \times \text{Mass}^{0.86}$ (ranging between 0.7 and 1).

A *Manduca* caterpillar has nine spiracles on each side of the body, so a total of 18 spiracles. Therefore, the volume of the tracheal system should scale with:

\[
\text{number of spiracles} \times \text{spiracle cross-sectional area} \times \text{length of tracheae} = 18 \times 6115.8 \times 105.52 \times \text{Mass}^{0.35+0.86} = 38 \times 109 \times \text{Mass}^{1.21} \text{(in micrometers}^3\text{)}, \text{or 0.038 Mass}^{1.21} \text{(in mL)}.
\]

The tracheal volume should scale approximately as $0.038 \times \text{Mass}^{1.21}$ (mass in grams, and volume in mL). This corresponds roughly to the measured scaling of average tracheal volume with average mass in each instar: $0.046 \times \text{Mass}^{1.16}$ (Figure 18)
3.3.5 Theoretical maximal oxygen delivery rate at each instar

For the purpose of this rough estimate, I assume that the tracheal system is a cylinder.
The rate at which oxygen molecules reach the tips of the tracheoles is given by Fick's law of
diffusion in cylinders, which describes how the oxygen supply will change as a function of
the length of the cylinder.

Fick's law:

\[ J = D \Delta C A / L \]

\( J = \) rate of mass transport \((\text{mass}/\text{time})\)
\( D = \) diffusion coefficient \((\text{area}/\text{time})\)
\( \Delta C = \) difference in concentration between the two ends of the cylinder \((\text{mass}/\text{volume})\)
\( A = \) cross-sectional area \((\text{area})\)
\( L = \) length of the cylinder \((\text{length})\)

**Dimensional analysis of Fick’s Law**

\[ J = D \Delta C A / L \]

Mass/time = area/time * mass/volume * area/length
\[ \text{Mass/time} = \frac{\text{area}}{\text{time}} \times \frac{\text{mass}}{\text{volume}} \times \frac{\text{area}}{\text{length}} \]

(dimensionless) = \( \frac{\text{area}^2}{\text{volume} \times \text{length}} \)

\( \text{area} = \text{length}^2 \)
\( \text{volume} = \text{length}^3 \)

(dimensionless) = \( \frac{\text{length}^3}{\text{length}^4} = 1 \)

The dimensions of Fick’s law are consistent.

In words, Fick’s law says that the amount of oxygen that will diffuse per unit time is
proportional to the product of (1) the diffusion coefficient \(D\), (2) the area \(A\) of the slab, and
(3) the concentration gradient across the slab (Vogel, 2003).
From the measurements presented above, we also know that:

\[ A \sim 3.5 \times 10^5 \text{ (Initial Mass)}^{0.86} \]
\[ L \sim 4.8 \times 10^3 \text{ Mass}^{0.35} \]

Therefore, the maximum rate of oxygen flow (mass/time) scales as follows:

\[ \text{O}_2 \text{ rate} \sim \text{Mass}^{0.86 \times 0.35} \]
\[ \text{O}_2 \text{ rate} \sim \text{Mass}^{0.50} \]

Therefore, the rate at which a given mass of oxygen travels per unit time scales approximately with Initial Mass\(^{0.50}\). This means that the theoretical ceiling of oxygen consumption rate for each instar scales approximately with the square root of the initial mass of the instar.

Even though the volume of the tracheal system scales hyperallometrically with mass, the oxygen delivery rate is not reset to the same size-specific rate at the beginning of each instar.

The hypoallometric scaling of maximum oxygen delivery rate could be a constraint on final size, because molting is not an indefinite solution to the problem of oxygen delivery to a growing body.

There will be a size beyond which molting will not relieve the constraint on oxygen delivery. This suggests that hypoxia could play a role not only in triggering one larval molt to the next but also in constraining the final body size.

### 3.4 Further questions

A) What determines the quantitative factor by which the larva increases the volume of its tracheal system at each molt?

The scaling relationship could be the result of opposing selective forces, some which push the tracheae to be larger, while others constrain them to remain small:

Hyperallometric scaling of tracheal volume means that as body size increases, tracheal volume occupies a larger proportion of total body volume, leaving less space for other
organs. The hyperallometric scaling thus constrains final body size, at the point where other body organs cannot fit in the space left unoccupied by the increasingly large tracheae. Also, the structure could become functionally compromised: as the tracheae occupy an increasing proportion of total body volume, the body volume will become hollow and therefore subject to breaking. For these reasons, it is advantageous to keep the tracheal system relatively small.

In contrast, as body size increases, metabolic demand increases, and the tracheal delivery system must be able to supply those increasing demands. The larger the tracheal system, the less constraint there is on metabolic rate. For this reason, it is advantageous to build a tracheal system as large as functionally possible.

There are functional reasons to keep the tracheal system small, and other functional reasons to build it large. The size of the tracheal system may be optimized to satisfy metabolic demand while leaving enough space for other organs.

B) Given that the spiracles scale hyperallometrically, and the theoretical respiration rate scales hypoallometrically, what is the larva sensing or measuring that makes the critical weight proportional to the initial weight?

Critical weight clearly scales almost perfectly isometrically with the initial weight of each instar. What about the structure of the tracheal system ensures this proportionality? What about the morphology or development determines the factor of 4.8 that relates the initial weight to the critical weight?
4. Respiration rates become constrained after critical weight

4.1 Introduction

Tracheae do not grow during each instar but increase in size discretely at the molt. This pattern of growth in discrete steps imposes a ceiling on the oxygen delivery rate at each instar. The respiration rate should reach this ceiling asymptotically in every instar. If the critical weight is the size at which oxygen delivery becomes constraining for further growth, the respiratory rate should plateau at the critical weight.

To test the hypothesis more specifically, I measured respiration rates and asked the following questions:

1) Is there a change in the pattern of oxygen consumption at the critical weight?
2) What is the maximum oxygen capacity permitted by the tracheal dimensions of each instar? Do the measured maximal respiratory rates match the theoretical respiratory ceilings calculated in the previous chapter?
3) How does respiration change in hypoxic conditions?
4) How does the oxygen consumption rate (metabolic rate) scale with body mass? The scaling of metabolic rate with body mass has been the subject of many empirical and theoretical studies (Banavar et al., 2001; Banavar et al., 1999; Glazier, 2010; West et al., 1997; West et al., 1999). Most of these studies focus on how supply-side constraints account for the hypoallometric scaling of metabolic rate relative to body mass.

West, Brown and Enquist and others have argued that the geometry of supply networks and the growth pattern of fractally branching networks constrain metabolic rates to scale hypoallometrically relative to body size, with an exponent of 0.75 (Banavar et al., 2001; Banavar et al., 1999; West et al., 1997; West et al., 1999). In their work (West et al., 1997), they assumed that supply networks grow in concert with the rest of the body and that the proportion of body volume occupied by the network remains constant. These assumptions
do not apply to growing larvae: because the tracheal system of insects grows discretely (increasing only at each molt), growth of the body and growth of the tracheal system are decoupled.

The dissociation between the increase in metabolic demand and the growth of supply networks makes insects a particularly good system for examining the contributions of supply-side constraints and intrinsic (unconstrained) metabolic demand in determining the measured metabolic rate. The metabolic rate at the beginning of each instar reflects the intrinsic metabolic demand unconstrained by supply. If metabolic rate is constrained by supply, then the size-specific metabolic rate should decrease within each instar as supply becomes increasingly constraining, and it should increase immediately after the molt to the next instar, when supply is no longer constraining.

4.2 Materials and Methods

I measured oxygen consumption for third, fourth and fifth instar larvae daily, from head capsule slip to head capsule slip, and to gut purge in the fifth instar. Measurements were done in a temperature-controlled insect rearing room at 25°C.

To do the respirometry, a potassium hydroxide respirometer was built as follows:
1) Make a 20% solution of KOH. The KOH absorbs all the CO₂ exhaled by the caterpillar.
2) Soak a wad of brown tissue paper and put at the bottom of a plastic falcon tube; cover with plastic/nylon mesh, to separate the larva from the KOH.
3) Record weight of larva, and then place larva in the tube.
4) Put a 1mL pipette through a rubber cork, and then close the tube with the cork.
5) Insert a drop of colored water into the 1mL pipette and wait a few minutes until the drop does not move, then record the position of the drop.
6) Record the level of the water every minute as the larva consumes oxygen.
Figure 25: Schematic representation of the respirometer.

Because the caterpillar is enclosed in an airtight container,

The respirometry data represent the amount of O$_2$ consumed (mL) per unit of time (min). I plotted these data as a function of the mass of the larva at the time at which the measurements were taken.
4.3 Results

4.3.1 Oxygen consumption before and after the critical weight

Oxygen consumption increases approximately linearly until the larva attains the critical weight. This is expected because as body mass increases, metabolic demand for oxygen increases, and the respiration rate is not yet constrained by supply. After critical weight, the...
oxygen consumption rate levels off. The slope of the respiration rate after critical weight does not differ from 0 in any of the measured instars (third, fourth, fifth). It is also more variable after the critical weight, which may reflect variation in the initial weights/tracheal dimensions of different individuals, and variation in each individual's ceiling of maximum oxygen delivery rate.

The change in the respiratory pattern after attainment of the critical weight is consistent with the hypothesis that the critical weight is the size at which the larva begins to sense a constraint on oxygen availability, and cannot continue to increase oxygen delivery rate in spite of increasing demand.

4.3.2 Oxygen delivery ceilings

What is the maximum oxygen delivery rate permitted by the tracheal dimensions of each instar? How does this compare to the theoretical oxygen delivery rate estimated by the dimensions of the spiracles?

Figure 27: The maximum respiration rate attained in each instar scales hypoallometrically with initial mass of instar
The ceiling of respiration rate scales with initial size of instar to the power of 0.85, which is higher than the predicted 0.5 scaling predicted from the morphological scaling pattern and theoretical calculations based on Fick's law. The discrepancy could be due to the fact that I assumed that the tracheal system resembles a cylinder when in fact it is a branching system. It could also be due to the fact that the scaling of spiracles is different for the prothoracic, first abdominal, and tail spiracles, and I did not take into account this variation in my estimate. Nevertheless, the theoretical and empirical scaling exponents both indicate that the maximal oxygen delivery rate scales hypoallometrically with body mass, which means that oxygen supply rates could constrain final body size.

Figure 28: Oxygen consumption in the third, fourth and fifth instars.

Respiration rate scales with Mass$^{0.80}$. 

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The fact that size-specific respiration rate is not reset at each instar implies that there is an upper size limit beyond which the respiratory ceiling will not be high enough to ensure continued growth. There are at least two ways in which respiratory structures could constrain final size:

1. Spatial constraints due to hyperallometric scaling of tracheal volume—the volume available for other body organs decreases as body size increases, until they cannot be reduced anymore;

2. Oxygen delivery constraints due to the hypoallometric scaling of oxygen flow rate—the ceiling of oxygen delivery does not scale isometrically with body mass, which means that as the larva increases in mass, its tissues must make do with less oxygen than before.
The decrease in size-specific growth rate within instars as well as across instars suggests that the respiration rate per unit mass is not reset to the same value at the beginning of each instar. Because there is no constraint on supply at the beginning of each instar, this graph suggests that the intrinsic size-specific demand for oxygen decreases across instars. In other words, one gram of fifth instar tissue has a lesser intrinsic demand for oxygen than one gram of third instar tissue.

I hypothesize that early in the instar, respiration rate is determined by the intrinsic demand of tissues, and as the larva progresses through the instar, respiration rate becomes increasingly constrained by the supply structures. The decrease in size-specific metabolic rate across instars, and specifically at the beginning of each instar when supply is not constraining, suggests that supply-side constraints cannot account for the decrease in size-specific metabolic rate. Instead, the intrinsic demand of cells appears to decrease through ontogeny.

The intrinsic demand for oxygen is driven by all cellular activities that require ATP. This intrinsic demand should be reflected in the density and activity of mitochondria, which are the site of ATP production and oxygen consumption. The higher the density of mitochondria, the higher the capacity for ATP production, and the higher the size-specific metabolic rate. (How the density and activity of mitochondria is regulated to match the demand for ATP is another interesting question, that is beyond the scope of this work).

What could account for the decrease in size-specific metabolic rate across instars? Two possibilities come to mind:
1) All cells equally decrease their metabolic rate.
   In the silkworm, *Bombyx mori*, the mitochondrial activity per unit mass decreases across instars (Blossman-Myer and Bursgren, 2010). I will test this hypothesis in *Manduca* by measuring mitochondrial activity.
2) As the body increases in size, the organs with lower metabolic rates occupy a relatively larger proportion of total body mass, than organs with higher metabolic rates. In the fifth instar of *Manduca*, the metabolic rate of the gut is approximately three times as high as that
of the fat body (Vandock et al., 2010). Therefore, if the gut occupies a smaller proportion of total body mass, this change could explain the leveling off of metabolism observed in the respiration measurements. This hypothesis will be tested by weighing the relative masses of total body and gut, and then determining if the proportion of gut to total body mass decreases with each instar.

4.4 Discussion

4.4.1 Summary

1) Respiration rate increases linearly with mass before the critical weight, and levels off after critical weight. This observation is consistent with the oxygen limitation hypothesis and with the idea that the critical weight is the size at which oxygen delivery becomes constraining for further growth.

2) The size-specific metabolic rate decreases across instars, and is not reset to the same initial value at the beginning of each instar. Because supply is not constraining at the beginning of each instar, this suggests a decrease in the intrinsic size-specific metabolic rate.

3) Because the maximal rate of oxygen delivery for each instar scales hypoallometrically with mass, molting is only a partial solution to oxygen limitation, and oxygen limitation may constrain final body size.

Most theoretical studies seeking to explain patterns of metabolic scaling focus on supply-side constraints, rather than on the determinants of intrinsic metabolic demand and what might cause this to change as body mass increases.

However it appears that demand, not supply, is the determinant of tracheal size. Indeed, within an instar, tracheole sprouting is sometimes observed in response to hypoxia. The degree of tracheal sprouting is functionally important as a signal to determine how much the tracheal system needs to increase in size at the next molt. Indeed, Wigglesworth (1954) showed that when he implanted a corpora allata (tissue with high oxygen demand) in a fourth instar *Rhodnius* larva, the insect adapted by sprouting new tracheae around the hypoxic tissue, and "At the next moult, the new 5th instar tracheae had formed to supply the implant."
The increase in diameter of the main tracheae over that upon the control side was detectable all the way back to the spiracle. Thus the proportions of the tracheal system as a whole are adjusted in response to new terminal growth." Wigglesworth concluded that "it has been shown that hypertrophy of the terminal tracheae results in an appropriate increase in diameter all the way to the spiracle."

4.4.2 Review of supply-side hypotheses for metabolic scaling

West, Brown and Enquist (1997, and subsequent papers on allometric scaling) argue that the hypoallometric scaling of metabolic rate is a necessary constraint imposed by the pattern of growth of a fractally branching supply network. They derive the 3/4 power scaling law based on the following premises: the terminal branches of the fractal supply network are invariant with body size, and the energy required to circulate fluid through the system is minimized. Other variants on supply network geometry and the constraints it imposes on metabolic rate have been proposed (Banavar et al., 2001, and others), showing that the 3/4 law could exist in other types of networks (not just fractally branching). All of these argue that metabolic rate is constrained by the geometry of the supply network.

