The Role of Threshold Size in Insect Metamorphosis and Body Size Regulation

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

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Abstract

The initiation of metamorphosis causes the cessation of the larval growth period which determines the final body size of adult insects. Because larval growth is roughly exponential, differences in timing the initiation of metamorphosis can cause large differences body size. Although many of the processes involved in metamorphosis have been well characterized, little is known about how the timing of the initiation of metamorphosis is determined.

Using different strains from *Tribolium castaneum*, *Tribolium freemani*, and *Manduca sexta* and varied nutritional conditions, I was able to document the existence of a threshold size, which determines when the larva becomes competent to metamorphose. Threshold size, however, does not dictate the exact timing of initiation. The exact timing for the initiation of metamorphosis is determined by a pulse of the molting hormone, ecdysone, but only after threshold size has been reached. Ecdysone pulses before the larva attains threshold size only cause the larva to molt to another larval instar. These results indicate the timing of metamorphosis initiation is controlled by two factors: (1) attainment of threshold size, at which the larva becomes competent to initiate metamorphosis and (2) the timing of an ecdysone pulse after attaining threshold size.

I hypothesize the attainment of threshold size, and therefore competence to metamorphose, is mediated by the effect of changing juvenile hormone concentrations

caused by the increase in size of the larva. While the larval body grows nearly exponentially, the corpora allata, which secretes juvenile hormone, grows very little if at all. The difference in relative growth causes juvenile hormone concentrations to gradually become diluted. When juvenile hormone concentrations fall below a threshold, changes in protein-protein binding occur that can cause changes in signaling networks and ultimately gene expression. These changes make the larva competent for metamorphosis.

I have demonstrated that only threshold size is consistently correlated with body size; other growth parameters such as growth rate, duration of instars, or number of instars do not consistently correlate with variation in body size. Using the black mutant strain of *M. sexta* I have shown that lower juvenile hormone titers correlate with lower threshold sizes. My hypothesis is consistent with the large body of literature indicating the involvement of juvenile hormone. I also hypothesize that the diversity of metamorphosis types in holometabolous insects can be explained by heterochronic shifts in the timing of threshold size and other developmental events related to metamorphosis. The heterochronic shifts affect not only the morphology of organs, but can also affect the overall phenotypic response of the larva to changes in the environment. The different phenotypic responses among species may make the more or less suited for certain types of niches.

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1. Introduction

"Size dictates the characteristics of all living forms. It is the supreme and universal determinant of what any organism can be or can do."

- - John Tyler Bonner, "*Why Size Matters: From Bacteria to Blue Whales*" (2006)

The size of an organism is perhaps one of its most obvious traits. Whether big or small, the size of an organism affects a myriad of things such as access to resources, conflict resolution, and even mate choice. Even though variation at the individual level exists, species usually have a general range in size that is characteristic for the species, the species specific size. Despite size being one of the most basic characteristics of any species, the processes that underlie how the proper species specific body size is achieved remain a mystery.

1.1 Body Size Regulation in Insects

Holometabolous insects are particularly good subjects for research on body size control because much of their development, physiology, and genetics are well understood. Holometabolous insects go through three distinct phases after hatching. The first phase, the larval phase, is where growth of the insect takes place. Because insects have a cuticular exoskeleton, the larva must molt and replace the exoskeleton several times to prevent this outer cuticular layer from limiting growth. The period between molts are referred to as instars. Although there are an average number of instars a larva will pass through for each species, the particular number for any

individual of that species often varies. At the conclusion of the larval growth phase, the larva will initiate metamorphosis and molt to the next life stage, the pupal stage. The pupa is a non-feeding developmental phase and therefore does not gain mass. Instead, the pupa relies on the stores accumulated during the larval growth phase. The pupa will eventually molt to the adult phase. Once an adult, the holometabolous insect will not molt again, therefore the size the adult as it emerges from the pupa will be the size of the insect for the remainder of its life. Because the adult size is determined by the pupal size, the size at the metamorphic transition from larva to pupa will determine the adult body size. Therefore, proper timing of the cessation of growth and the initiation of metamorphosis is vital to achieving a specific body size.

Metamorphosis is largely controlled by the activity of two insect hormones, ecdysone and juvenile hormone. Ecdysone is secreted by the prothoracic gland. Low background levels of ecdysone, which are typical throughout the lifetime of the larva, are necessary for normal growth. High titer surges of ecdysone initiate the molts that delineate instars, which is why ecdysone is known as the molting hormone. Additionally, a small peak of ecdysone during the last instar initiates the purging of gut contents in preparation for metamorphosis. The weight at the time of gut purging is typically the highest weight the individual organism will ever achieve. Ecdysone thus governs the timing of many important developmental events.

Juvenile hormone is secreted by the corpora allata, and affects what event ecdysone produces. When juvenile hormone levels are high, metamorphosis initiation is inhibited and ecdysone peaks lead to larval-larval molts. When juvenile hormone levels are low, ecdysone can initiate metamorphosis. Therefore, understanding the regulation of ecdysone and juvenile hormone will provide insight into how body size is controlled in holometabolous insects. There are currently two general hypotheses about how insects regulate body size. The first hypothesis claims the prothoracic gland, which produces ecdysone, acts as a size sensing organ and the other hypothesis postulates insulin acts as a metamorphosis initiating factor.

1.1.1 The Prothoracic Gland as a Size Regulator

In 2005, a cluster of papers came out showing altering the growth of the prothoracic gland in *Drosophila melanogaster* also altered final body size (Caldwell, et al.,; Colombani, et al.,; Mirth, et al.,). Although each of the papers used slightly different methods to alter the growth of the prothoracic gland, all generally found an inverse relationship between prothoracic gland growth and body size. As a result of this relationship, the prothoracic gland was hypothesized to sense and regulate body size. Despite their common findings, these papers disagreed about how the prothoracic gland affected body size. Caldwell, et al. found that development time was altered, Colombani, et al. found that growth rate was altered, and Mirth, et al., found changes in both development time and growth rate (2005). Because the prothoracic gland produces

ecdysone, which acts as a growth factor as well as the initiator of molting, the differences between these papers was attributed to the different ways in which the experimental conditions were affecting ecdysone production (Nijhout & Grunert, 2002). Even if the differences in way the prothoracic gland affected final body size were due to differences in experimental design, there remains doubt as to how broadly the conclusions from these papers can be taken.

Although the results of the 2005 papers were quite exciting, they possessed some inconsistencies with well known aspects of insect physiology. Perhaps chief amongst these inconsistencies was the complete disregard for the role of juvenile hormone. There are many papers showing that ectopic application of juvenile hormone, even in the final instar, inhibits pupation and just as many showing that removal of the corpora allata, the gland responsible for juvenile hormone production, can lead to precocious pupation (Parthasarathy & Palli, 2009; Fukuda, 1944; Kiguchi & Riddiford, 1978). Although such contradictory findings would usually suggest that either the large body of literature on juvenile hormone or the three papers proposing the prothoracic gland as the body size regulator must be wrong, this may not be the case. This apparent disagreement between the existing literature and the three 2005 papers is likely due to the choice of *D. melanogaster* as the model organism.

D. melanogaster is a poor model for understanding the role of juvenile hormone in larval development because *D. melanogaster* larvae become insensitive to juvenile

hormone in the second instar (Riddiford & Ashburner, 1991). This insensitivity within the second instar seems to occur very early developmentally when compared to the development of other insects, and is therefore probably a derived feature of *D. melanogaster*. This early insensitivity to juvenile hormone in *D. melanogaster* would make any role of juvenile hormone in insect development extremely difficult to detect in *D. melanogaster*. As such, it is not surprising that the 2005 papers did not find a role for juvenile hormone in body size regulation.