Others have argued that supply is constrained by the geometry of cell size (Kozlowski et al., 2003). Kozlowski et al. (2003) suggested that cell size, and by extension genome size, could explain scaling patterns of metabolic rate. When body size increases through an increase in cell size, the cell surface-to-volume ratio decreases. One unit of body mass will contain less cell membrane, and because most cellular energy is used to maintain ion gradients across membranes, the mass-specific metabolic rate will decrease. If all metabolic costs were related to cell surface, and size expansion was realized exclusively through cell size, then the standard metabolic rate would increase in proportion to body volume to the 2/3 power. When body size increases solely through an increase in cell number, then the metabolic rate per unit of body mass stays identical, and total metabolic rate scales proportionally with body mass. Because only part of the metabolic costs are related to cell surface and part to cell volume, the power will be higher than 2/3, but less than 1. Kozlowski et al. (2003) argued...
that the structure of the supply system is likely to be plastic, adjusting to meet the maximum metabolic demands.

Chown et al. (2007) also argued that the scaling of insect metabolic rate is inconsistent with the nutrient supply network model (e.g., West, Brown & Enquist 1997) which predicts a universal 0.75 scaling exponent, because intraspecific scaling exponents varied from 0.67 to 1.0 in their survey of 391 insect species from 16 Orders (Chown et al., 2007). Furthermore, they found that in species where body size increases were completely accounted for by an increase in cell number, the metabolic rate scaled isometrically with body mass, whereas in species where both cell size and cell number contributed to body size increase, the scaling exponents for metabolic rate were less than 1, consistent with the Kozlowski hypothesis.

The size and ploidy level of epidermal cells in *Manduca* increases through the larval and fifth instars (Kato et al., 1987). Cell division is caused by the ecdysone pulse before the molt, so the ploidy level is reduced at the molt. This suggests that the average cell size increases during each instar, and then is reduced at the molt. Therefore, large cells might be becoming hypoxic due to surface area/volume constraints.

At the beginning of the instar, the tracheal system has just been enlarged, and the cells have just undergone a wave of mitoses so they are relatively small and have a high surface area/volume ratio. Therefore, the metabolic rate is not constrained by either tracheal size or cell size. The metabolic rate measured at the beginning of each instar is therefore the intrinsic demand-driven metabolic rate, unconstrained by supply. The size-specific metabolic rates at the beginning of each instar decrease with each subsequent instar, even though they are not constrained by supply. Therefore, the decrease in size-specific metabolic rate as mass increases must be the result of a decrease in intrinsic metabolic demand.

The bottom line is that larger caterpillars consume less oxygen per unit mass than small ones, and this decrease is observed in absence of supply-side constraints. The WBE and the Kozlowski/Chown ideas are both supply-based hypotheses, although they differ over where
in the supply system the constraint on metabolic rate arises. However, it is also possible that the metabolic rate is driven by demand-side phenomena, or by changes in metabolic efficiency.

The fact that the intrinsic, unconstrained size-specific metabolic rate decreases across instars suggests that supply-side constraints cannot explain the scaling of metabolic rate. It suggests a change in the intrinsic metabolic rate, which is determined by mitochondrial activity. For example, in response to the increasing constraint on supply that occurs as the larva progresses through an instar, the mitochondria could become more efficient (produce more ATP per unit of oxygen consumed and reduce proton leakage), the cells could make do with less ATP per unit of mass, or the organs that have high metabolic demand could occupy a relatively smaller proportion of the total body mass. Once this adjustment in metabolic efficiency has been made, it would not be affected by the molt. In other words, the larva could become progressively more efficient at metabolizing energy, and these gains in efficiency would be conserved even as the absolute constraint on supply is relieved at each molt.

4.5 Conclusion

Consistent with Greenlee & Harrison (2005), who found that larvae come close to the ceiling on oxygen delivery late in each instar, the caterpillars approach the theoretical respiratory limit towards the end of the instar. Scaling of metabolic rate is inconsistent with the network supply model of metabolic scaling, and also inconsistent with the cell volume/surface area supply model. Instead, it appears that the intrinsic size-specific metabolic demand decreases throughout ontogeny.

The observations that SSRR is lower at the beginning of successive instars, and that the decrease is not accounted for by constraints on supply, suggest that this decrease must be explained by a change on the demand-side. I propose the following hypotheses (not mutually exclusive) to account for changes in the intrinsic oxygen demand:
1) Body composition changes: the body contains specialized cells with different intrinsic metabolic requirements; the organs with relatively low metabolic rate occupy a higher proportion of total body mass as the larva increases in size.

2) Each cell decreases its metabolic rate individually (all cells decrease their metabolic rate by the same degree).

These hypotheses will be tested in the following chapter.
5. The interaction between demand-side processes and supply-side constraints explains the hypoallometric scaling of metabolic rate

5.1 Introduction

The scaling of metabolic rate has been the subject of many empirical and theoretical studies (Banavar et al., 2001; Banavar et al., 1999; Chown et al., 2007; Glazier, 2010; Kozlowski et al., 2003; West et al., 1997; West et al., 1999; White et al., 2009). Not only is there controversy over the exact value of the scaling exponent; the reasons why metabolic rate scales hypoallometrically with body mass are incompletely understood.

West, Brown and Enquist (1997) (WBE) argued that the hypoallometric scaling of metabolic rate is a necessary constraint imposed by the pattern of growth of a fractally branching supply network. They derive a \( \frac{3}{4} \) power scaling law based on a distribution network with the following properties: the terminal branches of the fractal supply network are invariant with body size, the energy required to circulate fluid through the system is minimized, and the volume of the network occupies a constant proportion of the total body volume. Other variants on supply network geometry and the constraints it imposes on metabolic rate have been proposed (Banavar et al., 2001, and others), showing that the \( \frac{3}{4} \) law could exist in other types of networks (not just fractally branching). All of these studies are based on the idea that metabolic rate is constrained by the geometry of the supply network.

Others have argued that metabolic rate is constrained to scale hypoallometrically due to the geometry of cell size (Kozlowski et al., 2003). Kozlowski et al. (2003) suggested that cell size, and by extension genome size, could explain scaling patterns of metabolic rate. When body size increases through an increase in cell size, the cell surface-to-volume ratio decreases. One unit of body mass will contain less cell membrane, and because most cellular energy is used to maintain ion gradients across membranes, the mass-specific metabolic rate will decrease. If all metabolic costs were related to cell surface, and size expansion was realized exclusively through cell size, then the standard metabolic rate would increase in proportion to body volume to the \( \frac{2}{3} \) power. When body size increases solely through an
increase in cell number, then the metabolic rate per unit of body mass stays identical, and total metabolic rate scales proportionally with body mass. Because only part of the metabolic costs are related to cell surface and part to cell volume, the power will be higher than 2/3, but less than 1. Kozlowski et al. (2003) argued that the structure of the supply system is likely to be plastic, adjusting to meet the maximum metabolic demands.

Chown et al. (2007) showed that intraspecific scaling exponents varied from 0.67 to 1.0 in their survey of 391 insect species from 16 Orders (Chown et al., 2007), which is inconsistent with the universal ⅔ scaling exponent predicted by WBE. They found that in species where body size increases were completely accounted for by an increase in cell number, the metabolic rate scaled isometrically with body mass, whereas in species where both cell size and cell number contributed to body size increase, the scaling exponents for metabolic rate were less than 1, consistent with the Kozlowski hypothesis.

The WBE and the Kozlowski/Chown ideas are both supply-based hypotheses; they differ about the level in the supply system at which the constraint on metabolic rate arises. However, it is also possible that the metabolic rate is driven by demand-side phenomena. There is relatively little theory about what determines the intrinsic metabolic demand of cells/tissues/bodies. Intrinsic metabolic demand is driven by all cellular activities that require ATP. The more mitochondria a cell has, the more oxygen it can consume, and the higher the metabolic rate. Cytochrome c oxidase (COX) is a rate-limiting mitochondrial enzyme, and is required for ATP production from oxygen. Its activity is an accurate indicator of a tissue's metabolic potential (e.g., the maximum amount of oxygen that can be transformed into energy per unit of time).

5.1.1 Why insects are a good model system to study metabolic scaling

In insect larvae, the tracheal system grows discretely: it is fixed within instars and increases in size only at each molt. The body, in contrast, grows continuously. Thus, the growth of oxygen supply structures is decoupled from that of the tissues that demand oxygen. Early in
each instar (soon after the molt), the supply structures are over-built and supply exceeds demand; conversely, late in each instar, the supply structures become constraining for further growth due to inadequate supply relative to demand. Because of this mode of growth, insects are particularly well suited to study the effects of growth-induced changes in supply and demand on metabolic rate.

In *Manduca sexta* larvae, respiration rate levels off after the critical weight in each instar, which means that larvae become oxygen limited late in each instar (Greenlee and Harrison, 2005; Callier & Nijhout, 2011). Early in each instar, the tracheal system is large relative to the body, and respiration rate is not constrained by supply. Thus early in each instar the metabolic rate is primarily driven by intrinsic demand for oxygen, which is determined by the rate at which mitochondria can process oxygen. The rate limiting mitochondrial enzyme is cytochrome oxidase (COX). As the larva progresses through an instar, demand for oxygen increases, without a concomitant increase in supply. The larva thus becomes increasingly constrained, and the metabolic rate late in each instar represents the limit of the constraint on metabolic rate. After the molt, the metabolic rate once again represents the intrinsic demand of the tissue, unconstrained by supply.

To understand how the size-specific metabolic rate decreases across instars in *Manduca*, I obtained a developmental timeseries of (1) the proportion of total body mass accounted for by different tissues; (2) the metabolic rate of these tissues. Because Vandock et al (2010) found that the size-specific metabolic rate of the midgut is approximately three times higher than that of the fat body, I decided to focus on the gut tissue of *Manduca*.

To determine the proportion of gut mass relative to total body mass, I weighed the dry (empty) gut and the dry body (without gut or frass). To determine the contribution of mitochondrial density to the decrease in size-specific metabolic rate of *Manduca*, I used the same technique as Blossman-Myer and Burggren (2010) to measure the cytochrome c oxidase (COX) activity in different tissues from the second to the fifth instar.
Prediction. If gut proportion is the factor that accounts for the change in metabolic rate, then we would expect the gut proportion of total biomass to decrease. In contrast, if a decrease in mitochondrial activity accounts for the decrease in metabolic rate, then we would expect the size-specific mitochondrial activity to decrease. These two mechanisms are not mutually exclusive, and the decrease in metabolic rate could be due to a combination of these factors.

5.2 Materials and Methods

5.2.1 Tissue measurements.

Larvae were anesthetized under CO$_2$, weighed and dissected. The gut was removed, emptied, and weighed to the nearest 0.01g. The wet weight of the carcass (without the gut) was also recorded. The gut and carcass were placed on a tared aluminium foil and placed in a drying oven at 65°C for 48 hours (until the weight stabilized). The dry weights of guts and carcasses were recorded to the nearest 0.1 mg.

5.2.2 Mitochondrial isolation and cytochrome c oxidase measurements

5.2.2.1 Mitochondrial extraction (BioChain kit # KC010100)

1) Third instar larvae were weighed and then anesthetized with CO$_2$ and dissected. Gut was removed, emptied, rinsed in water, and weighed.

2) Gut tissue was rinsed in PBS for 10 minutes, and then transferred to 1.5 mL Eppendorf tubes on ice. 1 mL of mitochondria isolation buffer was added to each tube. Tissue was disrupted by sonication for 30 seconds.

3) The samples were centrifuged at 1000 rpm for 12 minutes. Pellet was discarded.

4) Supernatant was then centrifuged at 13000 rpm for 16 minutes, and the pellet was collected (supernatant discarded).

5) The pellet was resuspended in 500 uL mitochondria isolation buffer, and steps 3 and 4 were repeated.

6) Pellet was resuspended in 20 uL mitochondrial storage buffer.
7) 10 uL of each sample was set aside for the protein assay. 100 uL lysis buffer with 1x protease inhibitors was added to each sample.

5.2.2.2 Protein assay

1) Protein was determined using the BCA Protein Assay Kit (Pierce Product #23223; Rockford, IL). 50 parts of Reagent A (carbonate buffer containing bicicinonic acid (BCA)) and 1 part of reagent B (cupric sulfate solution) were mixed to create a working solution.
2) 200 uL of the working solution was added to each tube set aside in step 7.
3) The tubes were incubated at 37 degrees C for 30 minutes to allow the color to turn from apple green to purple.
4) The samples were diluted in spectrophotometer cuvettes: 700 uL of PBS was mixed with 200 uL of the sample.
5) The absorption of the diluted sample was read in the spectrophotometer at 562 nm.

5.2.2.3 Cytochrome c oxidase activity assay (BioChain kit #KC310100)

1) Spectrophotometer was set to read absorbance at 550nm.
2) Spectrophotometer was zeroed with 850 uL enzyme assay buffer, 100 uL enzyme dilution buffer, and 50 uL ferrocytochrome substrate solution.
3) The samples were prepared as follows: 850 uL enzyme assay buffer, 80 uL enzyme dilution buffer, and 20 uL of the sample. The cuvette was placed in the spectrophotometer. 50 uL ferrocytochrome substrate solution was added to the cuvette to initiate the reaction, and then absorbance was read at 5 second intervals for 60 seconds.
4) The rate of change of absorbance was calculated. The rate of the reaction indicates the cytochrome c oxidase activity in the tissue.

5.2.2.4 Calculation of mitochondrial activity

The absorbance of each sample was read at 5 second intervals for 60 seconds. The rate of change of absorbance, which indicates enzymatic activity of cytochrome c oxidase, was determined by calculating the rate of change from t = 0 to t = 25s. This rate was then
divided by the absorbance of the sample determined by the protein assay, which is proportional to the amount of protein in the sample. The final number obtained indicates cytochrome c oxidase activity per gram of protein.

**5.3 Results**

In the third and fourth instars of *Manduca*, the empty gut mass is a constant proportion of total body mass: about 10-15% of total dry biomass. Thus the decreasing size-specific metabolic rate cannot be accounted for by a decrease in the proportion of the gut, a highly metabolically active tissue.

In the fifth instar, however, the empty gut mass decreases from about 15% of total dry body mass (for larvae up to about 4 grams) to 8% of total body mass (larvae >10 g). Thus the proportion of total body mass represented by midgut is almost halved during the fifth instar. The decrease in the proportion of gut mass relative to body mass can at least partially account for the drop in size-specific metabolic rate in the fifth instar.

![Figure 30: Proportion of gut mass relative to body mass](image)
In *Bombyx mori*, the gut represents 10% of biomass in the third instar, 14% of biomass in the fourth instar, and 8% of biomass in the fifth instar (measured as dry gut weight and dry body mass; Blossman-Myer and Burggren, 2010, Figure 4F). It is uncertain at what masses within each instar these data were determined. However, the numbers for *Bombyx mori* and *Manduca sexta* are in the same ballpark (8-15%).

**Figure 31: Mitochondrial activity per unit of protein decreases across instars**

Blossman-Myer and Burggren (2010) found that in *Bombyx*, whole-body mitochondrial density decreases across instars. They found that COX activity was about 2.25 units per gram of body mass in the third instar, 1 unit in the fourth instar, and 0.5 units in the fifth instar (Blossman-Myer and Burggren 2010, Figure 6). Consistent with their results, my measurements show that mitochondrial density in the *Manduca* gut decreases as the larva increases in mass.