Finally, the canalization of *D. melanogaster* for three instars may also explain the apparent inconsistencies between the existing literature and the three 2005 papers. It is often believed that each species has a set number of larval instars it must past through before pupating; however, it is actually quite common for species to have a variable number of instars (Esperk et al. 2007). Despite the variability in number of larval instars among other insects, *D. melanogaster* is invariant for having three larval instars. In insects with variable instars, there must be a way for the insect to determine when it is in the last instar. Because *D. melanogaster* does not have a variable number of larval instars, it is unclear if such a mechanism for determining which instar is the final instar even exists. Any mechanism that allowed an insect to determine when it is the final instar would also affect body size. Whereas insects with variable numbers of instars could compensate for changes in growth rate or duration of instars by having more or fewer instars, *D. melanogaster* could not. Therefore the growth rate within instars and the

timing of molts would be especially important in *D. melanogaster*, perhaps more so than in insects with variable numbers of instars. Although this inflexibility of *D. melanogaster* in number of instars is not representative for the majority of insects, it may explain the results in the 2005 papers for *D. melanogaster*.

Understanding some of the specific biological traits of *D. melanogaster* provides the basis for the resolution of the apparent disagreement between the existing literature and the three 2005 papers. With an understanding that *D. melanogaster* shows early juvenile hormone insensitivity and is invariant in its number of larval instars, it is completely reasonable to accept that body size is largely controlled by processes associated with prothoracic gland growth in *D. melanogaster*. The conclusions about the body size regulation via prothoracic gland growth, however, will be specific to species with invariant numbers of instar and early juvenile hormone insensitivity, which is not the majority of insects. It is therefore necessary to continue studying body size regulation in order to find the mechanism at work in the many juvenile hormone sensitive species with variable numbers of larval instars.

1.1.2 Insulin as the Metamorphosis Initiating Factor

The hypothesis that insulin is acting as a Metamorphosis Initiating Factor (MIF) has gained popularity, despite the evidence that directly contradicts the hypothesis. The impetus for a MIF came from work on eye development in *Manduca sexta* (MacWhinnie, et al., 2005; Allee, et al., 2006). Because it was found that imaginal tissue behaved

differently in the fifth (final) instar than previous instars, the novel factor MIF was contrived. Later work invoked MIF as a way for nutritional cues to inhibit juvenile hormone in imaginal tissue and induce metamorphosis specifically in the fifth (final) instar (Truman, et al., 2006). It is now believed that those nutritional cues were the work of insulin, making insulin the MIF. When insulin is applied to fifth (final) instar *M. sexta* wing disc in vitro cultures, insulin increased the expression of the pupal gene *broad* of the wings at 48 hours (Koyama, et al., 2008). This increase in *broad* expression was taken as an initiation of metamorphosis, hence MIF.

Unfortunately, insulin does not act as a MIF in the fourth instar because *broad* expression, or any indicator of metamorphosis, is not affected by presence or absence of insulin (Koyama, et al., 2008). It seems at contradictory that insulin can only initiate metamorphosis in what is already the final instar, and is yet considered to be the factor that initiates metamorphosis. It seems more likely that insulin is acting as a growth factor in the final instar and thus increasing broad expression by accelerating developmental growth. Until insulin can initiate some feature indicative of metamorphosis in a non-final instar, I will find the designation of insulin as a "metamorphosis initiating factor" to be completely unsubstantiated.

1.2 An Alternative Hypothesis for Size Regulation

In light of the shortcomings of the current hypotheses on body size determination in insects, I have sought to develop an alternative hypothesis that may be

more broadly applied to many different groups of insects. For developing a new hypothesis, I will utilize the red flour beetle, *Tribolium castaneum*, as my main model organism, but I will also use its sister species, *Tribolium freemani*, and the classical physiological model insesct, *M. sexta*, as well. By employing multiple model organisms, it is my goal to develop a broad hypothesis that can explain body size regulation in many different insect groups. In the following chapters I will present my research in this area, some implications for development and ecology, and some general conclusions about my work.

In Chapter 2, I examine the physiological basis for the body size difference seen in the sister species *T. castaneum* and *T. freemani*. By performing interspecies hybrid crosses, I show that the physiological change associated with the known X-linked effect on body size corresponds to changes in threshold size. Other factors, such as growth rate, duration or number of instars and initial size are different among the different hybrid progeny but do not correlate with the X-linked body size difference. Using the *Giant* strain of *T. castaneum*, I also show that threshold size does vary within the *T. castaneum* species naturally. I propose that selection on such threshold size variation in the ancestor of *T. castaneum* and *T. freemani* could have been the genesis of the body size difference between these sister species.

In Chapter 3, the effects of threshold size on number of instars within a species are examined and a molecular mechanism for threshold size is proposed. I will show

that *T. castaneum* larvae undergo six or seven instars not because of the duration of any particular instar or differences in growth rate, but because of attaining their threshold size at different times relative to the molting cycle. Using a known juvenile hormone titer hypomorph, the *black* strain of *M. sexta*, I show that changes in juvenile hormone are linked to changes in threshold size. I then propose that juvenile hormone levels are at least partially controlled by the dilutive affect of the rapid larval body growth and minimal, if any, growth of the juvenile hormone producing gland, the corpra allata. Because juvenile hormone inhibits the juvenile hormone receptor, *methoprene tolerant*, from dimerizing, the titer of juvenile hormone within the body acts as a regulator *methoprene tolerant* activity in a concentration dependent manner, mediated by body size growth.

In Chapter 4, I examine the diversity present in extant holometabola, insects that go through complete metamorphosis. I address the perplexing similarities seen among many derived metamorphosis types and address how such convergence arose. By comparing different independently derived metamorphosis types to a more primitive type, I show that many of the features associated with derived metamorphosis types are actually present in the primitive metamorphic form. I then propose that the similarities seen between the independently derived metamorphosis types may be due to convergent heterochronic shifts in the developmental processes present in the primitive metamorphosis type. Physiological changes relevant to such heterochronic shifts are

discussed, particularly changes in threshold size. I then use a model to explore the how changes in the relative developmental timing of threshold size can affect the phenotypic landscape for body size. I then relate these changes to life history events to explain why extant holometabola have such a diversity of metamorphosis types.

In Chapter 5, I conclude with some thoughts about threshold size. I will also provide some additional data that did not fit well within the other chapters and hints at how much more there is to learn about the mechanism behind threshold size. I will finish with some future directions as well as the challenges that remain for the field of insect physiology, both empirical and theoretical.

2. A Physiological Basis for Interspecific Size Difference

Although even closely related species can differ in body size, the physiological and developmental basis for such size differences remains unknown. The growth of holometabolous insects has been extensively studied and thus provides a good framework investigating the control of body size. After hatching, holometabolous insects go through three distinct phases; the larva, pupa, and adult. For holometabolous insects, growth in body size occurs in the larval phase. Due to the size limiting constraints of the non-growing exoskeleton, the larva must molt to manufacture a larger exoskeleton and allow for continued growth. The interval between these molts is referred to as an instar.

Although the number of instars is generally thought to be species specific, there can often be individual variation in number of instars within the species (Esperk et al. 2007). The variation in instar number is controlled by a physiological mechanism known as threshold size. Threshold size is the size at which the larva determines the final larval instar and can be measured by weight or head capsule size (Kingsolver, 2007). In *Manduca sexta*, the instar that reaches or exceeds threshold size at its start is the final larval instar (Nijhout, 1975). However, within the longicorn beetle, *Psacothea hilaris*, threshold size can occur within the final instar itself (Munyiri et al., 2004). Within the last instar, the larva will initiate metamorphosis by purging its gut contents and finding a suitable location for pupation. Both the molting events as well as gut purge are under

the control of the molting hormone ecdysone. The weight at the time the gut is purged is the maximal weight for the larva. Because the pupa does not feed, the insect does not grow in mass. The pupa will then molt to become an adult. Adult holometabolous insects do not molt, and this means that the maximal size of the larval stage ultimately determines the body size of the adult. Thus determining the factors that control the maximal larval size will also identify factors that control maximal adult body size.

The size difference between two closely related species of the flour beetle genus *Tribolium* provides us with an ideal system for studying the physiological and developmental basis of body size determination. *Tribolium castaneum* and *Tribolium freemani* are sister species that diverged roughly 11.6 to 47.0 million years ago and exhibit a substantial size difference (Angelini & Jockusch, 2007). With an average maximal weight at the time of gut purge of 4.5 mg, *T. freemani* is more than twice the average maximal weight at the time of gut purge of 2.1 mg for *T. castaneum*. Because two closely related but differently sized species often differ in many traits that may affect body size, it is often difficult to distinguish which traits are correlated or causative to differences in body size. Hybridizations between differently sized species can provide insight into the underlying regulation of size by placing both species' genes in a common cellular environment. Despite their difference in size, *T. castaneum* and *T. freemani* can be crossed to produce sterile but viable hybrid offspring. In *Tribolium*, males are heterogametic (XY) and only receive the genetic content of the X-chromosome

from their maternal parent whereas homogametic females (XX) receive and X-chromosome from both parents. When these sister species are crossed, their progeny display a strong X-linked effect on body size (Nakakita et al. 1981; Brownlee & Sokoloff, 1988). Interspecies hybrid males have body size phenotypes similar to their maternal species lineage from which they inherited their X chromosome. Hybrid females have a phenotype intermediate to that of the parental lineages. The altered growth traits associated with this X-linked effect, however, have not been identified. By identifying the similarities and differences between the hybrid offspring, I have determined which growth traits have changed in linkage with the X-chromosome.

My results have provided me with evidence of a novel mechanism for the evolution and control of body size in insects. Although I expected to find changes in growth rate, number of instars, or duration of instars that were X-linked and correlated with differences in the body size of hybrid progeny, none were consistent with X-linkage. I found that only threshold size was consistently correlated with the body size phenotypes of the hybrid progeny. This indicates threshold size was one of the main evolutionary targets changed in the evolution of the different sizes of *T. castaneum* and *T. freemani*.

2.1 Materials and Methods

2.1.1 Species and Hybrid Growth Data

The *T. freemani* strain, *T. castaneum* wild type *TIW1* strain, and the *T. castaneum* body size mutant strain *Giant*, were obtained from Dr. Beeman of GMPRC, USDA-ARS. Interspecific and intraspecific crosses were created using 20 virgin males and 20 virgin females of *T. castaneum* from the *TIW1* strain and *T. freemani*. All rearing was done at 30°C and 40% relative humidity. Crosses were allowed to mate for 5 days preceding egg collection. For egg collection, adults were transferred to egg collection medium (95% flour and 5% yeast by weight, fine mesh sifted) and eggs were collected after 24 hours (Egg Day 0). Eggs were monitored daily for three days for any early hatching larvae, which were removed. All larvae used for the experiment hatched on Egg Day 3, Larval Day 0. Twenty newly hatched individuals from each cross were picked at random for the experiment. Each individual larva was reared in a 1.5 ml microcentrifuge tubes with four small ventilation holes in the cap containing the standard medium (95% flour and 5% yeast by weight). Each larva was individually weighed daily (including Larval Day 0) and examined for indications of molts, which were also recorded. All weight measurements were taken using a Cahn 25 Automatic Electrobalance. On the day of pupation, individuals were weighed and sexed.

To control for the effects of handling, 20 random newly hatched larvae from each cross were raised in similar conditions and concurrently with the daily weight measurement experiments. These larvae were not weighed daily, only observed with minimal disturbance for pupation. On the day of pupation, individuals were weighed

and sexed. Comparison of these and the daily weight experiments showed the effect of daily handling did not significantly alter overall developmental period nor pupal weight (data not shown).

2.1.2 Calculation of Growth Rates

To determine a growth rate for overall development and individual instars, the standard exponential growth equation (weight = initial weight (x) e $^{\text{growth rate (x) time}}$) was fitted to the data using the least R squared method. For final instars, the day of gut purge was used as the last day for fitting as it marks the initiation of metamorphosis and the cessation of feeding and growth.

2.1.3 Threshold Size Determination

To determine threshold size, individual progeny of various weights from intraspecific strain crosses and interspecific crosses were obtained from uncrowded group culture conditions on standard media. Progeny were tested by starvation on cellulose (AlphaCel) until they pupated or died. Control larvae were placed on standard media to detect any aberrant death not due to experimental conditions. All pupating larvae were sexed. The outcome of the threshold size experiments were ordered by weight at starvation and then percent pupating was determined in a moving average of eight individuals. Threshold size is the point at which 50% of starved larvae pupate. For the interspecies crosses, PeakFit (Systat Software) was used to find the midpoint of the transitional parts of the graph. Briefly, the data were smoothened before taking the

first derivative. The baseline for the derivative was fixed at the start and end points. It was then analyzed to find the Gaussian amplitude peaks, which were used as the threshold sizes for the corresponding progeny.

2.2 Results

2.2.1 Beginning and End Weights

My results show that males from the hybrid crosses are more similar to the maximal weight at gut purge of the maternal species, whereas females from hybrid crosses show an intermediate phenotype (Figure 1). Although the males from the *T. freemani* male crossed to *T. castaneum* female are larger than males from the pure *T. castaneum* cross, they are still within the wild type range of *T. castaneum* and are not statistically different from the females of the pure *T. castaneum* cross. Weight at hatching was correlated to the maternal species of the cross but did not consistently correlate with maximal weight at the time of gut purging (Figure 2). Therefore, the initial weight at hatching does not determine the phenotypic differences in body size of these crosses.

2.2.2 Instar Comparisons

Progeny from the *T. freemani* male with *T. castaneum* female cross all had seven larval instars, whereas in the reciprocal cross males had seven larval instars and females had six larval instars. In the *T. freemani* male with *T. castaneum* female cross, males and females differed statistically in the duration of their second instars (Figure 3). In the

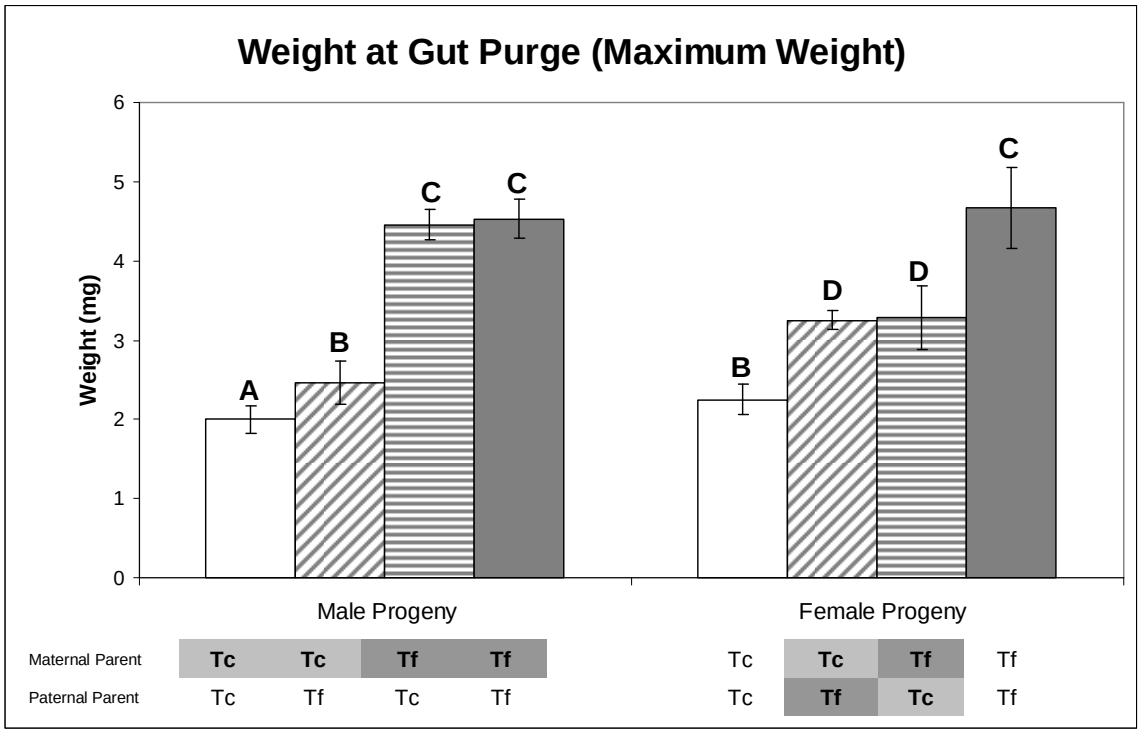

Figure 1: Weight at Gut Purge (Maximum Weight) of Progeny. Bars show average weight at gut purge for progeny produced by the genotypes indicated below the bar (Tc for *T. castaneum* and Tf for *T. freemani*). There is no statistical difference between bars with the same letter above them. Error bars represent ±1 standard deviation.