The density of COX decreases within and across instars, which indicates that there are fewer mitochondria per gram of protein as body mass increases. There are two possible explanations for this observation: (1) the mitochondria are becoming more efficient, by producing more ATP per unit of oxygen consumed; or (2) the body is becoming more efficient in its consumption, requiring less ATP to carry out living functions.
5.4 Discussion

In early instars, the decrease in size-specific metabolic rate is accounted for by a decrease in COX density; in the final instar, it is due to both a decrease in COX density and a decrease in the proportion of gut mass (highly metabolically active tissue) relative to body mass. Large larvae consume less oxygen per unit mass than small larvae, and this is due to a decrease in the size-specific COX activity. Larger larvae must either by produce more ATP per unit of oxygen consumed (by decreasing mitochondrial proton leakage), or by make do with less ATP per unit mass by becoming more efficient in ATP utilization.

5.4.1 Regulation of efficiency of ATP production

This brings up the question of how mitochondrial efficiency is regulated. Production of ATP can be decoupled from oxygen consumption by a family of uncoupling proteins that allow protons to leak from the outer to the inner membrane of the mitochondria, bypassing the ATP synthase channel (Boss et al., 2000; Rolfe and Brand, 1997). Thus, the efficiency of a mitochondrion can be modulated by regulation of its "leakiness" and mitochondria that leak a lot would require more oxygen than a "watertight" mitochondrion to produce the same amount of ATP.

Mitochondrial leakage may seem like a waste of energetic resources and oxygen. There are several proposed functions for proton leakage (Rolfe and Brand, 1997):

1. Proton leakage is means of heat production. Mammals in cold climates could use the mitochondrial proton leak to produce heat rather than ATP. This function is unlikely to apply to ectotherms such as Manduca.

2. Proton leakage increases the potential for regulation of ATP production. If ATP demand suddenly spikes, the uncoupling proteins can stop the leakage, and more ATP can be instantaneously produced; if ATP demand is low, the protons are permitted to leak. This advantage at the regulatory level may have a cost, however: Mitochondria are a source of
reactive oxygen species that are thought to contribute to ageing (Lane, 2002). Animals with more efficient mitochondria, e.g. mitochondria that produce lots of ATP and few reactive oxygen species per unit of oxygen consumed, will have a longer lifespan (Lane, 2002). This explanation could ostensibly apply to Manduca larvae, but does not explain why the efficiency should change across ontogeny. To explain the change across ontogeny, there would need to be a system that measures oxidative damage, and, as damage accumulates, adjust the rate of mitochondrial leakage accordingly.

5.4.2 What drives the increasing efficiency of metabolism as animals increase in size?

The fact that COX activity per unit mass decreases within and across instars could be accounted for by gains in mitochondrial efficiency in response to within-instar supply-side constraints. Within each instar, the larva increases in size so metabolic demand increases, but supply does not. This constraint induces metabolic changes in efficiency of ATP production or ATP consumption. These gains in efficiency would be retained across molts, thus leading to a decrease in COX density through ontogeny.

Within each instar, larvae become increasingly metabolically constrained. Larvae have evolved several solutions to the constraint on oxygen supply: (1) structural adaptations: enlargement of supply structures by molting; (2) biochemical adaptations to use oxygen more efficiently (less mitochondrial proton leakage, for example). The biochemical adaptations are conserved across molts, and this accounts for the decrease in the intrinsic demand for oxygen as the larva grows. Importantly, the biochemical and structural adaptations to oxygen constraints occur on different time scales; biochemical adaptations are rapid relative to structural adaptations.

The two adaptations to metabolic constraints that occur on different timescales could account for the general hypoallometric scaling of metabolic rate. Biochemical adaptations can occur rapidly in response to the geometrical constraints on oxygen supply associated with growth. In contrast, structural adaptations occur with a time lag (due to the time
necessary to build new supply structures). The gains in efficiency at the biochemical level are retained even after the structures are enlarged. This “ratcheting” mechanism could account for the generally observed decrease in the intrinsic metabolic demand of tissues as animals increase in size.

5.5 Summary

The size-specific metabolic rate decreases because the mitochondrial density decreases across instars. In the fifth instar, the decreasing proportion of highly metabolically active tissue (e.g., the gut) also contributes to the decrease in size-specific metabolic rate. The metabolic rate adjusted for cytochrome c oxidase activity accounts for the decrease in size-specific metabolic rate in the early larval instars, but the fifth instar has a different metabolic pattern, perhaps due to preparations for metamorphosis and a switch to lipids as primary oxidation substrate.

The fact that COX activity per unit mass decreases within and across instars could be accounted for by gains in mitochondrial efficiency in response to within-instar supply-side constraints. These gains in efficiency would be retained across molts, thus leading to a decrease in COX density through ontogeny. I hypothesize that this interaction between supply-side constraints and the mechanisms that regulate intrinsic demand could explain the hypoallometric scaling of metabolic rate.
6. A mathematical model for growth and molting

6.1 Introduction

In this chapter I derive a model for growth and molting built and parameterized based on measurements in previous chapters. The model will be a test of how well the mechanisms of growth and molting are understood in Manduca, and whether the known principles, when formalized into a mathematical framework, are sufficient to reproduce experimentally measured growth curves in normoxia and to predict growth under hypoxia. The model will be used to predict critical weights under hypoxia and these predictions will be compared to measurements in the following chapter.

6.1.1 Development of a mechanistic growth function

Although descriptive sigmoidal functions have been used to model growth in different organisms for a long time (von Bertalanffy, Gompertz, etc.), these do not represent the underlying mechanism of growth that I hypothesize is operating in Manduca. Instead of using an existing growth function, I chose to create a sigmoidal growth function for growth based on the changing relationship of the body and tracheal system in Manduca.

The model is inspired from the "Metabolic-level boundaries" hypothesis of Glazier (2010). His main idea is that when metabolic demands are high, metabolic scaling should be primarily limited by fluxes of resources, wastes, or heat across surfaces, which scale as $M^{2/3}$ (Glazier, 2010). In contrast, when metabolic demands are low and amply met by surface-dependent processes, metabolic scaling should be more related to the energy demand required to sustain tissues, which is directly proportional to tissue mass or volume (scales as $M^1$). Glazier assumes that the size-specific intrinsic metabolic demand is invariant with respect to body mass, which we know is not the case in Manduca. Nevertheless we begin with this model and tweak it later to accommodate this known pattern in Manduca.

In a freshly molted larva, the supply systems are more than adequate to satisfy the relatively low metabolic demand, so demand is determined by the energy required to sustain the
tissues. Furthermore, in a freshly molted larva, most cells have just undergone mitosis (triggered by the pulse of ecdysone that initiated the molt), so cells are diploid and relatively small, and diffusion is adequate to supply oxygen to the cells. Thus metabolism is not constrained by supply systems, and should scale with $M^1$.

In contrast, late in the instar, demand has increased but the dimensions of the tracheal system have not, and cells have undergone multiple rounds of endomitosis. Therefore, the tracheal supply system may impose a ceiling on the maximum oxygen delivery rate, and the delivery of oxygen to larger cells may be constrained by surface-area-dependent diffusion. The supply systems at multiple scales become increasingly constraining with respect to delivery of necessary nutrients, so respiration rate and metabolic rate (and therefore growth rate) scale with $\text{Mass}^{1-x}$, where $x$ increases as the larva progresses through the instar.

### 6.2 Model description

#### 6.2.1 Growth

1) Early in the instar, respiration rate is not constrained by surface-dependent processes, so it scales with $\text{Mass}^1$.

2) As the larva progresses through the instar, respiration rate asymptotically approaches the ceiling imposed by the dimensions of the tracheal system, so the scaling of respiration rate with body mass does not keep up with mass, and decreases through the instar.

3) I assume that growth rate scales with metabolic rate; the growth function is based on how metabolic rate changes through the instar.

4) $R_{\text{max}}$, the maximum oxygen consumption rate permitted by the tracheal system of given dimensions, is a function of the initial weight of the instar, because the dimensions of the tracheal system are set at the beginning of the instar and cannot increase until the next molt.

I formalize the growth pattern described above with the following growth equation:

$$
\frac{dM}{dt} = aM \left( \frac{R_{\text{max}} - bM}{R_{\text{max}}} \right)
$$
At the beginning of the instar, $b*M$ is small; therefore the exponent limit tends toward 1. As the larva grows larger, $b*M$ becomes large, the exponent decreases, and the growth curve levels off.

Table 5: Parameters for model of growth and molting

<table>
<thead>
<tr>
<th>Dimension</th>
<th>Meaning</th>
<th>Notes</th>
<th>Value</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>None</td>
<td>Non-dimensionalized Mass</td>
<td>Non-dimensionalization necessary for consistency of units</td>
<td></td>
</tr>
<tr>
<td>Rmax</td>
<td>Mass/time</td>
<td>Max. oxygen supply rate in the instar</td>
<td>Determined by the size of the tracheal system, which is set at the beginning of each instar</td>
<td>$0.0061M_o^{1.07}$ (normoxia) $0.0038M_o^{1.04}$ (hypoxia) $M_o$ represents the initial mass of instar</td>
</tr>
<tr>
<td>a</td>
<td>Growth constant</td>
<td>None</td>
<td>0.047-0.057</td>
<td>Fit to growth curves</td>
</tr>
<tr>
<td>b</td>
<td>Mass/time</td>
<td>Metabolic demand</td>
<td>0.00031 - 0.00049</td>
<td>Fit to growth curves</td>
</tr>
<tr>
<td>Critical Ratio</td>
<td>none</td>
<td>Point at which molt is triggered</td>
<td>0.17-0.25</td>
<td>Determined by experimentally measured critical weight</td>
</tr>
</tbody>
</table>
Figure 32: $R_{\text{max}}$ is the scaling of maximum oxygen flow with the initial mass of instar. Data is from Greenlee and Harrison (2005).

### 6.2.2 Molting

Due to the decoupled growth of tracheae and body, oxygen availability decreases through each instar. The molt is triggered when $(bM/R_{\text{max}}) > \text{Critical ratio}$, or when demand/supply exceeds the critical ratio. When the larva attains the critical ratio, either it triggers molting immediately, if a photoperiodic gate is open, or it triggers molting upon opening of the next photoperiodic gate.

I parameterized the model based on growth curves of control larvae (25°C, normal food, 16L:8D, normoxic). Below is the model fit to the growth trajectory of a cohort of control larvae. The black lines are measured growth curves and the yellow lines are simulated growth curves. To parameterize the model, I simulated growth curves for a wide range of parameter values, and then eliminated the simulated growth curves that did not fit the following features correctly: the initial size, the critical weight for each instar, the final size, and the duration of the growth period.
Figure 33: Measured (black) and simulated (orange) growth trajectories for normoxic larvae

The following graph shows the same growth data and simulations but transformed as size-specific growth rate. The size-specific growth rates decrease across instars, which indicates that molting does not reset the size-specific growth rate to the same initial value at the beginning of each instar. The model reproduces this pattern in the decrease if the size-specific metabolic rate across instars. The fact that the size-specific growth rate decreases across instars indicates that molting does not allow the larva to continue growing indefinitely; there will be a size at which the size-specific growth rate falls to zero, providing a theoretical limit on body size.
6.3 Model predictions

6.3.1 Predicting the shift in critical weight under hypoxic conditions

In hypoxic conditions, larvae should attain the critical ratio of supply to demand at a smaller size. Therefore, hypoxic larvae should have a reduced critical weight and initiate molting at a smaller size. I assume that (1) supply has been reduced, whereas the demand for oxygen is unchanged; (2) the critical ratio is unchanged in hypoxia. I expect that the larvae will molt at the size that corresponds to the same critical ratio, and that the size at which the critical ratio is reached will be smaller because Rmax has decreased.

For the first approximation, I assume that the intrinsic demand for oxygen is not modulated as a function of external oxygen tension. I simulate growth and molting in hypoxia by keeping all parameters estimated in Table 7 are unchanged, except Rmax: in hypoxia, Rmax scales as 

$$0.0038M_o^{1.04},$$

rather than

$$0.0061M_o^{1.07}$$

(data from Greenlee and Harrison, 2005).
To estimate the hypoxic critical weight, I solve the following equation for M:
\[ \frac{M}{R_{\text{max}}} = \text{critical ratio} \]

### Table 6: Predicted critical weights in hypoxia

<table>
<thead>
<tr>
<th></th>
<th>Normoxia</th>
<th>Hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>R_{\text{max}}</td>
<td>0.0061 (M_o^{1.07})</td>
<td>0.0038 (M_o^{1.04})</td>
</tr>
<tr>
<td>Predicted critical weight 4(^{\text{th}}) instar (g)</td>
<td>0.95</td>
<td>0.53</td>
</tr>
<tr>
<td>Predicted critical weight 5(^{\text{th}}) instar (g)</td>
<td>6.04</td>
<td>3.30</td>
</tr>
</tbody>
</table>

The model predicts that the critical weight of the fourth instar in hypoxia is 0.53g. The fourth instar larvae should therefore molt at or just above 0.53 g. The model predicts that the critical weight for the fifth instar in hypoxia is 3.30g, so fifth instar larvae should molt at or just above 3.30 g.

These predictions will be compared to the observed data from critical weight experiments presented in the following chapter.
6.3.2 A comment on the effect of temperature on body size

Insects have a smaller body size when reared in high temperatures and a larger body size when reared in lower temperatures. The reasons for this are not completely understood. One explanation is that temperature shortens the growth period more than it increases the growth rate, resulting in a smaller final size (Davidowitz et al., 2003). Another possible explanation is that temperature shifts the critical weight. Because metabolic demand is increased in high temperatures, the critical ratio of supply to demand will be reached at a slightly smaller body size, and therefore molting and/or metamorphosis will be triggered at a smaller body size. In contrast, in lower temperatures, metabolic demand is reduced, so the critical ratio of supply to demand will be reached at a slightly larger body size, and therefore molting and/or metamorphosis will be initiated at a larger body size.

The effect of temperature on critical weight has not been determined experimentally due to the small effect on size that is expected, and thus the large number of larvae necessary to use in order to have enough statistical power to demonstrate a shift in critical weight. With
respect to the model, the effects of temperature would be complicated because temperature would affect not only $R_{\text{max}}$ (because diffusion is faster in higher temperatures), but it would also affect the growth parameter, $a$, and the metabolic demand parameter, $b$.

### 6.4 Discussion

The model formalizes a metabolic boundaries hypothesis, as applied to ontogeny (rather than to inter-species comparisons, as the hypothesis was formulated in (Glazier, 2010)). The model is able to reproduce normoxic growth curves and predict the hypoxic critical weight in the fourth instar, using four parameters. These predictions will be compared to measurements presented in the following chapter.

*The limitations of the model.* It is not at present possible to estimate the model parameters experimentally because their physiological meaning remains too abstract. Also, I assume that the intrinsic demand for oxygen is independent of supply, whereas it is more realistic to expect that demand is modulated as a function of external oxygen tension. Correcting the model to include this process (which is complex and incompletely understood) would potentially complicate it more than improve it.

**$R_{\text{max}}$** represents the size of the respiratory system in the current instar and is determined by the initial size of the instar. However, this parameter does not shed light on the developmental process by which the tracheal system coordinates its growth from instar to instar; it is only a scaling parameter. The tracheal size of the next instar is likely determined by the deficit of oxygen sensed in the previous instar, but the process by which this is determined is not clear.

**$b$** represents the decrease in the scaling exponent of metabolism with mass as the larva progresses through the instar. It is not understood why the exponent does not reset to 1 at the beginning of each instar.
a is the growth constant and represents how metabolism is converted into body mass. The growth rate is a function of many environmental factors (temperature, nutrition, etc.) as well as intrinsic factors (genetics). The model does not explicitly factor in the contributions of these influences on growth rate.