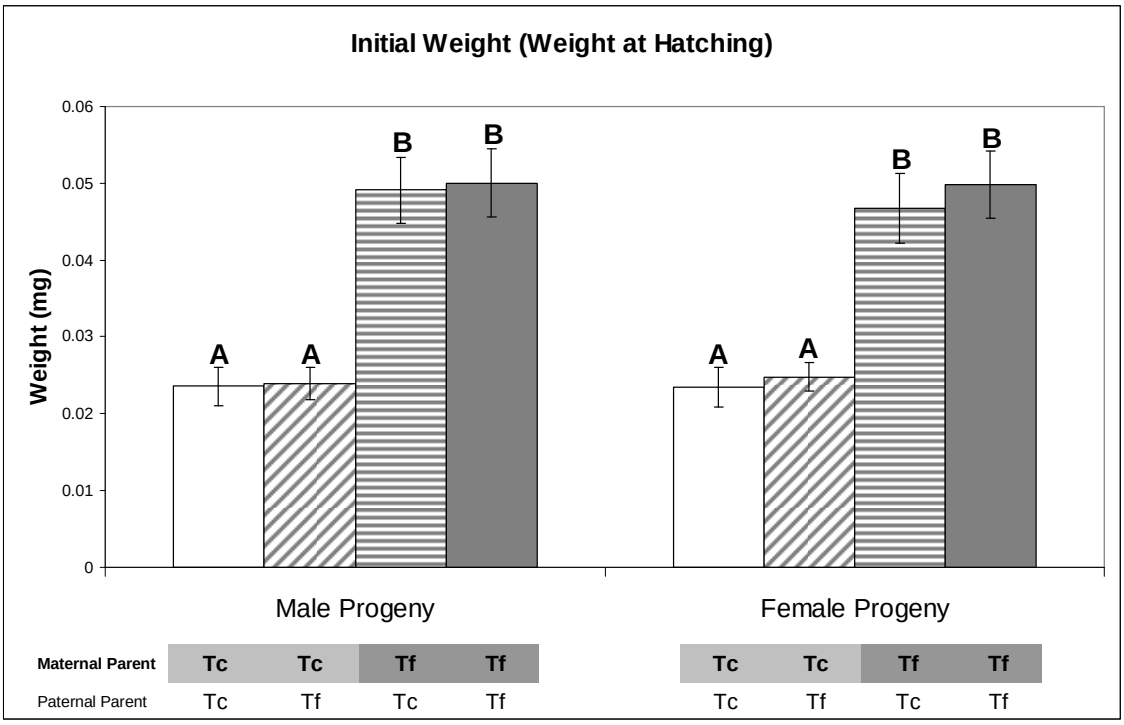

Figure 2: Initial Weight (Weight at Hatching) of Progeny. Bars show average weight at hatching for progeny produced by the genotypes indicated below the bar (Tc for *T. castaneum* and Tf for *T. freemani*). There is no statistical difference between bars with the same letter above them. Error bars represent ±1 standard deviation.

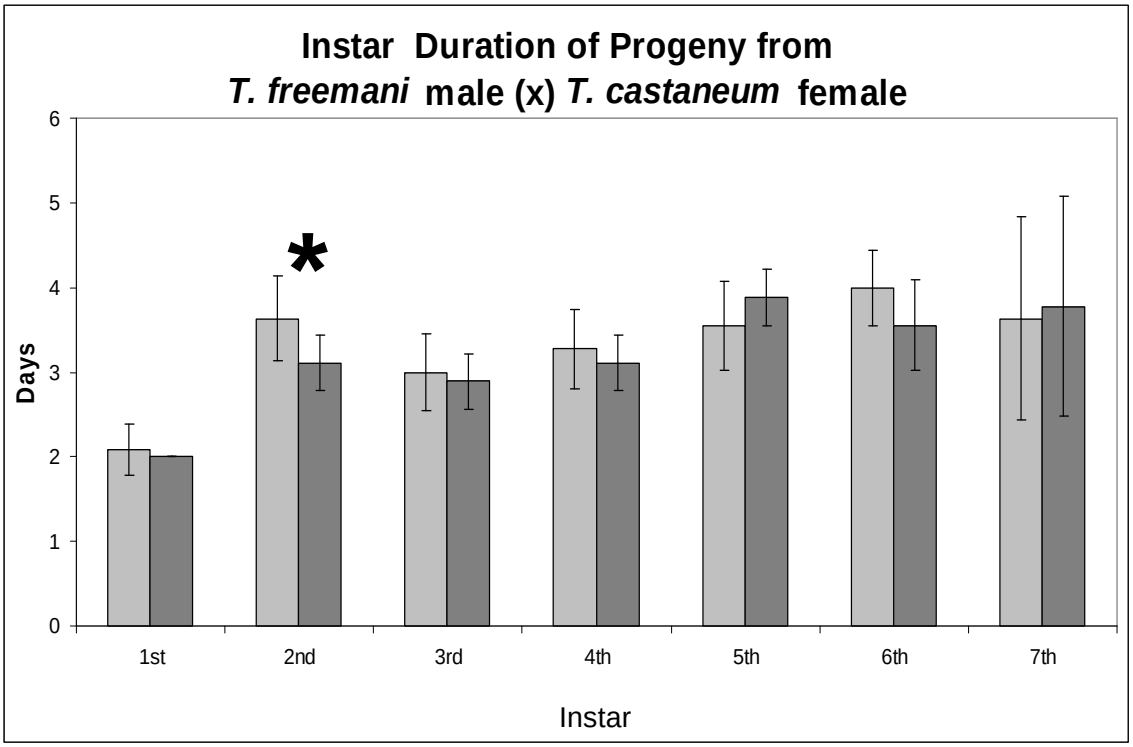

Figure 3: Instar Duration of Progeny from *T. freemani* male and *T. castaneum* female interspecies cross. Male (light grey) and female (dark grey) bars show average instar duration in days. Instars in which males and female statistically differ are denoted with an asterisk. Error bars represent ±1 standard deviation.

reciprocal cross males and females were not statistically different when non-final instars were compared (Figure 4). There was a statistical difference from the progeny of the *T. castaneum* male with *T. freemani* female cross in the duration of their sixth instars; however it must be noted that this comparison is between a final instar for the females and a penultimate instar for the males and is likely due to the different biological processes that take place during final and non-final instars (i.e. gut purge versus molting).

My analysis of growth rates within each instar revealed that in the *T. freemani* male with *T. castaneum* female cross, males and females were statistically different in the second and fourth instars and generally females tended to have higher growth rates than males whereas the reciprocal cross showed no differences between males and females (Figures 5 and 6).

I also compared the hybrid females from the interspecies crosses to each other because they had similar maximal weight phenotypes at the time of gut purging. Although the hybrid females from the *T. freemani* male with *T. castaneum* female cross had seven larval instars, the reciprocal cross only had six larval instars. I found no statistical difference in growth rates of non-final instars (Figure 7). The duration of each instar was the same for the female hybrid progeny of the reciprocal interspecies crosses, except for the duration of the first instar (Figure 8).