**Critical Ratio** is the point at which molt is triggered. The setpoint at which there is not enough oxygen could be determined by the molecular affinity of oxygen to an enzyme (see Appendix for a summary of what is known about the biochemistry of oxygen sensing). It is not known how this threshold is determined. Nor is it known how the sensation of hypoxia, which is initially local, is transferred to the brain, where the decision to initiate molting is made.

Nevertheless, the model captures what is known about the processes of growth, metabolism and molting in *Manduca*. It was used to make predictions about the critical weight which will be tested against data in the next chapter. It will be used to make specific, quantitative predictions for how larvae should respond to hypoxic conditions when transferred to hypoxia in mid-instar (cf. Chapter 8), and therefore help to identify specific points in larval physiology that still need to be understood.
7. Critical weight is reduced in hypoxia

7.1 Introduction

If oxygen limitation is the cue to initiate molting, then we should be able to alter the critical weight by changing the external oxygen tension. In hypoxia, the supply of oxygen will be drastically reduced, whereas the intrinsic demand for oxygen remains the same (per unit mass). Therefore, the larva will attain the critical ratio of supply-to-demand at a smaller size, and should initiate molting at a smaller size. Conversely, larvae reared in hyperoxic conditions should reach their ceiling at a larger size and therefore have a larger critical weight.

In the previous chapter, I made quantitative predictions for the hypoxic critical weights in the fourth and fifth instars. To test these predictions, I measured growth curves and also did a classical critical weight experiment using starvation methods in normoxia (21% oxygen) and hypoxia (5% oxygen) for fourth and fifth instar larvae.

7.2 Materials and Methods

7.2.1 Normoxic critical weights

Larvae of the fourth and fifth instars were weighed to the nearest 0.01g at body masses between 0.40 and 1.40 g for the fourth instar and 4.00-9.00 g for the fifth instar, and about 50% were allowed to continue feeding while the rest were starved by removing their food and replacing it with a block of 3% agar. Larvae were checked daily for slippage of the head capsule (4th instars), or exposure of the dorsal vessel (5th instars). In *Manduca*, head capsule slippage occurs 13-16 hours after the secretion of ecdysone (Truman, 1972) in the 4th larval instar, and exposure of the dorsal vessel occurs approximately 12 hours after secretion of ecdysone in the 5th instar (Truman, 1972; Nijhout 1974). The mass at starvation, date of starvation and the date of head capsule slippage or exposure of the dorsal-vessel were recorded for each larva. The critical weight is calculated as the smallest weight at which there is no statistically significant difference between feeding and starved animals in the
timing of head capsule slippage or dorsal vessel exposure, respectively. Each point represents the results of a study using approximately 100 animals (fourth instar) and 140 animals (fifth instar). Starved larvae were given a block of agar to avoid dehydration.

7.2.2 Hypoxia and hyperoxia

Larvae were introduced into a plexiglass glove box (Scienceware, Pequannock, NJ). After larva were introduced, the glove box was sealed and flushed for 60 minutes with a mixture of either 5% oxygen: 95% nitrogen, or 40% oxygen: 60% nitrogen (National Welders, Durham, NC). After the appropriate level of oxygen was achieved the flow of gas was reduced to 250 mL/min for the duration of the experiment. A ToxiRaeII oxygen analyzer (San Jose, CA) was used to continuously monitor oxygen levels in the glove box. A ScoutPro electronic balance (Ohaus, Parsippany, NJ) within the glove box was used to weigh larvae daily to the nearest 0.01 grams. As with the normoxic starved larvae, hypoxic starved larvae were given a block of agar to prevent dehydration.

7.2.3 Critical weight in hypoxia

Critical weight experiments in hypoxia were conducted in the same manner as in normoxia except that larvae were in the glovebox under 5% oxygen. Approximately 70 animals (fourth instar) and 60 animals (fifth instar) were used for the critical weight experiment in hypoxia.

7.3 Results and discussion

7.3.1 Critical weights in normoxia

Normoxic critical weights were measured in normoxic second, third, fourth and fifth instars. All instars were found to have a size at which starvation no longer delays the timing of the molt, which is the operational definition of the critical weight. The critical weight of each instar in *Manduca* is always 4.5 times the initial weight of the instar, which suggests that the critical weight is actually determined by a structure that is set at the beginning of the instar.
Figure 36: Raw data for the critical weights in the second, third, fourth and fifth instars.
7.3.2 Growth in hypoxia and hyperoxia.

Hypoxic fourth instars molt in the range of 0.4-1.1 grams, with the peak of the distribution around 0.5g. Because the critical weight in the normoxic fourth instar is 0.95g, this shows that the majority of the hypoxic fourth instars must have molted below the normoxic critical weight. By definition, then, a large fraction of hypoxic fourths initiated their molt at a reduced critical weight.
Figure 38: Fourth instar larvae are much smaller when reared in hypoxia and slightly but significantly (p=0.008) larger in hyperoxia

In contrast to the hypoxic fourth instar larvae which are much smaller than the normoxic ones, hyperoxic fourth instar larvae are only slightly, but significantly, larger than normoxic larvae. The fact that hyperoxia did not have as dramatic an effect as hypoxia suggests that perhaps increasing the external oxygen tension is not sufficient to increase oxygen levels in the cells. Hypoxia creates a constraint at the level of oxygen delivery from the spiracles to the tracheoles, but hyperoxia does not relieve the constraint on growth.

Several explanations are possible. If each cell at the beginning of the instar has a tracheole to supply it, two constraints come into play: (1) as cell division progresses there are a greater number of cells "sucking" on the same tracheole; (2) as cells grow, the diffusion distances increase, and diffusion of oxygen to the entire cell becomes more difficult.

The constraint could lie at the level of diffusion of oxygen from the tips of the tracheoles to the cells, or it could be that the biochemical enzymes that process oxygen (such as cytochrome oxidase) are already saturated and cannot process oxygen any faster, even with additional oxygen supply. The effects of hyperoxia reported here are consistent with previous studies that have shown that the effects of hyperoxia are often variable and sometimes nonlinear (Frazier et al., 2001; Harrison et al., 2006).
7.3.3 What is the rate-limiting step in oxygen delivery?

If growth is constrained by oxygen supply late in each instar, the question still remains: at what level of the supply system does the constraint arise? Specifically:

a) Does it arise from the changing relationship between the tracheal system and the body mass, where the rate-limiting step would be the diffusion of oxygen from spiracle to tracheole? The tracheal system is fixed in size during each instar, whereas the body increases in mass, so it is possible that the tracheal dimensions become constraining for growth.

b) Does it arise from the liquid phase that separates the air in the tracheoles from the inside of the cell that it supplies (oxygen diffuses much more slowly through water than through air)? Larvae are able to control the amount of oxygen received by each tissue by adjusting the thickness of the liquid phase at the tips of the tracheoles (Wigglesworth, 1953). It so, it is possible that it is not the path of oxygen through the air in the tracheae that is rate limiting; rather it could be the liquid phase at the tracheoles.

c) Does the constraint lie in the geometry of growing cells, whose surface area-to-volume ratios could become constraining for diffusion as the cell increases in size? Chown et al. (2007) and Kowlowski et al. (2003) have suggested that as cells become larger, the surface area-to-volume ratio becomes constraining for exchange of oxygen, nutrients and waste. Several studies (Kato et al., 1987; Kato and Riddiford, 1987) have shown that in *Manduca*, cells grow and undergo endomitosis during each instar, and they divide late in the instar in response to the ecdysone pulse that triggers the molt. Thus, cells are relatively small during the early part of the instar, and become larger late in the instar. The larger cells maybe subject to constraints due to their decreasing surface area/volume ratios.

Constraints at different hierarchical levels of the supply system could operate at different times and size and in different environmental conditions. For example, early in the instar, the fluid at the tips of the tracheoles could be rate-limiting, but the dimensions of the tubes could become rate-limiting late in each instar. It is also possible that in hypoxia the tubes are rate-limiting, whereas in hyperoxia, the fluid at the tips of the tracheoles is rate-limiting.
The duration of instar is approximately the same in hypoxic and normoxic conditions. These observations contrast with normoxic, poorly fed larvae (40% diet), which molt at approximately the same size as control-fed larvae, but have a significantly extended instar duration because it takes much longer for them to attain the normoxic critical weight. These contrasting findings indicate that the effects of hypoxia are specific to hypoxia and not the result of general maltreatment.

Figure 39: Fourth instar duration in hypoxia, normoxia and hyperoxia

Table 7: Fourth instar durations in hypoxia, normoxia and hyperoxia

<table>
<thead>
<tr>
<th></th>
<th>Mean Instar duration</th>
<th>St. Dev.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypoxic (N=37)</td>
<td>3.2 days</td>
<td>0.50</td>
</tr>
<tr>
<td>Normoxic (N=29)</td>
<td>3.0 days</td>
<td>0.28</td>
</tr>
<tr>
<td>Hyperoxic (N=29)</td>
<td>3.1 days</td>
<td>0.41</td>
</tr>
</tbody>
</table>
Table 8: Average final size of instar under different conditions (mass in grams)

<table>
<thead>
<tr>
<th></th>
<th>Normoxic control</th>
<th>100% diet, 5% O₂</th>
<th>50% diet, normoxic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fourth</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>instar</td>
<td>1.46 (N = 15)</td>
<td>0.64 (N = 24)</td>
<td>1.32 (N = 57)</td>
</tr>
<tr>
<td>Fifth</td>
<td>11.25 (N = 10)</td>
<td>5.52 (N = 15)</td>
<td>8.69 (N = 25)</td>
</tr>
</tbody>
</table>

Table 9: Average instar duration (days) under different conditions

<table>
<thead>
<tr>
<th></th>
<th>Normoxic control</th>
<th>100% diet, 5% O₂</th>
<th>50% diet, normoxic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fourth</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>instar</td>
<td>3.04 (N = 23)</td>
<td>3.18 (N = 63)</td>
<td>4.40 (N = 57)</td>
</tr>
<tr>
<td>Fifth</td>
<td>4.83 (N = 23)</td>
<td>8.57 (N = 15)</td>
<td>7.24 (N = 25)</td>
</tr>
</tbody>
</table>

The effects of hypoxia are distinct from those of malnutrition or general maltreatment in the fourth instar. In contrast, poorly fed fourth instar larvae molt slightly smaller but are significantly delayed relative to controls. In the fifth instar, both hypoxic and malnourished larvae have significantly extended instar durations. This is likely due to the fact that JH levels tend to remain high in poorly growing larvae (Fain and Riddiford, 1975), and metamorphosis cannot be initiated until JH clears from the hemolymph. Thus, in the fifth instar both hypoxic and malnourished larvae probably have high JH levels for an extended period of time, which prevents them from initiating metamorphosis at the same time as controls.

Hypoxic fifths molted in the range of 4 to 8 grams, with the peak distribution around 5.5 grams. They molted only slightly below the normoxic critical weight of 6.5 grams. The observation that hypoxia had a more drastic effect in the fourth instar than in the fifth may reflect the additional regulation that occurs in the fifth instar in preparation for the molt. Larvae might be sensing hypoxia, but unable to initiate molting until JH clears from the hemolymph, which allows the transition to pupal development.
Figure 40: Hypoxic fifth instars are much smaller than normoxic controls. Hyperoxic fifth instars are not significantly larger than controls.

Figure 41: Fifth instar duration under hypoxia, normoxia and hyperoxia

Table 10: Fifth instar duration in hypoxia, normoxia and hyperoxia

<table>
<thead>
<tr>
<th></th>
<th>Mean Instar Duration</th>
<th>St. Dev.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypoxia (N=18)</td>
<td>8.2 days</td>
<td>1.26</td>
</tr>
</tbody>
</table>
The instar duration of hypoxic fifth instars was almost double that of control, normoxic fifth instars. This suggests that the fifth instar larvae took longer to attain the hypoxic fifth instar critical weight. Alternatively, because the fourth instar larvae are not molting delayed relative to controls, this suggests that a different mechanism might be operating in the fifth instars.

Hyperoxic fifth instars were not significantly larger than normoxic fifths, perhaps because raising the external oxygen level does not relieve the constraint on oxygen supply at the level where it lies; it could also be due to oxygen toxicity. Alternatively, the fact that there was no detected effect on size may be due to the additional regulatory mechanisms (JH regulation) that exist in the fifth instar.

Because hyperoxia did not have a dramatic effect on size, it would have been difficult to detect a shift in the critical weight in hyperoxic conditions. I chose instead to focus on the critical weight in hypoxia.

### 7.3.4 Critical weights in hypoxia

I attempted to determine the critical weight of 4th and 5th instar larvae under 5% hypoxic conditions by placing larvae in hypoxia at the beginning of the instar under study (4th or 5th instars were done separately), starving hypoxic larvae at various weights, and comparing their time to the next molt (4th instar) or to the gut purge (5th instar) with larvae that were feeding normally. In both instars, I found that starved hypoxic larvae were always delayed relative to fed hypoxic controls: there was no size at which starvation did not delay ecdysone secretion.
Figure 42: Critical weight experiments in normoxia and in hypoxia, in the fourth and fifth instars.

Starved larvae that failed to reach the critical size eventually molt by some mechanism that evidently does not involve size-dependent oxygen limitation. In both hypoxia and normoxia such larvae were delayed by the same amount of time.

A candidate mechanism that is independent of the brain and could account for these observations is provided by the “leaky prothoracic glands” hypothesis (Nijhout, 1976; Truman, 1972) (see section 1.6). It is a well-established fact in insect endocrinology that, although in the normal molting cycle the secretion of ecdysone is driven by PTTH from the
brain, molting and ecdysone secretion can occur in brainless animals, though usually with significant delay (Fukuda, 1944; Judy, 1972; McBrayer et al, 2007; Nijhout, 1976; Truman, 1972). The leaky prothoracic glands hypothesis proposes that the prothoracic glands secrete ecdysone at a basal rate, and when the effects of this low level of ecdysone accumulate above a threshold level, the molt is triggered (Nijhout, 1976).

I propose that during normal growth the brain triggers a molt when a critical size is attained, but under stressful conditions that inhibit growth, such as hypoxia or starvation, the critical size is never reached and the brain is not in control. Instead, either autonomous activity of the prothoracic glands, or stimulation of the glands by extra-cerebral factors, possibly insulin, induces the molt via the gradual accumulation of ecdysone.

### 7.4 Conclusion

One interpretation of the data presented in this chapter is that hypoxia decreased the critical weight, and hyperoxia did not increase it. The hypoxic fourth instar larvae molted below the normoxic critical weight, and at the same time as the normoxic larvae. Thus, by definition they must have initiated molting at a smaller size than normoxic larvae, implying a lower critical weight in hypoxia. The hypoxic fifth instar larvae molted smaller than the normoxic fifth instar larvae, and were also delayed relative to normoxic fifths, most likely because high levels of JH prevented the larvae from transitioning to the wandering stage even after reaching the critical ratio of supply to demand.
Figure 43: I tested whether lack of oxygen is the trigger that initiates the molting cascade

An alternative interpretation of the data is that, because the hypoxic starved larvae were delayed relative to hypoxic fed larvae at all sizes (in both the fourth and fifth instars), there is no critical weight, as it is operationally defined, in hypoxia. If this is the case, the hypoxic larvae are not molting via the critical weight mechanism but via an alternative, size-independent mechanism. One candidate mechanism for triggering size-independent molting is the leaky prothoracic glands (section 1.6), which will be further explored in Chapter 9.

The first interpretation is that hypoxia lowers the critical weight; the second interpretation is that hypoxia disrupts the critical weight mechanism altogether, inducing the larvae to use a size-independent back-up mechanism to trigger ecdysone secretion. If the first interpretation is correct, then larvae transferred to hypoxia in mid-instar should be induced to molt precociously; if the second interpretation is correct, then larvae transferred to hypoxia in mid-instar should be disrupted from the critical weight mechanism and instead rely on a size-independent back-up mechanism, which will make them delayed relative to normoxic controls.
8. Mid-instar transfer into hypoxia does not trigger molting

8.1 Introduction

To whether hypoxia triggers molting, fourth instar larvae were transferred from normoxia to hypoxia at increasing sizes through the fourth instar. I expected that larvae transferred into hypoxia would molt at a smaller size, and sooner, than their normoxic equivalents.

I simulated the instar durations and final sizes of larvae transferred into hypoxia by using the model presented in chapter 6, and modifying it to simulate growth in hypoxia. Larvae should grow normally in normoxic conditions until either of the following:

(a) They are transferred to hypoxia
   a. If transferred below the hypoxic critical weight, they continue to grow until attainment of the hypoxic critical weight and then secrete PTTH at the next photoperiodic gate.
   b. If transferred above the hypoxic critical weight, they are induced to secrete PTTH at the next photoperiodic gate.

(b) They attain the normoxic critical weight and are induced to secrete PTTH at the next photoperiodic gate.

I assume that the brain is always competent to secrete PTTH, and that the prothoracic gland is always competent to secrete ecdysone. Failure to induce molting upon transfer to hypoxia could indicate that the brain-PG axis is not always competent for signal transduction. For example, there could be a refractory period after a molt, during which the prothoracic gland cannot secrete ecdysone. In addition, there might be nutritional requirements: the prothoracic gland requires sufficient nutrition (sensed via insulin and TOR signaling pathways) to secrete a pulse of ecdysone. These factors are not taken into account in the simulations.
8.2 Predicted and measured size at L4-L5 molt

I used the model to predict final sizes under two hypotheses:

1. If critical weight is unchanged in hypoxia, final sizes should be at or above the normoxic critical weight.
2. If critical weight is lower in hypoxia, final sizes should be smaller than the normoxic critical weight, if larvae were transferred to hypoxia early enough in the instar.

![Figure 44: Predicted final sizes of fourth instars if the critical weight is (orange) or isn't (yellow) shifted under hypoxia](image)

The x-axis represents the weight at which it was transferred into hypoxia, and the y-axis represents the size at which it molted. The black dotted lines represent the normoxic and hypoxic critical weights. If the critical weight is not changed under hypoxic conditions, then all larvae should molt at or above the normoxic critical weight, as shown in yellow. If the critical weight is lower in hypoxia, then we would expect final sizes to be distributed as shown by the orange line. The interrupted lines are due to the photoperiodic gating of PTTH secretion.
Each white dot represents a larva that was transferred into hypoxia. The thick black line is the normoxic critical weight and the gray band is the range of normoxic final sizes. The data show that hypoxic larvae, when transferred sufficiently early in the instar, molt well below the normoxic critical weight, which suggests that they are initiating their molt at a reduced critical weight.
8.3 Predicted and measured instar durations

Next, I used the model to predict instar durations for larvae transferred to hypoxia. Larvae placed into hypoxia before the hypoxic critical weight should follow the hypoxic control curve, and initiate molting when they attain the hypoxic critical weight. I expected that larvae put into hypoxia after the hypoxic critical weight should be triggered to molt immediately or at the next photoperiodic gate; they should take longer than the hypoxic controls, but molt faster than the normoxic controls. Larvae put into hypoxia after the normoxic critical weight should molt on the same schedule as the normoxic controls.
Figure 47: Predicted instar durations upon transfer to hypoxia

The normoxic control represents the time to head capsule slippage for a larva at the size indicated on the x-axis. The hypoxic control represents the time to head capsule slippage for a larva that is hypoxic for the entire duration of the instar. The hypoxic larva is slightly delayed relative to normoxic, because it has a slower growth rate, and it molts at a smaller final size.

The yellow curve represents larvae that are transferred from normoxia to hypoxia at the size indicated on the x-axis. Before the hypoxic critical weight, mid-instar transfer larvae should molt on the same schedule as hypoxic controls. Conversely, after the normoxic critical weight, mid-instar transfer larvae should molt on the same schedule as the normoxic controls. When larvae are transferred to hypoxia above the hypoxic critical weight but below the normoxic critical weight, they should be induced to molt immediately, and therefore molt before the normoxic controls of equivalent size.

The data do not agree with the model prediction. Larvae transferred to hypoxia after the hypoxic critical weight but before the normoxic critical weight take much longer to molt than predicted by the model. Because these larvae are past the critical ratio of oxygen
supply/demand, it is possible that they are inducing a stress response that interferes with the molting process. Larvae transferred into hypoxia either before the hypoxic critical weight, or after the normoxic critical weight, behave as expected.

Figure 48: Data from larvae that were transferred to hypoxia in mid-instar

Transferring fourth instar larvae from normoxia into hypoxia does not accelerate the molt, but rather delays it significantly. Thus, the transfer to hypoxia does not immediately trigger the molting cascade.
If the transfer to hypoxia were the signal that initiated the molt, then there should be a constant time to the molt upon transfer to hypoxia, regardless of the size at which the larva was transferred. Instead, we observe that the larger the larva is at the time of transfer, the longer the duration of the instar, up until about 0.95 grams, which is the normoxic critical weight: after 0.95 g, the larvae have made the decision to molt, and neither hypoxia nor starvation affect the timing of the molt.

8.4 Discussion

8.4.1 A possible explanation

Mid-instar transfer to hypoxia may trigger a stress response in the development of the tracheal system. Tracheal cells for the next instar develop around the existing functional tracheal system, which does not grow during the instar. The new tracheal cells will form the new larger tracheal system, which will become functional when the old tracheae are shed during the molt. In normal development, the new tracheal cells have the entire instar to develop and form functional trachea.
In hypoxia, the new tracheal cells proliferate in order to produce a hypertrophied tracheal system for the next instar, to compensate for low oxygen levels. The proliferation of tracheal cells induced by the transfer to hypoxia will be proportional to the lack of oxygen; since larger larvae need more oxygen, their tracheal cells proliferate even more than the tracheal cells of smaller larvae. In addition, the molt is inhibited during the proliferation of tracheal cells, until the new tracheal system is sufficiently developed to become functional. Thus, a sudden transfer to hypoxia induces a compensatory proliferation of tracheal cells which must form a functional tracheal system before the molt can be triggered. Because the necessary time to make a functional tracheal system depends on the degree of proliferation of cells, and because larger larvae are induced to proliferate more tracheae than smaller larvae, the larger larvae (but still below the normoxic critical weight) have the longest delay in molting.

If this explanation (hypothesis) is correct, it suggests that the trigger to molt requires not only that the larva senses a lack of oxygen, but also that the new tracheal cells are sufficiently developed to become a functional tracheal system.

The hypoxia hypothesis alone does not explain the observations, so an additional or alternative molting mechanism must exist. Other evidence that an alternative molting mechanism exists comes from starvation experiments. Larvae that are starved before the critical weight eventually molt (even though they never attain the critical weight); this indicates that they must be molting via an alternative molting mechanism. This mechanism produces molting sizes and times that are highly variable, which suggests that this mechanism is not as tightly regulated as the critical weight mechanism. These are signatures of the leaky prothoracic glands mechanism (section 1.6).

8.4.2 Two alternative molting mechanisms

Larvae reared in hypoxia are not a uniform cohort, but are actually of two kinds, depending on when they were transferred to hypoxia. Larvae that are transferred before the hypoxic critical weight will grow until attaining the hypoxic critical weight, then molt using the critical
weight mechanism. Similarly, larvae that grow in normoxic conditions until they attain the normoxic critical weight use the critical weight mechanism. Larvae transferred in between the hypoxic and normoxic critical weights instead use the leaky prothoracic glands mechanism.

Larvae that use the critical weight mechanism will molt when they attain the hypoxic critical weight. Larvae using the leaky prothoracic gland mechanism will molt when they accumulate enough ecdysone credits, regardless of what size they are.

There are two molting mechanisms, one size-dependent, and one that depends on the sufficient accumulation of low levels of ecdysone. How and when do the larvae choose between which mechanism they will use?

Is it possible that the larva is always accumulating ecdysone-stimulating factor and that it will molt by whichever mechanism triggers the molt first. For example, under normal growth conditions, the larva attains the critical weight before accumulating a lot of ecdysone-stimulating factor, so the critical weight mechanism is used. If it is prevented from attaining the critical weight by starvation or ligation, then it is constrained to wait until it has accumulated enough ecdysone-stimulating factor.

The transfer experiment suggests that transferring larvae in mid-instar disrupts the critical weight mechanism and forces larvae to fall back on the leaky prothoracic glands to secrete enough ecdysone to eventually trigger a molt.

8.4.3 Prothoracicostatic factors

The fact that instar durations are extended dramatically in larvae that are transferred from normoxia to hypoxia suggests that the larva may be secreting factors that actively inhibit molting until conditions become more favorable. Several prothoracicostatic factors are known: the prothoracicostatic peptide (PTSP), Bommo-myosuppressin (BMS), and three
FMRF-amide related peptides (BRFa) that are produced by neurons that communicate directly with the prothoracic glands (Marchal et al., 2010; Van de Velde et al., 2009).

PSTP is a myoinhibitory peptide (inhibits various kinds of visceral muscles) isolated in *Bombyx mori* and it has the same sequence as a myoinhibitory peptide isolated from the ventral nerve cord of *Manduca* (Hua et al., 1999). PSTP interferences with PTTH-stimulated ecdysteroidogenesis, probably by inhibiting S6 kinase, which prevents S6 phosphorylation that is required for PTTH–stimulated ecdysteroidogenesis (Marchal et al., 2010). Bommo-myosuppressin has a stronger prothoracicostatic effect than PSTP (Yamanaka et al., 2005).

BRFa is expressed mainly in the neurosecretory cells of thoracic ganglia, whose neurons deliver BRFa directly to the prothoracic gland (Yamanaka et al., 2005).

### 8.5 Conclusion

The hypoxia hypothesis predicted that if larvae were transferred to hypoxic conditions in mid-instar, they would be triggered to molt. This was not what I observed. Instead, larvae transferred to hypoxia were even more delayed than control normoxic larvae, suggesting that the mid-instar transfer to hypoxia had interrupted the normal molting process.

From this I can conclude the following:

1. Putting larvae in hypoxia from the beginning of the instar may not affect their molting process, if molting is triggered by a timing mechanism, but a mid-instar transfer to hypoxia interrupts the normal molting process, and causes the larvae to molt via an alternative mechanism.

2. Hypoxia alone does not trigger molting, although it does affect the size at which larvae molt and the timing of molting.

The mechanism of leaky prothoracic glands will be further examined in the following chapter.
9. Leaky prothoracic glands

9.1 Delayed ecdysone peaks occur in brainless larvae

Nijhout (1976) showed that isolated abdomens of *Manduca* can be induced to molt if exposed to a sustained but continuously low ecdysteroid titer. Thus the ecdysone peak is not necessary to trigger the molt; if the ecdysone titer is above a relatively low threshold level for a sufficient amount of time, the molt will be triggered. Nevertheless, it is not known if the steady state infusions of ecdysone are representative of the leaky ecdysone phenomenon that occurs in suboptimally growing larvae.

In *Calpodes* (Dean and Steel, 1982), neck-ligated larvae show a peak of ecdysone, although it is delayed relative to the controls. A similar delayed peak in ecdysone titers has been observed in headless *Precis coenia* (Kremen and Nijhout, 1989; Kremen and Nijhout, 1998) and in PTTH-ablated *Drosophila* (McBrayer et al., 2007). Thus, in a variety of different species, the prothoracic gland can eventually secrete a peak of ecdysone in absence of the head (PTTH stimulation).

The question, then, is whether this peak is secreted autonomously by the prothoracic gland or whether it is stimulated by ecdysone-stimulating factors (other than PTTH, which is only found in the head). To understand the mechanism of leaky prothoracic glands it is necessary to measure ecdysone titers in starved and ligated larvae, and compare these titers to those of control larvae. Larvae starved or ligated below 6.5 g are forced to molt by leaky prothoracic glands because they never attain the critical weight. Any differences between starved and ligated larvae will be due to the activity of the brain.

9.2 The role of the brain

9.2.1 The brain is unnecessary for ecdysone secretion, but is required for correct timing of the ecdysone pulse

Ecdysteroid titers in neck-ligated and PTTH-ablated larvae show a gradual rise which is not caused by PTTH secretion. The ecdysone peak is delayed relative to control larvae, which
indicates that the brain and PTTH are necessary for correct timing of ecdysone secretion, but not necessary for the delayed rise in ecdysone. The following are alternative mechanisms that could account for the delayed, gradual rise in ecdysteroid titer:

(1) There is a source of ecdysone other than the PG which, in absence of PTTH, secretes ecdysteroids. This possibility can be excluded because thorax-ligated larvae undergo developmental arrest and never expose the dorsal vessel (Nijhout, 1976). Therefore, the prothoracic gland must be the source of ecdysone.

(2) Some part(s) of the nervous system other than the brain may secrete low levels of PTTH. This seems unparsimonious and we have no evidence that PTTH is secreted elsewhere (for example, from the ventral nerve cord).

(3) The catabolism, sequestration or excretion of ecdysteroids is depressed. At present there is little information regarding these aspects. It is interesting to note that ecdyson oxidase, the enzyme which degrades ecdysone, requires oxygen in order to function. In hypoxic conditions, it is possible that the enzyme does not function as well and therefore causes rapid accumulation of ecdysone in the hemolymph, which would trigger the molt to happen prematurely.

(4) There is spontaneous production of ecdysteroids by the PG. Given enough time and nutrition, the prothoracic gland might secrete enough ecdysone autonomously to trigger molting.

Neck-ligation physically blocks PTTH from attaining the prothoracic gland and inducing ecdysone secretion. Thus ligated larvae molt by a mechanism that is independent of the head. Larvae starved before the critical weight are prevented from attaining the critical weight and therefore molt via a size-independent mechanism too. Starved larvae are still subject to the prothoracicotropic activity of the brain because the brain is not physically isolated from the prothoracic glands as it is in ligated larvae. Any difference between starved and ligated larvae is due to this activity of the brain.
Figure 50: Time to wandering for starved, ligated and control larvae

The ligation curve shows a different pattern than the starved. At very small sizes the starved and ligated curves (black and blue) are identical which indicates that at those sizes the brain is not active. Then around 4 grams the ligation curve becomes delayed relative to the starved curve, which indicates that at this size, the brain begins to have ecdysone-stimulating activity.

The critical weight by definition the size at which the starved and control curves meet. At the critical weight, the brain has had sufficient ecdysone-stimulating activity to induce molting at the normal time. At critical weight, the ligated larvae are still delayed relative to the starved larvae. This is because PTTH is secreted 24 hours after critical weight in the fifth instar. Nevertheless, the ligated larvae are still delayed relative to starved larvae, because PTTH has not been secreted yet. Thus the brain is still necessary after critical weight if the larvae are to molt on time.
From this graph we can distinguish three important timepoints:

1. separation of the ligated vs the starved curves: at this size (4 g), the brain becomes important, and has prothoracicotropic activity separate from the PTTH pulse (but we don't know what the brain is doing or secreting at this stage)

2. starved and fed curves come together: at this point the brain as made the decision to initiate molting but PTTH is not secreted until 24 hours later

3. ligated and fed curves come together: PTTH is secreted and the molting cascade is in progress.