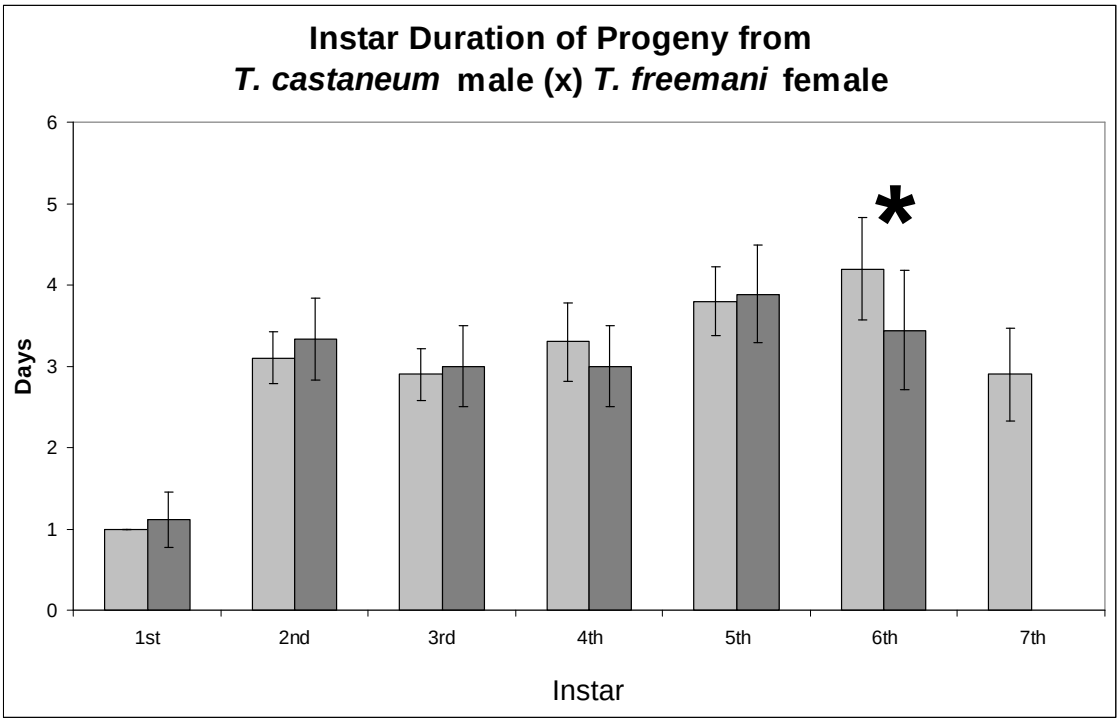

Figure 4: Instar Duration of Progeny from *T. castaneum* male and *T. freemani* female interspecies cross. Male (light grey) and female (dark grey) bars show average instar duration in days. Instars in which males and female statistically differ are denoted with an asterisk. Error bars represent ±1 standard deviation.

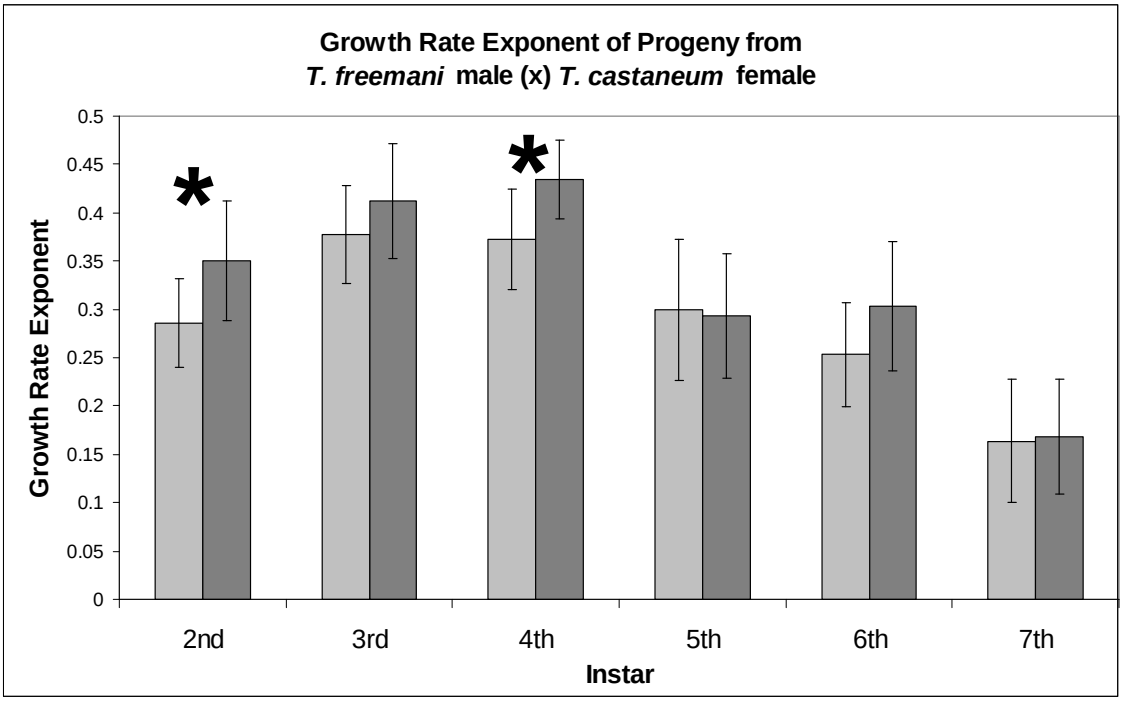

Figure 5: Growth Rate Exponent of Progeny from *T. freemani* male and *T. castaneum* female interspecies cross. Male (light grey) and female (dark grey) bars show average growth rate exponent for an instar. Instars in which males and female statistically differ are denoted with an asterisk. Error bars represent ±1 standard deviation.

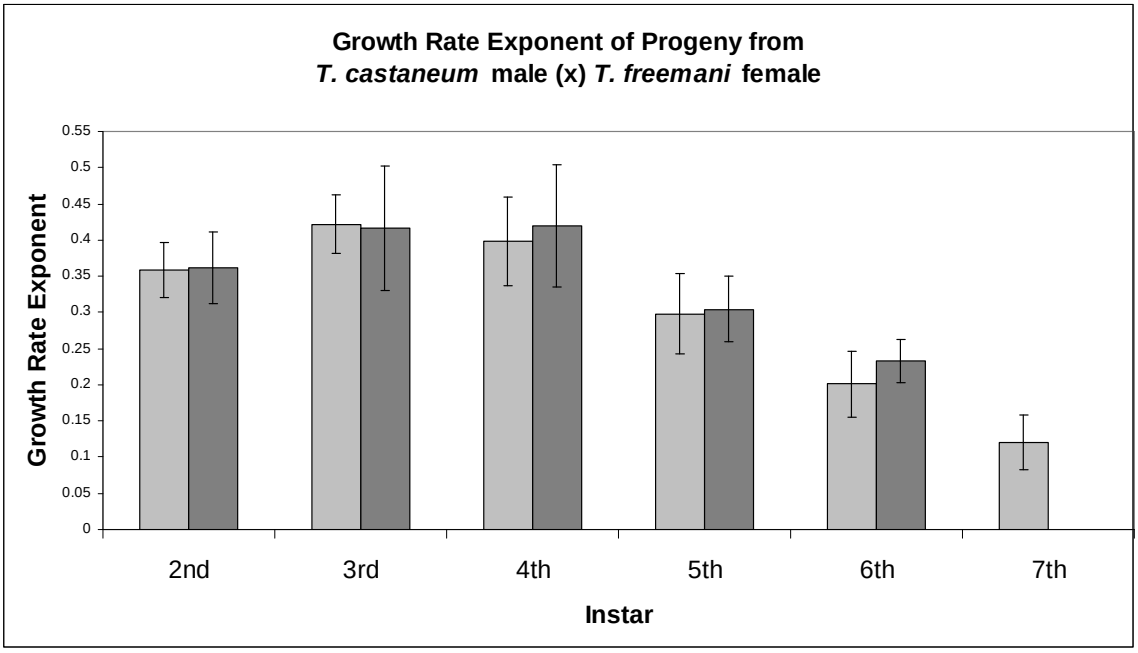

Figure 6: Growth Rate Exponent of Progeny from *T. castaneum* male and *T. freemani* female interspecies cross. Male (light grey) and female (dark grey) bars show average growth rate exponent for an instar. Instars in which males and female statistically differ are denoted with an asterisk. Error bars represent ±1 standard deviation.

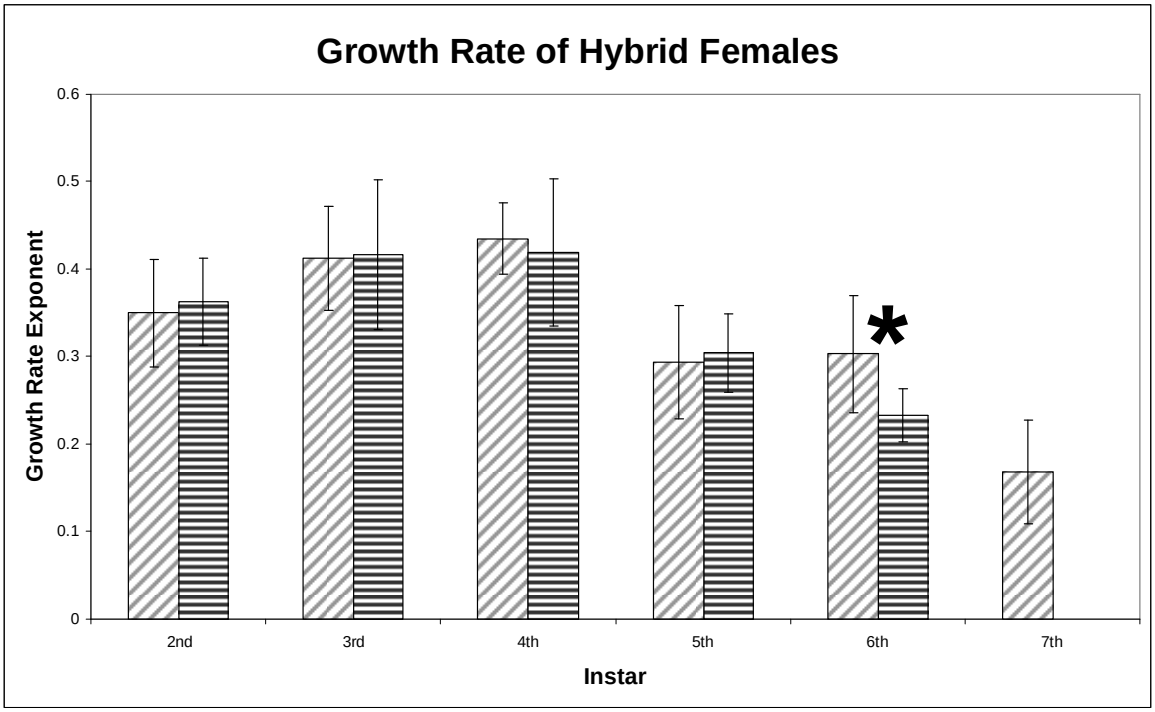

Figure 7: Growth Rate Exponent of Female Progeny from *T. freemani* and *T. castaneum* interspecies cross. Bars show the average growth rate exponent of female hybrid progeny from *T. freemani* male and *T. castaneum* female cross (diagonal stripes) and *T. castaneum* male and *T. freemani* female cross (horizontal stripes). Instars with statistical differences are denoted with an asterisk. Error bars represent ±1 standard deviation.

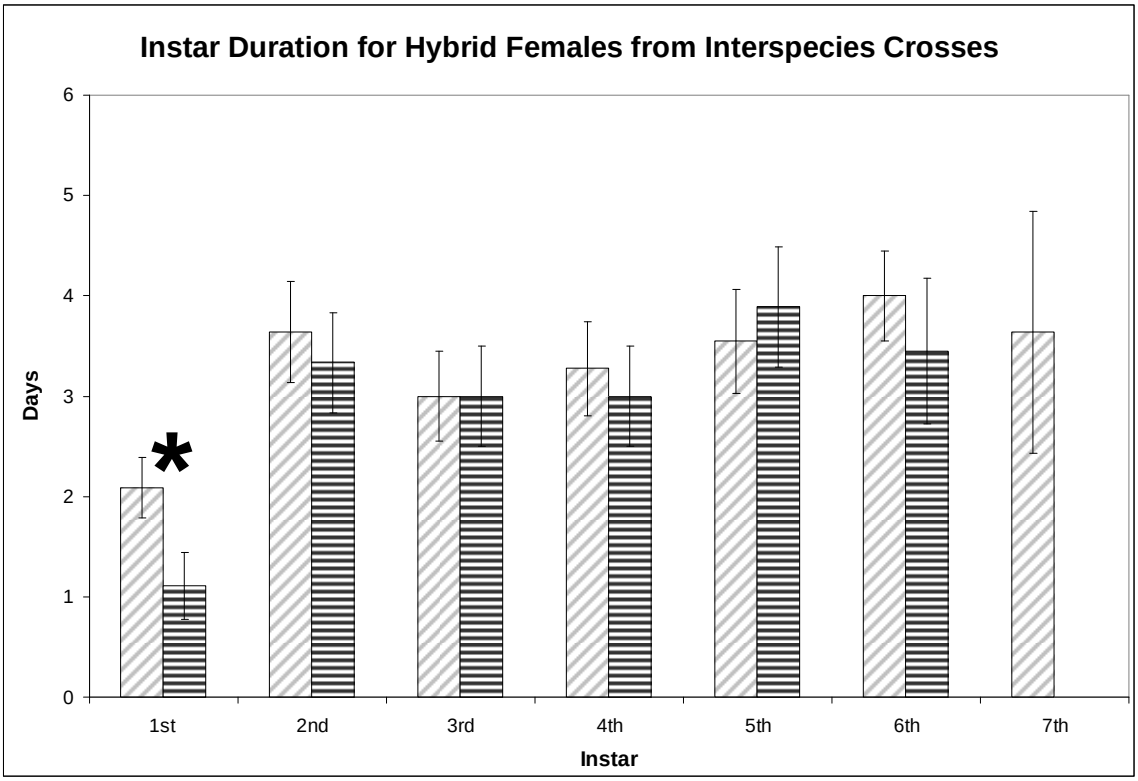

Figure 8: Instar Duration of Female Progeny from *T. freemani* and *T. castaneum* interspecies cross. Bars show the average instar duration of female hybrid progeny from *T. freemani* male and *T. castaneum* female cross (diagonal stripes) and *T. castaneum* male and *T. freemani* female cross (horizontal stripes). Instars with statistical differences are denoted with an asterisk. Error bars represent ±1 standard deviation.

2.2.3 Threshold Sizes

2.2.3.1 Threhsold Sizes for Non-Hybrid Strains

I determined threshold size for the wild type *TIW1* (1.46 mg) and the *Giant* (3.06 mg) strains of *T. castaneum* as well as the threshold size for *T. freemani* (4.53 mg) (Figure 9). Threshold size is the physiological mechanism by which the larva determines the final larval instar. In *M. sexta*, threshold size must be passed before the start of the last instar (Nijhout, 1975). However, in *Tribolium* species, I found that threshold size can be surpassed within the last instar. These findings are consistent from what is known in the longicorn beetle, *P. hilaris* (Munyiri et al., 2004). *Tribolium* larvae when starved below threshold size are unable to molt to become a pupa but could molt to additional larval instars, in some cases multiple times. Because my starvation experiment affected the ability to pupate, but not to molt in general, our results indicated that threshold size, which regulates the type of molts (larval-larval versus larval-pupal), was being measured.

2.2.3.2 Threshold Sizes for Interspecies Hybrid Progeny

The threshold size experiments revealed sex differences among interspecies hybrid progeny. In my threshold size experiments using the *T. castaneum* wild type strain, *T. castaneum Giant* strain, and *T. freemani* wild type strain, I found sharp transitions in the percent pupation curves (Figure 9). In the reciprocal interspecies