The important thing to note is that although the critical weight has previously been considered a developmental point or switch, this graph suggests that it is instead the end of a process during which the brain gradually stimulates ecdysone secretion. The critical weight is the size at which the brain has secreted enough ecdysone-stimulating factor to cause the caterpillar to molt on time. In addition, the fact that ligated larvae eventually molt suggests that there are ecdysone stimulating factors in the abdomen, or that the prothoracic gland has a low level of autonomous activity.

Prothoracic glands of *Manduca* that are cultured in vitro secrete ecdysone at a very low basal level, but do not produce a “peak” of ecdysone similar to that observed in ligated or starved larvae (Walsh and Smith, 2011). This suggests that the glands are not autonomously stimulated to secrete ecdysone but that there are prothoracicotropic substances from elsewhere in the body (not the brain). Good candidate prothoracicotropic substances include insulin, and other neurosecretory substances that could be secreted from the ventral nerve cord.

To further understand the process of ecdysone secretion in control, starved and ligated larvae, I measured ecdysone titers of larvae in these conditions. In an attempt to figure out what factors might cause the prothoracic gland to secrete ecdysone, I also injected ligated larvae with insulin and ventral nerve cord extracts.
9.3 Materials and methods

1) Hemolymph collection
Larvae were starved or ligated in the following weight classes: 6.0-6.5g, and 7.5-8.0g. The date and time of starvation/ligation was recorded as well as the weight. Hemolymph was collected from larvae, and the date and time of hemolymph collection was recorded.

To collect the hemolymph, larvae were anesthetized under CO$_2$ and then one anterior proleg was cut open. The hemolymph was dropped onto a square piece of parafilm and immediately pipetted into a 1.5mL Eppendorf tube. 100 µL of hemolymph was pipetted into 900 µL of ice-cold methanol. This mixture was vortexed and then centrifuged for 3 minutes at 13000 rpm. The pellet was discarded and the supernatant was stored in the freezer for further processing.

Sample preparation for ecdysteroid radioactive immunoassay
(See DCC protocol)
Samples were aliquoted into 400 uL aliquots, each in duplicate. Standards representing 100, 50, 25, 12.5, 6.25, 3.1 ng/mL of ecdysteroid were also prepared.
Samples were evaporated in the Speed-Vac to remove the methanol.
The radioactive edysone was added and left for three hours to compete with the edysone in the samples to react with the antibody.
The samples were chilled on ice for 5 minutes, and then 500 uL of dextran coated charcoal solution was added to each sample. The charcoal was allowed to sit in the sample for 5 minutes and then the samples were centrifuged for 3 minutes at 13000 rpm. 500 uL of the supernatant was placed into 10 mL of scintillation fluid. The samples were read on the scintillation counter (H3) for 5 minutes.

Insulin injections.
Human recombinant insulin was diluted 1:3 with lepidopteran saline. Fifth instar larvae were ligated between 5.5 and 6.5 g and injected with 50 uL of insulin daily, until they dorsal-
vesseled. The number of days from the first injection to dorsal-vessel exposure was compared to that of control larvae and sham-injected larvae.

**Serotonin injections.**
Serotonin was diluted into a 1mM solution. Fifth instar larvae were ligated between 5.5 and 6.5 g and injected with 20 µL daily until they dorsal-vesseled.

**Preparation of extract from thoracic and abdominal ganglia.**
Larvae between the sizes of 7.0-8.5g were selected and deeply anesthetized in CO₂. Larvae were dissected, and the gut was removed to reveal the ventral nerve cord. The thoracic ganglia were collected separately from the abdominal ganglia. 10 animals’ worth of thoracic ganglia were placed in 400 µL of insect saline. The same was done for the abdominal ganglia. The ganglia were macerated inside the eppendorf tube, sonicated for 30 seconds, and centrifuged for 10 seconds. 50 µL of supernatant was injected into ligated larvae (6.0-6.5g) every day, starting the day after ligation, until they dorsal-vesseled. The timing of dorsal-vessel exposure was compared to that of controls and sham-injected larvae.

**9.4 Results**
In ligated or starved larvae, ecdysone titers were measured in order to determine what the ecdysone profile of “leaky PG” look like. The graph shows that ecdysone titers eventually rise enough to trigger exposure of the dorsal vessel.
The ecdysone titers gradually rise and eventually trigger the exposure of the dorsal vessel (required ecdysone titer between 20-50 ng/mL). Larvae were either starved or ligated between 6.0-6.5 grams, just below the critical weight. Almost all starved larvae exposed their dorsal vessel by the fourth day after starvation. Ligated larvae took a little bit longer, many exposing the dorsal vessel on the fourth or fifth day after ligation. Since starved and ligated larvae were identical in all respects except that starved larvae have access to the secretions of the brain, the difference between starved and ligated larvae (the slightly faster rise in ecdysone titer in the starved larvae) is due to prothoracicotropic activity of the brain. This prothoracicotropic activity is different from the pulse of PTTH that occurs after critical weight, because these larvae were starved or ligated before critical weight and therefore never secrete a pulse of PTTH.

Insulin and serotonin failed to significantly accelerate the timing of ecdysone secretion relative to control ligated larvae. This indicates that neither insulin nor serotonin, at the dose
administered and at the size of larvae tested (5.5-6.5g), increased ecdysone secretion. The thoracic and abdominal extract likewise did not appear to have a prothoracicotropic effect.

![Graph showing timing of exposure of the dorsal vessel for larvae injected with various candidate prothoracicotropic substances.](image)

**Figure 52**: Timing of exposure of the dorsal vessel for larvae injected with various candidate prothoracicotropic substances.

### 9.5 Discussion

We found that the brain has prothoracicotropic activity aside from the normal pulse of PTTH. The prothoracicotropic activity of the brain occurs over a range of sizes and time; it is not a definite event but represents an accumulation of smaller effects over a span of time.

I failed to detect ecdysone-stimulating activity of insulin, serotonin, thoracic or abdominal extracts. The fact that insulin failed to have prothoracicotropic activity is surprising because in *Drosophila*, upregulation of insulin signaling in the prothoracic gland accelerates ecdysone secretion. However, my results are consistent with those of (Walsh and Smith, 2011).

The fact that thoracic and abdominal extracts were unable to accelerate ecdysone secretion indicates that the ventral nerve cord does not have substances with prothoracicotropic
activity. This suggests that the brain is the only part of the nervous system that secretes prothoracicotropic substances. The rise of ecdysone titers in ligated larvae could be caused by ecdysone-stimulating factors in the abdomen.

It is possible that the leaky prothoracic gland mechanism is an evolutionarily primitive mechanism that was subsequently modified to canalize final body size. The critical weight mechanism could have evolved to control and modify the leakage of the prothoracic gland in order to (a) canalize final size, (b) canalize instar durations and therefore the total development time, or (c) ensure optimal growth conditions by triggering the molt when oxygen supply/demand is no longer adequate, instead of persisting to grow in low oxygen conditions (which would reduce the growth rate, and possibly lead to a longer development time). In other words the size sensing mechanism could ensure optimal metabolic supply to demand ratios in order to allow the larva to attain its final size in the best metabolic conditions possible allowed by the environment.

In contrast, the leaky prothoracic gland mechanism ensures that the larva will complete its growth and eventually become adult, although neither the final size nor the development time are tightly regulated. The leaky PG mechanism does not coordinate the changing metabolic supply/demand ratio with the molting cycle. The PTTH signaling mechanism, controlled by the brain, could have evolved to coordinate molting with metabolic supply/demand, which would ensure that the larva spends most of its intermolt growth periods unconstrained by oxygen supply.
10. A mathematical model for leaky glands

In order to understand and quantify the relative contributions of brain and abdomen in stimulating ecdysone secretion, we have created a simple mathematical model to represent the role of the brain, the abdomen, and nutrition in stimulating ecdysone secretion from the prothoracic gland.

10.1 Outline of the model

In this model, the fed larvae exhibit the timing due to the contributions of the brain and abdomen with nutrition; starved larvae show the contributions of the brain and abdomen without nutrition; neck-ligated larvae show only the contribution of the abdomen (without nutrition).

The total accumulation of ecdysone-stimulating factor is given by the following expression:

\[(\text{time fed}) \times (\text{rate of accumulation while fed}) + (\text{time starved or ligated}) \times (\text{rate of accumulation while starved or ligated}) = X,\]

where \(X\) represents the total amount of ecdysone stimulating factor necessary to trigger a molt.

Therefore, the time from starvation or ligation to the molt is given by:

\[\text{TimeStarvedOrLigated} = \frac{-rateFed \times TimeFed + X}{rateStarvedOrLigated} \]

Furthermore, I hypothesize that the rate of secretion of ecdysone-stimulating factor can be partitioned between secretion from the brain and secretion from the abdomen under different conditions:

\[rateFed[t] = BRAIN[t] + ABDOMEN[t],_\text{with}_\text{nutrition}\]
\[rateStarved[t] = BRAIN[t] + ABDOMEN[t],_\text{without}_\text{nutrition}\]
\[rateLigated[t] = ABDOMEN[t],_\text{without}_\text{nutrition}\]
\[rateAllatectomized[t] = BRAIN[t] + ABDOMEN[t],_\text{without}_\text{JH}\]
Little is known about the rates of secretion of ecdysone stimulating factor from the brain (represented by the function BRAIN[t]) and the abdomen (function ABDOMEN[t]); however, it is likely that these rates are influenced by factors such as nutrition and juvenile hormone titer.

Given these unknowns, I hypothesize that the rate of secretion of ecdysone-stimulating factor from the brain and the abdomen can be represented by sigmoid functions, and that they are a function of nutrition, JH levels, and the time of starvation or ligation. I hypothesize that the rate of secretion under starvation or ligation is constant for any given individual, and that this constant value is determined by the time at which the larva is starved or ligated.

10.1.1 The ecdysone-stimulating activity of the brain

At the beginning of the instar, the brain activity is initially low because JH inhibits the activity of the brain. As JH titers drop, the ecdysone-stimulating activity of the brain increases. The higher the ecdysone-stimulating activity of the brain, the lower the critical weight, and vice versa. Let’s suppose that the rate at which the brain secretes ecdysone-stimulating factor plateaus at a certain level. Then, the ecdysone-stimulating activity of the brain can be represented by sigmoid, which I express as a Gompertz function:

$$BRAIN[t] = ae^{-be^{\frac{t}{M_i}}}$$

Where

\(a\) represents the maximal rate of secretion of ecdysone stimulating factor from the brain.

This in turn will be a function of the ratio of oxygen supply to demand, which depends on body size. The more oxygen becomes constraining, the lower the critical weight. The more the brain stimulates ecdysone stimulation, the lower the critical weight. Therefore, the model predicts that the activity of the brain should vary in concert with the constraint on oxygen supply.
b represents the displacement of the sigmoid;

$1/JH$ represents the effect of juvenile hormone: when JH is high, the sigmoid rises slowly (representing slower rates of secretion), and when JH is low, the sigmoid rises quickly (faster rates of secretion); and

t represents the time from the beginning of the instar.

The lower limit of JH is 1, which represents total absence of JH. Thus, in an allatectomized larva, the brain will secrete ecdysone stimulating factor at the following rate:

$$AllatectomizedBRAIN[t] = ae^{-be^{-t}}$$

10.1.2 The ecdysone-stimulating activity of the abdomen

Little is known about the ecdysone-stimulating activity of the abdomen. Indeed, the brain is usually portrayed as the main player in controlling ecdysone secretion; however, the observation that ligated larvae eventually molt shows that the abdomen must secrete ecdysone-stimulating factor, possibly at a lower rate than the brain. I suspect that the ecdysone-stimulating activity of the abdomen is modulated by nutrition.

The larva does not eat during the molting period of approximately 24 hours, so when it emerges from the molt, it is in a fasted state, and the secretion of ecdysone-stimulating factor from the abdomen is low. As it begins to feed, the rate of secretion from the abdomen increases until the gut becomes full. When the gut is full, the absorption of nutrition becomes saturated, and the rate of secretion of ecdysone-stimulating factor also levels off. Thus, the activity of the abdomen can also be described as a sigmoid, and expressed with a Gompertz function:

$$ABDOMEN[t] = ce^{-de^{-nutrition+t}}$$
where
c represents the maximal rate of secretion of ecdysone stimulating factor from the abdomen;
d represents the displacement of the sigmoid;
nutrition represents the effects of nutrition: when nutrition is good, the sigmoid rises relatively quickly (representing higher rates of secretion), and when nutrition is poor, the sigmoid rises slowly (slower rates of secretion), and
t represents the time from the beginning of the instar.
The lower limit of nutrition is 1, which represents starvation. Thus, in a starved larva, the abdomen will secrete ecdysone stimulating factor at the following rate:

\[ StarvedABDOMEN[t] = ce^{-dt} \]

Therefore, the rates of secretion of ecdysone-stimulating factor are the following:

\[ rateFed[t] = BRAIN[t] + ABOMEN[t] \]
\[ rateStarved[t] = BRAIN[t] + StarvedABDOMEN[t] \]
\[ rateLigated[t] = StarvedABDOMEN[t] \]
\[ rateAllatectomized[t] = AllatectomizedBRAIN[t] + ABOMEN[t] \]

10.2 Model predictions

10.2.1 Control conditions

The fed larvae are represented in blue, the starved in red, and the ligated in yellow.

First, I fitted the parameters so that the model replicated the observed data: The fed larvae take approximately 100-125 hours (4-5 days) to expose PTTH secretion, from the beginning of the instar. Larvae that are starved or ligated at the beginning of the instar take the same amount of time. The starved curve meets the fed curve around 6 grams, which is the critical weight. The ligated curve remains delayed until PTTH secretion, around 8-9 grams.
10.2.2 Decreasing oxygen availability decreases critical weight

Parameter \( a \) represents the maximum rate of secretion of ecdysone-stimulating factor from the brain, and this rate will depend on the relative oxygen supply and demand. When oxygen is highly constraining, the larva should molt soon and at a small size (lower critical weight), so the activity of the brain should be high (\( a \) should be high).
Thus, as predicted the parameter that represents the activity of the brain as a function of oxygen supply and demand regulates the critical weight.
10.2.3 Gradual dilution of nutrition

Davidowitz et al. (Davidowitz et al., 2003) examined the effect of nutrition on critical weight and found that as the diet was gradually diluted, the critical weight became undetectable (their Figure 4 is reproduced below). In 100% fed larvae, the critical weight was 6.5g, in 60% diet larvae the critical weight was 6 grams, and in 40% diet larvae the critical weight was undetectable. I interpret this to mean that under 40% diet, larvae are not using the critical weight mechanism but instead are using the “leaky prothoracic glands” mechanism.

Figure 56: Figure 4 from Davidowitz et al. (2003)
I simulated this experiment with my model by varying the parameter that represents nutrition. Under low nutrition conditions, the fed curve approaches the starved curve, such that the critical weight indeed becomes indistinguishable.

Figure 57: The critical weight becomes undetectable under low nutrition conditions.

Under medium nutrition conditions, the critical weight decreases ever so slightly, but nutrition does not have a strong effect on the critical weight, as shown by the Davidowitz experiment and confirmed in the model.
### 10.2.4 Allatectomy

Allatectomized larvae are larvae in which the corpora allata, which produces JH, is removed. Thus, in these larvae, JH is prematurely removed. I simulated the timing of exposure of the dorsal vessel as a function of the size at which allatectomy was performed (green curve). To my knowledge, this experiment has not been performed in the lab. However, Nijhout (Nijhout, 1975) showed that larvae allatectomized at the beginning of the fifth instar do not accelerate the timing of exposure of the dorsal vessel; they take approximately 4 days.

![Simulation of larvae allatectomized at various sizes](image)

**Figure 58: Simulation of larvae allatectomized at various sizes**
The simulation shows that larvae allatectomized at the beginning of the instar should molt around the same time as fed larvae (as shown by Nijhout (Nijhout, 1975)). The model predicts that larvae that are allatectomized around 4-5 grams should expose their dorsal vessel sooner than fed intact larvae of the same size.

10.2.5 Interaction between allatectomy and starvation

Yui Suzuki performed allatectomies and starved larvae at the beginning of the fifth instar. He refed the larvae for a brief period, and found that the allatectomized larvae always exposed their dorsal vessel four days after this feeding period (pers. comm.; in prep for publication).

To simulate the experiment, I need to simulate larvae that are allatectomized at the beginning of the fifth instar, and starved for a varying amount of time, then refed for a fixed period, and then starved again until exposure of the dorsal vessel.

The rate at which the brain secretes from larvae that are allatectomized at the beginning of the fifth instar is $a e^b$.

The rate at which the abdomen secretes from larvae that are starved from the beginning of the instar is $c e^d$.

When larvae are refed for a period of $P$ hours, the abdomen secretes at the fed rate for this period. The total amount of ecdysone-stimulating factor secreted during this period is given by the integral:

$$\int_0^P ABDEOMEN[t]dt = \int_0^P ce^{-de^{e^d}t} dt$$

Therefore, the total amount of ecdysone-stimulating factor that has been secreted in an allatectomized, starved, larva that has been refed for a period $P$, is given by:
\[(ae^{-b} + ce^{-d})x + \int_{0}^{x} ce^{de^{-dm}} dt\]

where \(x\) represents the duration of the instar.

According to Yui’s experiment, this amount of ecdysone stimulating factor should trigger the larva to molt in approximately four days. This prediction will have to be tested against more specific data.

### 10.2.5 Comparison between *Drosophila* and *Manduca*

In *Drosophila* the critical weight and minimum viable weight are almost synchronous (McBrayer et al., 2007; Mirth and Riddiford, 2007). Furthermore, larvae that are starved after critical weight actually accelerate their molt relative to fed controls, due to a “bail out” response. This switch-like behavior of *Drosophila* at the critical weight contrasts with that of *Manduca* whose critical weight curves are less sharp. To investigate the biological reasons for these differences I investigated which parameters affect the sharpness of the curves around the critical weight.

Parameter \(d\) is the main parameter that affects the sharpness of the critical weight curves; as \(d\) becomes larger, the curves become sharper. Parameter \(d\) influences the rate of secretion from the abdomen; the larger \(d\) is, the lower the rate of secretion from the abdomen. Thus, this suggests that the smaller the contribution of the abdomen, the sharper the curves. This suggests that in *Drosophila*, the abdomen contributes relatively little ecdysone-stimulating factor compared to the brain, whereas in *Manduca*, the abdomen contributes relatively more.
Figure 59: *Manduca* critical weight curves slope gently.
Figure 60: *Drosophila* critical weight curves are sharper.

### 10.3 Discussion

I have created a model that incorporates what is known about the critical weight and oxygen sensing, as well as reproducing the known data about molt initiation in ligated and starved larvae that use the “leaky prothoracic glands” mechanism. The model correctly reproduces the effects of oxygen and nutrition on the critical weight.

In normal growth conditions, larvae use the critical weight mechanism, but in poor or stressful growth conditions, larvae use size-independent mechanisms to initiate the molt. The size-independent mechanism is less well regulated and larvae tend to molt on a more
variable schedule, whereas the critical weight and PTTH mechanism is much less variable
and more predictable. This suggests the possibility that the critical weight and PTTH
mechanism may have evolved as an additional regulatory layer on top of the “basal”
mechanism of slow accumulation of ecdysone-stimulating factor. The PTTH mechanism
could provide the larva with more refined control over the time of day at which the molt
occurs, and thus allow it to molt at the optimal time to avoid predation, for example.

If this hypothesis is correct, we should be able to see varying degrees of this kind of
regulation depending on the ecology of species. Species such as Drosophila in which
development must happen quickly due to the ephemeral nature of the larval environment
molt on a timing mechanism; larvae of Precis coenia which are not synchronous with each
other evidently do not have a mechanism that tightly regulates the timing of molting and
developmental stages. These different species might partition the control across the brain
and abdomen in different ways as well.

10.4 Future work

The model is based on hypotheses about the origins and rates of secretion of ecdysone-
stimulating factor. The next avenues to pursue are the following:

1) Identify and measure ecdysone stimulating factors in the hemolymph and test whether the
measured activity corresponds to that predicted by the model. We have done preliminary
experiments attempting to extract PTTH from the ventral nerve cord to test whether PTTH
is present in the abdomen and could be stimulating ecdysone secretion. The experiments
showed no evidence of PTTH in the ventral nerve cord; thus it is not likely that PTTH exists
in the abdomen. Instead, insulin is a likelier candidate, and it is secreted by the fat body.

2) Although oxygen sensing plays an important role in sensing critical weight and initiating
the molting process, it is still unknown how oxygen sensing mechanistically interacts with
the processes that determine the timing of hormone secretion.
11. Future work

Until now, there have been two largely independent avenues of research in size regulation: molecular genetic mechanisms have been investigated in *Drosophila*, whereas physiological mechanisms have been studied in *Manduca*. Recent work in *Drosophila* has explored how nutritional signals, mediated through insulin and TOR signaling, influence the timing and the size at which the endocrine cascade that initiates metamorphosis is triggered (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005). How these processes are linked to the critical weight and the mechanism of size-sensing remains an open question. In *Manduca*, complementary information is available: we have recently found that the larva senses its size via a decrease in oxygen supply relative to demand (Callier and Nijhout, 2011, accepted), but the link between oxygen sensing and the endocrine signals that trigger metamorphosis remains to be explored. The proposed research will clarify the genetic and physiological mechanisms by which oxygen sensing regulates the timing of the hormonal signals that initiate metamorphosis and determine final body size.

In *Drosophila* there is a small peak of ecdysone that coincides with critical weight. For this reason the work on critical weight in *Drosophila* has focused on understanding the regulation of ecdysone titers. Regulation of ecdysone secretion by the prothoracic gland has been extensively studied at the molecular level (Caldwell et al., 2005; Colombani et al., 2005; Layalle et al., 2008; Mirth et al., 2005; Shingleton, 2005; Walkiewicz and Stern, 2009). These studies have shown that ecdysone secretion rate can be nutritionally modulated via the insulin/IGF signaling (IIS) pathway— the pathway that regulates growth with respect to nutrition in all animals. Activation of the IIS pathway in the prothoracic gland alone accelerates ecdysone secretion while inhibition of the pathway decreases ecdysone secretion.

PTTH, which is secreted from the brain several hours after the larva attains critical weight, is the classical regulator of ecdysone secretion from the prothoracic gland. It is not clear how PTTH and insulin signaling interact, and whether either is sufficient for ecdysone secretion. Because there are multiple inputs to the process of ecdysone secretion, the field is moving away from the view of PTTH as the trigger that sets in motion an irrevocable series of
events that lead to the molt. Rather, the role of the prothoracic gland to integrate various signals and become competent to secrete ecdysone is becoming more prominent.

**Approach**

Previous work in *Manduca* and *Drosophila* shows that oxygen levels impact body size, but the mechanisms by which oxygen levels affect size are not known. I will use the molecular tools available in *Drosophila* to examine the effects of hypoxia specifically on the dynamics of ecdysone synthesis, secretion and degradation (Aim 1). Canonically, ecdysone secretion from the prothoracic glands is regulated by PTTH; however, recent work on size regulation in *Drosophila* has shown that insulin is also a critical input for the regulation of ecdysone secretion. It is not known how insulin and PTTH interact, and whether they are both necessary, or individually sufficient, to trigger ecdysone secretion. I will use genetic and physiological tools available in each species to investigate the necessity and sufficiency of PTTH and insulin in the regulation of ecdysone secretion from the prothoracic gland (Aim 2).

**Aim 1: Molecular mechanisms of sensing hypoxia and their relation to ecdysone secretion**

Peck and Maddrell (Peck and Maddrell, 2005) hypothesize that “the proximate mechanism setting limits to size during development is probably the onset of cellular hypoxia”. Nevertheless, the physiological and molecular mechanisms by which hypoxia affect critical weight and the neuro-endocrine signals that terminate growth are not known. My preliminary data establish hypoxia as an important regulator of critical weight, and hence final body size, in *Manduca*. The tools necessary to elucidate these mechanisms are not readily available in *Manduca*. They are, however, available in *Drosophila*. Due to the novelty of my studies on the effect of oxygen levels on critical weight in *Manduca*, I do not yet know what role oxygen levels play in regulating critical weight in *Drosophila*. Consequently, I will (1) test whether hypoxia decreases body size in *Drosophila* by decreasing the critical weight, (2) elucidate the molecular mechanisms responsible for the effect of hypoxia on body size, and
(3) functionally manipulate these molecular mechanisms to alter critical weight in *Drosophila* larvae.

I will use methods well established in *Manduca* to determine whether oxygen levels in *Drosophila* mediate attainment of critical weight. Specifically, I will determine whether *Drosophila* larvae become increasingly hypoxic in the late stages of their final instar and whether attainment of critical weight coincides with the size at which the tracheal system is no longer sufficient to satisfy oxygen demand. I will then determine whether, as in *Manduca*, changes in oxygen level alter the size at which larvae attain critical weight. If oxygen does not alter the critical weight in *Drosophila*, I will investigate the other mechanisms by which oxygen reduces body size in *Drosophila* (see Aim 2).

The effects of hypoxia are likely to be mediated by HIF-1 (Hypoxia Inducible Factor-1): extensive research has identified HIF-1 as the primary mediator of the response of growth and development to changes in oxygen levels in *Drosophila* and all animals (Berra et al., 2006; Bracken et al., 2003; D’Angelo et al., 2003; Hirota and Semenza, 2005; Huang et al., 1998; Khanna et al., 2006; Majmundar et al., 2010; Millonig et al., 2009; Qutub and Popel, 2007; Romero et al., 2007). HIF-1 consists of a heterodimer of two transcription factors: HIF-1α (*sima* in *Drosophila*) and ARNT (aryl hydrocarbon receptor nuclear translocator) (*tango* in *Drosophila*). Under normal oxygen conditions HIF-1α is rapidly degraded. This degradation is blocked under hypoxic conditions. As a result, the protein accumulates, migrates to the nucleus, and the HIF-1α-ARNT complex initiates transcription of target genes.

I expect that HIF-1 is involved in the response to hypoxia and molt initiation. Specifically I hypothesize that HIF-1 becomes increasingly activated at the point at which the size of the tracheal system is no longer sufficient to satisfy oxygen demand, and that this increase in activation coincides with attainment of critical weight. I further hypothesize that it is activation of HIF-1 in brain or the prothoracic gland that is responsible for attainment of CW and the synthesis of ecdysone that stops larval growth.
Because HIF-1 is mainly regulated at the level of protein degradation, not mRNA expression, I will use western blot analysis to quantify changes in HIF-1α throughout development in Drosophila. I will also assay HIF-1α accumulation in vivo using the ODD-GFP reporter construct. The ODD (oxygen-dependent degradation) domain of HIF-1α is sufficient and necessary to initiate degradation of HIF-1α in normoxic conditions. Like HIF-1α, the ODD-GFP fusion protein accumulates under normoxic conditions and so I can use its accumulation as a proxy for HIF-1α accumulation. Finally I will assay the activity of HIF-1 using the LDH-lacZ reporter construct, which undergoes HIF-1α mediated transcription of β-galactosidase in normoxic conditions. I have access to all the antibodies and transgenic flies necessary to conduct these experiments.

Once I have established that HIF-1 accumulation is correlated with attainment of critical weight, I will manipulate HIF-1α expression and activity in the brain and the prothoracic gland. I will test the hypothesis that HIF-1α activity in the brain and/or prothoracic gland is sufficient and necessary to accelerate critical weight attainment in Drosophila larvae in hypoxic conditions. To test whether HIF-1α activity is sufficient to reduce critical size, I will measure critical size in larvae mutant for fatiga (fga). Fatiga is the Drosophila homolog of HIF propyl hydroxylase (HPH), which degrades HIF-1α in normoxic conditions. Loss of fga therefore results in the accumulation of HIF-1α independent of oxygen levels – fga-mutants essentially have constitutively active HIF-1. If HIF-1α is sufficient to reduce critical weight, I should see a reduction in critical weight in larvae where fga expression is knocked down either globally or in the brain/prothoracic gland alone. To test whether HIF-1α is necessary to reduce critical size I will determine whether larvae mutant for HIF-1α are able to reduce critical size in hypoxic conditions. If HIF-1α is necessary to reduce critical weight in hypoxic conditions, I should see no reduction in critical weight in hypoxic larvae where HIF-1α expression is knocked down either globally or in the brain/prothoracic gland alone.
Whilst I hypothesize that the brain and/or prothoracic gland is the locus of control for the oxygen regulation of critical weight, it is straightforward to explore the involvement of other tissues, for example the fat body, trachea, etc, using additional organ-specific GAL4 drivers to drive expression of HIF-1α.RNAi and fig.RNAi. I have access to all the transgenic flies necessary to conduct these experiments.

These experiments will determine whether oxygen affects the critical weight in *Drosophila* and whether the effects of oxygen on body size are mediated by HIF. They will also provide training opportunities for me to learn techniques in *Drosophila* developmental genetics.

*Alternative strategies.* The critical weight of *Drosophila* might not be regulated by hypoxia in the same way that it is in *Manduca*. In the event that *Drosophila* larvae are not using oxygen limitation to trigger metamorphosis, it follows that some other mechanisms are. I plan to explore these alternative mechanisms in Specific Aim 2.

**Aim 2: Establishing how multiple inputs (insulin and PTTH) are integrated by the prothoracic gland to regulate the rate of ecdysone secretion and timing of metamorphosis initiation.**

*Manduca* and *Drosophila* that are starved below the critical weight (but above the minimum viable weight) will eventually molt, after some delay (Davidowitz et al., 2003; Nijhout and Williams, 1974b). I hypothesize that this delayed molt is due to a mechanism that allows larvae growing under suboptimal nutritive conditions to molt and metamorphose, even at a smaller than optimal body size. In both *Manduca* and *Drosophila*, this delay is longer when larvae are starved at progressively lower weights. We have found that neck-ligated larvae of *Manduca* likewise will molt, albeit after some time (Callier and Nijhout, unpublished). Thus a delayed molt can occur in the absence of the brain. This is actually a very old observation in insect physiology going back to the classical studies of Fukuda (1944) who showed that brainless *Bombyx* will eventually molt (Fukuda, 1944). *Drosophila* larvae in which the PTTH neurons have been ablated likewise have a delayed metamorphosis (McBrayer et al., 2007). This raises the question of how larvae with a disrupted PTTH-ecdysone axis are able to molt.
In normal growth conditions, PTTH is the signal that causes the prothoracic gland to secrete ecdysone and initiate molting or metamorphosis. However, insulin also plays a role in regulating ecdysone secretion, as shown by the precocious initiation of metamorphosis in Drosophila larvae in which insulin signaling is upregulated in the prothoracic gland.