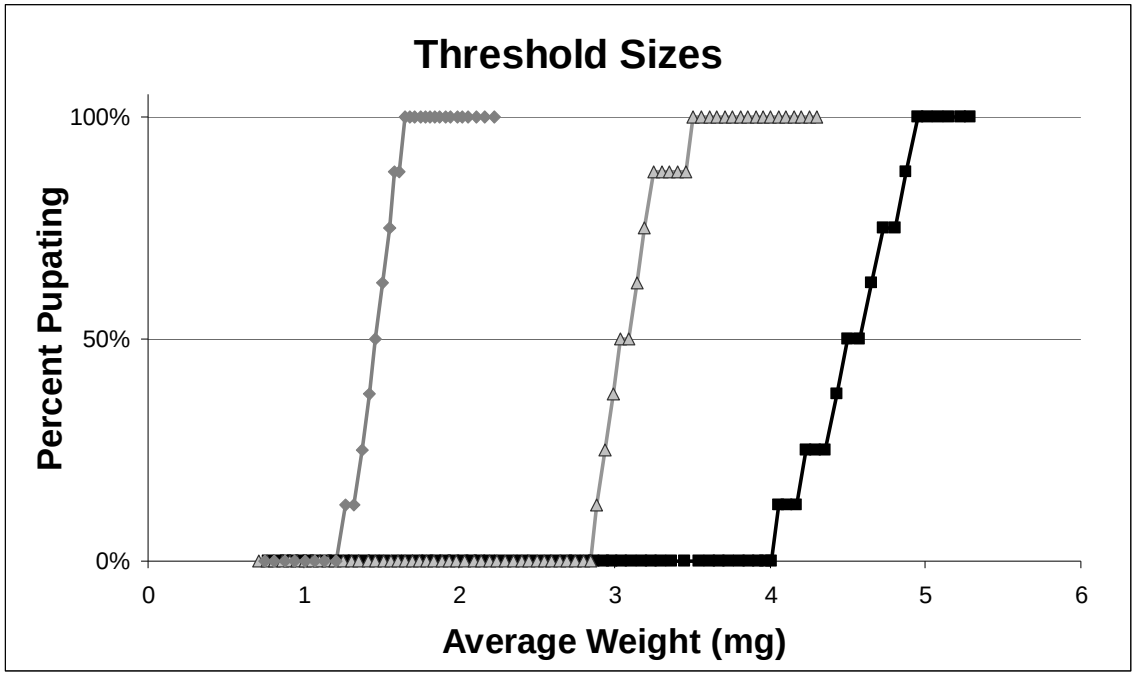

Figure 9: Threshold Sizes of *T. freemani* and the *TIW1* and *Giant* strains of *T. castaneum*. The percent pupating for *T. freemani* (squares) and the *TIW1* (diamonds) and *Giant* (triangles) strains of *T. castaneum* indicate that threshold size occurs at 4.53 mg, 1.46 mg, and 3.06 mg for the respective strain.

crosses, by contrast, the percent pupation curves had two sharp transitions with an intermediate plateau between these transitions (Figures 10 and 11). When the pupae from the experiment were sexed, because *Tribolium* cannot be sexed as larvae, I found that the weight ranges the males and females pupated differed. When the hybrid progeny from the *T. freemani* male with *T. castaneum* female cross were starved, hybrid males could pupate at weights above 1.96 mg while hybrid females could only pupate at weights above 2.838 mg (Figure 8). In the reciprocal interspecies cross, the hybrid female progeny could pupate at weights above 2.645 mg while hybrid males could only pupate at weights above 3.461 mg (Figure 9).

Although threshold size is usually defined as the point where 50% of the progeny are pupating, my findings suggested that males and females were responsible for different transitions in the curves. In the hybrid progeny of both interspecies crosses, one sex made up the recovered pupae of the transition at the lower weight as well as the intermediate plateau. In the transition of the curves at the higher weights, both sexes were being recovered as pupae. I interpreted this finding to mean that the two sexes of the hybrid progeny had different threshold sizes. Therefore, the midpoint of the transition in the curve at the lighter weight would correspond to the threshold size of the sex being recovered at lower weights. Similarly, the midpoint of the transition in the curve at the higher weights was therefore the threshold size of other sex not recovered at lower weights. The hybrid threshold sizes , combined with the threshold sizes for the

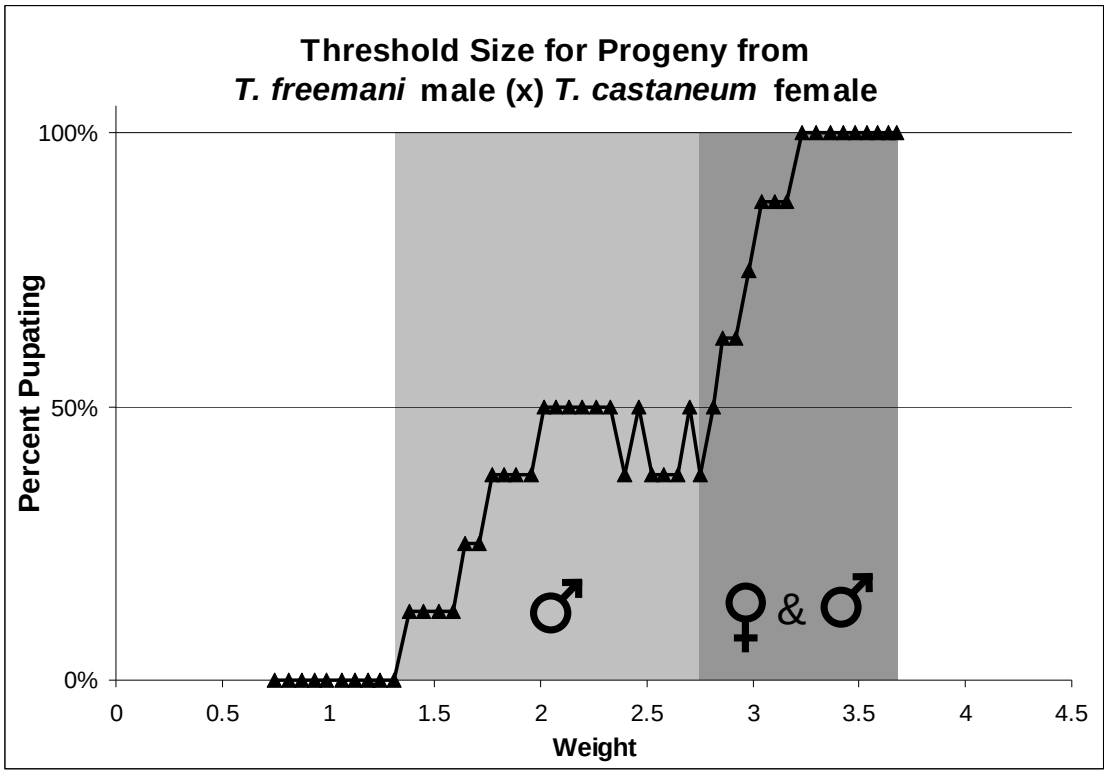

Figure 10: Threshold Sizes of Progeny from *T. freemani* male and *T. castaneum* female interspecies cross. The line represents the percent pupating. Light grey area represents the weight range where all pupating larvae were male, while the dark grey area represents the weight range where pupating larvae were of both sexes.

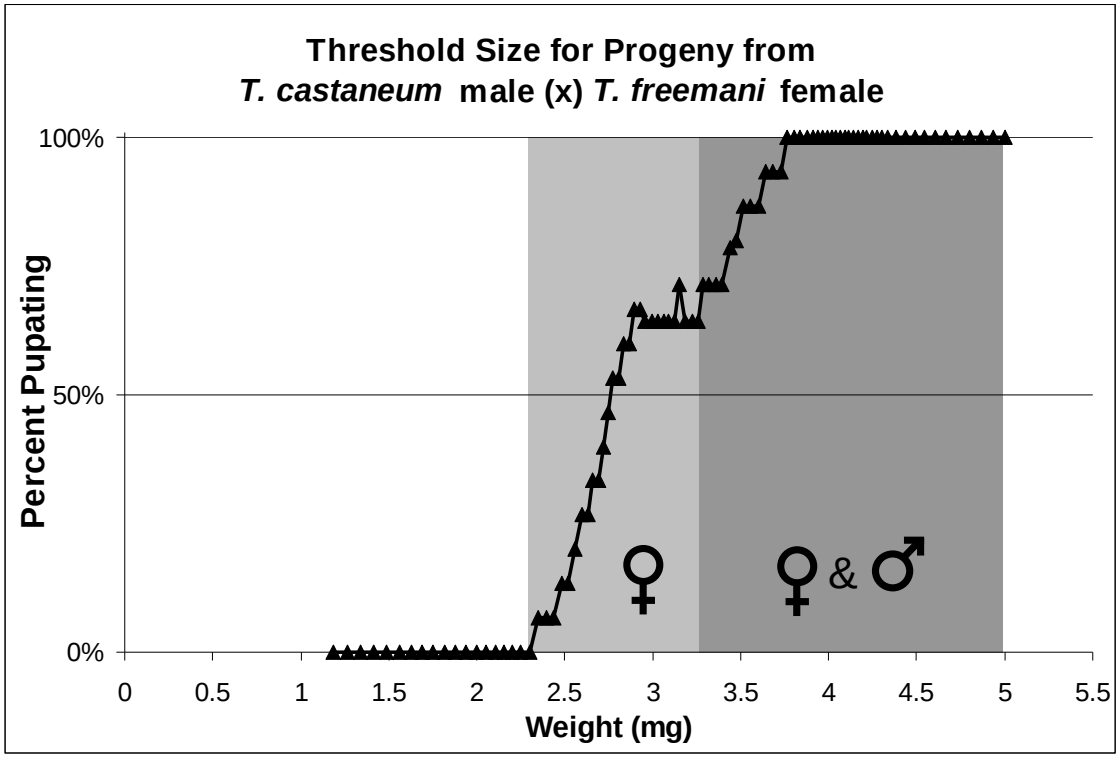

Figure 11: Threshold Sizes of Progeny from *T. castaneum* male and *T. freemani* female interspecies cross. The line represents the percent pupating. Light grey area represents the weight range where all pupating larvae were female, while the dark grey area represents the weight range where pupating larvae were of both sexes.

wild type pure species crosses where no sex differences were detected are graphed together in Figure 12. These results indicate that hybrid males of the interspecies crosses had threshold sizes similar to that of the maternal parent whereas hybrid females had an intermediate threshold size. The threshold size results are consistent with the phenotypic size results of both crosses and show an inheritance pattern consistent with an X-linked trait.