PTTH and insulin are both known to stimulate ecdysone secretion from the prothoracic gland, but it is not known whether they are necessary or sufficient for regulating ecdysone secretion. They may act additively (independently) as well as interactively (for example, insulin signaling primes the prothoracic gland to respond to the PTTH signal). If PTTH and insulin act both additively and interactively, there is redundancy built into the system so that the larva has multiple ways of achieving the transition to the pupal stage. The experiments proposed below will investigate the interactions between PTTH and insulin, and shed light on the “back up” mechanisms that come into play when larvae are prevented from attaining the critical weight or have a disrupted brain-prothoracic gland axis.

**Determining the role of PTTH in Manduca**

To understand the alternative mechanism used by starved larvae that never attain the critical weight, I must establish whether PTTH is actually secreted in pre-critical weight starved larvae. I have an antibody for PTTH (O'Brien et al., 1988) and recently verified its efficacy. Using ELISA, I will measure the PTTH titers in the fifth instar of Manduca in control larvae and starved larvae, to determine whether starvation affects the timing or quantity of PTTH secretion. If PTTH is not secreted in starved larvae, then there must be another ecdysone-stimulating factor, possibly bombyxin (the Manduca homolog of insulin). If PTTH is secreted, I will determine whether the delay in ecdysone secretion is due to delayed PTTH or whether PTTH is secreted on time but the prothoracic gland is unable to respond due to starvation.

To test whether bombyxin is the ecdysone stimulating factor that eventually causes brainless larvae to secrete ecdysone, I will inject isolated bombyxin into neck-ligated larvae and
determine whether this causes precocious ecdysone secretion. I will also culture prothoracic
glands in vitro and determine whether bombyxin primes the prothoracic glands to respond
to PTTH, to quantify whether bombyxin and PTTH act additively or interactively with
respect to ecdysone secretion. This is similar to the experiments of (Walsh and Smith, 2011)
but using bombyxin rather than human insulin. Culturing the glands in vitro will allow me
to control the levels of PTTH and insulin independently.

The experiments proposed above will allow me to quantify to what degree bombyxin and
PTTH act additively or interactively to trigger ecdysone secretion from the prothoracic gland
in Manduca. These experiments will allow me to produce a developmental profile of PTTH
and ecdysone under fed, starved and neck-ligated conditions. I am already trained in the
methods to measure ecdysone titers, but I will gain new skills in culturing glands in vitro and
measuring PTTH using the ELISA assay.

Understanding the interaction between PTTH and insulin in Drosophila

I will use epistatic analysis to explore the roles of IIS and PTTH in regulating critical weight
in Drosophila. I will increase IIS in the prothoracic gland and determine to what extent (if
any) ablation of the PTTH-producing neurons delays development. Conversely, I will
increase PTTH-expression in PTTH-specific neurons and determine to what extent (if any)
down-regulation of IIS in prothoracic gland delays pupariation.

First, I will drive PTTH over-expression in these neurons (using a PTTH neuron specific Q-
system driver to express QUAS-PTTH) and decrease insulin signaling in the prothoracic
gland (using a prothoracic gland GAL4 driver to express UAS insulin), and determine
whether the prothoracic gland secretes enough ecdysone for pupariation. If driving PTTH is
not sufficient for pupariation, this would demonstrate that PTTH is insufficient (and we
already know it is unnecessary, because larvae in which the PTTH-neurons have been
ablated are able to pupariate, with a delay).
I will also do the converse experiment: Using the prothoracic gland-specific driver \textit{phm-GAL4} to drive expression of \textit{UAS-PI3K} (Mirth et al., 2005), I will increase insulin signaling in the prothoracic gland in PTTH-neuron-ablated larvae and in intact larvae, and determine whether PTTH-neuron-ablated larvae are delayed relative to intact larvae. (I will use the Q-system to genetically ablate the PTTH-neurons in flies where I am using UAS-GAL4 to drive PI3K expression in the prothoracic gland). If PTTH-neuron-ablated larvae are delayed relative to intact larvae, this would indicate that insulin signaling does not abrogate the need for a PTTH signal. If the two groups are synchronous in the timing of pupariation, this would indicate that insulin signaling in the prothoracic gland “short circuits” the PTTH signal.

If insulin signaling alone is \textit{sufficient} for causing ecdysone secretion in the absence of PTTH, I would next investigate whether it is \textit{necessary}. To show that insulin signaling is necessary, I will starve larvae and block insulin signaling (genetically in \textit{Drosophila} and using wortmannin injections in \textit{Manduca}) in larvae that lack PTTH secretion. To block PTTH secretion, I will use the Q-system to genetically ablate the PTTH-neurons in flies, and I will neck-ligate \textit{Manduca} larvae. If these larvae never pupariate or show signs of initiating metamorphosis, this would indicate that insulin signaling is both necessary and sufficient to induce ecdysone secretion from the prothoracic gland. If larvae are able to pupariate or transition to wandering, this would indicate that insulin signaling is sufficient but not necessary, and suggest that there must be other ecdysone-stimulating factors involved in regulating ecdysone secretion.

These experiments will allow me to determine the additive or interactive effects between insulin and PTTH, and elucidate to what degree there is redundancy in the mechanisms that trigger ecdysone secretion and initiate metamorphosis. These experiments will also provide training opportunities to make transgenic flies and learn methods of genetic analysis.
Appendix A: Molecular basis of oxygen-sensing in *Drosophila*

**A1. Oxygen sensing in *Drosophila***

*Drosophila* is one of the main organisms where the molecular mechanisms of oxygen-sensing have been studied. Oxygen-starved cells express Branchless FGF, which triggers tracheole sprouting (Ghabrial et al., 2003; Jarecki et al., 1999). Low oxygen stimulates the expression of branchless gene, and Branchless FGF guides the tracheoles to cells lacking oxygen.

How is low oxygen sensed by the cells, and how does low oxygen trigger the expression of branchless? The expression of branchless is regulated by the hypoxia-inducible factor (HIF), the main transcription factor involved in the hypoxic response (Ghabrial et al., 2003). Hypoxia-inducible factor (HIF) is a transcription factor that controls many hypoxia-responsive genes. HIF transcription factor functions as a heterodimer, with subunits alpha and beta (Irisarri et al., 2009). 3 HIF-prolyl4 hydroxylases control the abundance of HIFalpha in response to oxygen concentration. The beta-subunit is constitutively expressed. Tracheole sprouting depends on stabilization of the alpha subunit; in normoxic conditions, HIF-1alpha is rapidly degraded by the proteasome (Centanin et al., 2008; Hirsila et al., 2003; Irisarri et al., 2009). The oxygen-dependent regulation of the alpha-subunit is exerted through several mechanisms:

1) Control of protein degradation. Hypoxia-Inducible Factor (HIF) is hydroxylated at 2 proline residues by HIF prolyl-hydroxylases (PHD), that require iron (Fe) and oxygen (O2) as cofactors. Hydroxylation marks HIF for degradation by E3 ubiquitin ligase. In normoxia, HIF has a very short half life, but in hypoxia, HIF is stabilized, and can act as a transcription factor for many hypoxia-responsive genes. At low oxygen levels, hydroxylation is prevented, ubiquitination does not occur, and HIFalpha is stabilized. The enzymes that catalyze prolyl hydroxlation, termed PHD1, PHD2, and PHD3, are considered bona fide oxygen sensors because they require O2 as cofactors to degrade HIF-alpha. This provides a
mechanism by which hypoxia can quickly change gene expression profile of a cell when it becomes hypoxic.

2) Recruitment of transcriptional coactivators. HIF requires transcriptional coactivator P-300. Oxygen-dependent hydroxylation of a specific asparagine residue next to the HIF alpha-C terminal end by factor-inhibiting HIF (FIH) prevents interaction with P-300. In hypoxia asparaginyl hydroxylation is prevented and interaction with P-300 can take place, which in turn allows HIF-dependent transcription.

3) Regulation of subcellular localization. Drosophila HIFalpha homolog, Sima, is localized mainly in the cytoplasm in normoxia and accumulates in the nucleus upon hypoxic exposure. Nuclear import depends on a bipartite nuclear localization signal mapping next to the C-terminus of the protein. Nuclear export is mediated by a CRM1-dependent nuclear export signal localized in the oxygen-dependent degradation domain (ODDD). CRM1-dependent nuclear export requires oxygen-dependent hydroxylation of a specific prolyl residue in the ODDD, and the activity of the von Hippel Lindau tumor suppressor factor. prolyl hydroxylation and VHL activity are necessary for nuclear export of Sima/HIFalpha. Sima nuclear export is an oxygen-regulated step. In normoxia, HIFalpha is hydroxylated and exported, but in hypoxia the export signal is no longer present and HIFalpha accumulates in the nucleus where it can regulate target genes.

In sufficient oxygen conditions, HIF-1alpha is hydroxylated and degraded by PHD1,2,3. There is a negative feedback loop in this system: PHD1,2,3 are transcriptional targets of HIF-1alpha. Under high oxygen conditions, cells reset their normoxic set point by downregulating expression of PHD, and under low oxygen conditions, cells reset their normoxic set point by upregulating expression of PHD (Khanna et al., 2006). Thus, the normoxic setpoint of cells is not fixed to a specific oxygen partial pressure, but is adjustable because of the feedback loop between HIF and the PHDs that degrade it.
When cells cultured at 20%, 10%, and 5% oxygen are exposed to 1/2 of their original oxygen concentration, HIF is equally induced in all cases, independent of absolute oxygen levels (Millonig et al., 2009). They also show that HIF-1alpha is completely degraded via PHD at any given level of sustained hypoxia. A fast onset of hypoxia led to rapid induction of HIF1, whereas a slower onset of hypoxia led to a "slower but more prolonged expression" of HIF. The complete degradation of HIF1 during prolonged hypoxia is mediated by PHDs. Millonig et al. (2009) show that cells sense changes in oxygen levels, independent of absolute oxygen partial pressures.

Hypoxia has two effects on the PHDs (D'Angelo et al., 2003):
1) lack of oxygen represses enzymatic activity, because PHDs require oxygen as cofactor;
2) lack of oxygen causes accumulation of HIF, which activates transcription of PHDs. The upregulation of PHD transcription might function to stop hypoxic signaling in reoxygenated cells.
The feedback loop between HIF and its degradation enzymes PHDs might cause oscillations in the levels of HIF and PHDs, especially after a sudden transfer from normoxia into hypoxia. Oscillatory changes in enzyme activity might prevent the larva from sensing the precise decrement in oxygen relative to the beginning of the instar that specifies attainment of critical weight.

Tracheole sprouting depends on the stabilization of HIF-alpha. The terminal tracheal cells (tracheoles) are more sensitive to hypoxia than any other cell type. The threshold to induce HIF-alpha-driven gene activation in these cells is near the normoxic oxygen level (Centanin et al., 2008). Analysis of the prolyl-hydroxylases that target HIF-alpha shows that their Km's are just above the concentration of dissolved oxygen in air, which suggests that they would be sensitive to any decrease in oxygen concentration (not in the saturated region of Michaelis Menten kinetics, but in the region of the steepest slope) (Hirsila et al., 2003).

Interestingly, HIF-1alpha can be induced in normoxic conditions by insulin signaling (Dekanty et al., 2005). Insulin signaling causes HIF-1alpha to accumulate by preventing its degradation and promoting its nuclear localization (Dekanty et al., 2005). The effect of insulin on HIF-1alpha is mediated by the PI3K and TOR pathways (Dekanty et al., 2005). Hypoxia also causes the dephosphorylation and inactivation of TOR, 4EBP1, and S6K (Brugarolas et al., 2004; Martin, 2005). Hypoxia-induced inhibition of TOR occurs independently of HIF-1alpha, but how TOR is regulated by hypoxia is not known.

I hypothesized that if larvae were indeed becoming more hypoxic as they progressed through the instar, then HIF activity should be upregulated later in each instar. To test this hypothesis, I sampled tissue from larvae in the early, middle and late fourth and fifth instars and used a Western blot to estimate HIF protein levels.

**A2. Methods**

Western blot.
Ginger Hunter in the Kiehart lab helped me to do the Western blot.

Larvae of the fourth and fifth instars were chosen at the following weights:
4th instar: 0.22g, 0.53g, 1.49g
5th instar: 1.94g, 2.00g, 6.48g, 10.28g, 12.48g

A razor blade was used to cut the animal just in front of the first abdominal spiracle. For the 4th instars, the head section was conserved. For the fifth instars, the head section was sliced dorsally in half to reduce the amount of material.

The material was put on ice and added 50mL of buffer, and then ground up and boiled for 4 minutes. The eppi tubes were centrifuged, and then 10 microL of material was again diluted with 10 microL of buffer. Lanes were loaded on the gel with 10 uL of ladder, and 5 uL of all other material. The gel ran for approximately 3 hours at 0.20 volts, and then the protein was transferred to a nitrocellulose membrane overnight.

The membrane was tested with Ponceau dye to see the protein, and then the protein was blocked in 3% BSA for 1 hour.

The membrane was rinsed with TBST for 10 minutes.

The membrane was then incubated in primary antibody for HIF-1alpha (test) and actin (control) overnight in the cold room.

The membrane was washed for 10 minutes in TBST and then incubated with secondary antibody for 75 minutes. The secondary antibody for HIF-1alpha is rabbit HRP, and the secondary antibody for actin is mouse HRP.

The membranes were washed, and then treated with chemiluminescent products to develop the film.

HIF-1alpha is a 120 kDa protein and it should show bands at ~97 kDa and ~120kDa on the gel, according to the Novus Biologicals information sheet.
A3. Results

Figure 62: Western blot results
The content of the lanes is as follows, from the ladder (right) all the way to the left-most lane:

- early fourth (0.2g)
- middle fourth (0.6g)
- late fourth (1.3g)
- early fifth (2g)
- early fifth (2g)
- critical weight fifth (6.5g)
- late fifth (10g)
- late fifth (12g)

The western blot did not work as expected. The bands do not show dramatic differences at each stage. There are several possible explanations for this:

1. The antibody specific to Drosophila HIF-alpha may not bind to Manduca HIF-alpha, and the bands represent antibody binding to another protein(s) in Manduca;
2. There is no detectable variation in HIF activity at different stages within and between instars.

Larvae from the early, middle and late fourth instar were sampled, as well as larvae of early, middle and late fifth instar. I was expecting to see a change through each instar, indicating that the larva was getting more hypoxic as it progressed through the instar. However, the protein bands on the gel showed bands at approximately 150 kDa and 50 kDa, and the thickness or density of bands did not change significantly from one stage of development to another. The most likely explanation is that the HIF antibody that was tested in Drosophila does not work in Manduca, and the bands that we see are not HIF.

Because these inconclusive results would require a significant amount of time and work to troubleshoot, I decided not to pursue molecular work and focus instead on morphology, physiology, and modeling of tracheal function and growth.
References


Biography

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EDUCATION

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Callier, V. and H. F. Nijhout, (2011). Control of body size by oxygen supply reveals size-dependent and size-independent mechanisms of molting and metamorphosis, PNAS.


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