2.2.3.3 Interacton of Threshold Size with Overall Growth

Threshold size interacts with the overall growth of the larvae. Although threshold size in *M. sexta* must be attained by the start of the last instar to determine the final instar, in *Tribolium* species threshold size can be surpassed within the last instar, similar to *P. hilaris*. In the female hybrid progeny, threshold size was clearly passed near the midpoint of the last instar in both interspecies crosses (Figures 13 and 14). The growth curves of the hybrid males from both crosses indicate that the hybrid males had attained threshold size very early in their last instars (Figures 13 and 14). Differences in the environmental conditions could affect the growth rates and therefore the size of the larvae at which it initiates molting. Changes in the relative timing for the attainment of threshold size and the initiation of molts could change the resulting final body size. This interaction of threshold size with the timing of molts may be why some previous studies found hybrid males from these interspecies crosses to be the more or less similar to the size of the maternal species (Nakakita et al. 1981; Brownlee & Sokoloff, 1988).

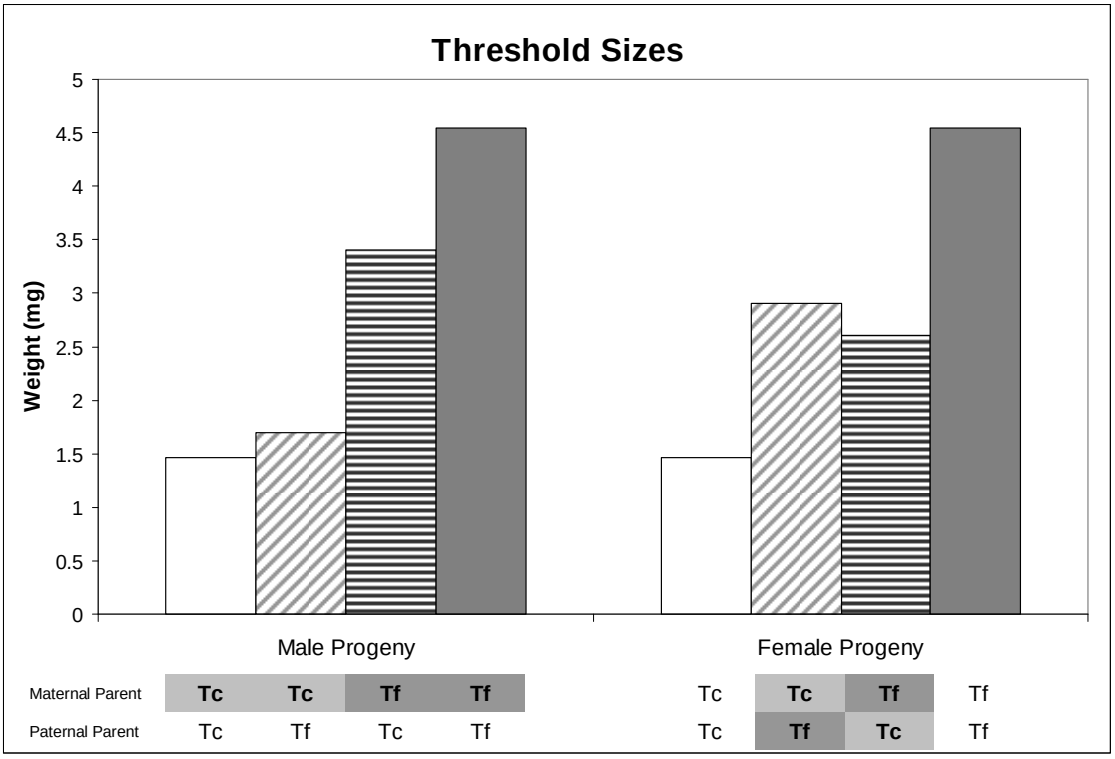

Figure 12: Threshold Sizes of Progeny. Bars show the calculated threshold sizes for progeny produced by the genotypes indicated below the bar (Tc for *T. castaneum* and Tf for *T. freemani*).

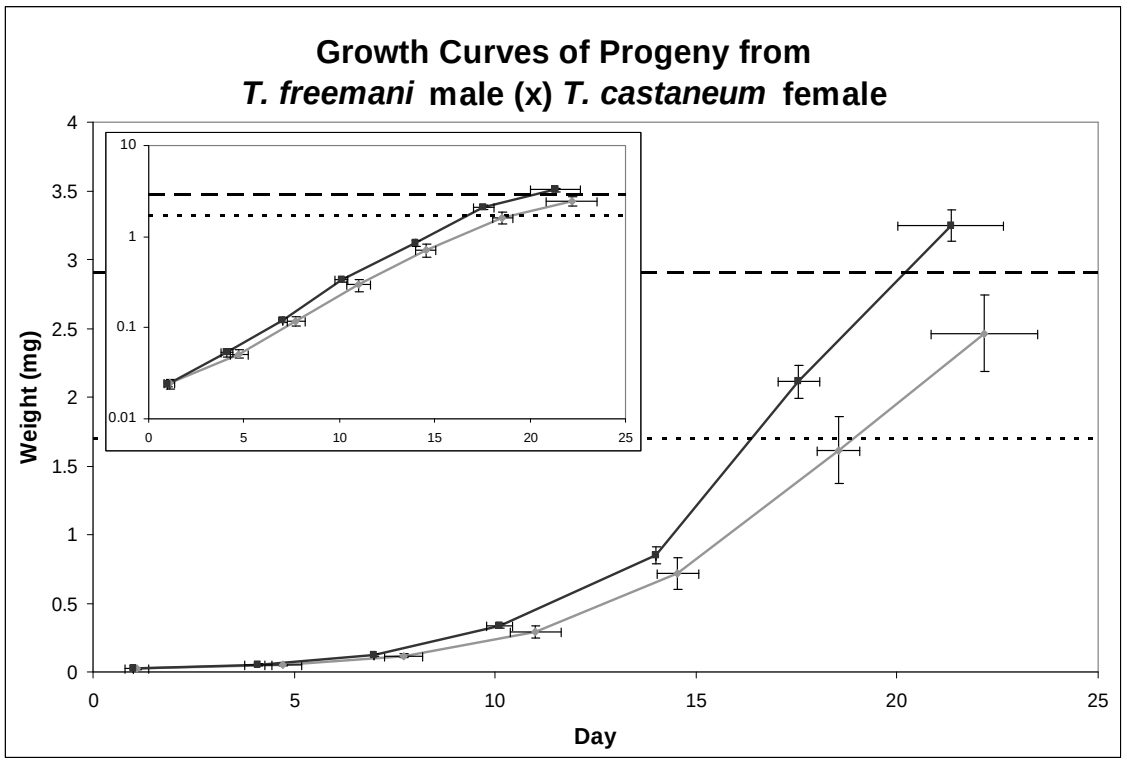

Figure 13: Average Growth Curves of Progeny from *T. freemani* male and *T. castaneum* female interspecies cross. Points depict the final weight of an instar with the exception of the final instar, for which weight at the time of gut purge (maximal weight) was used. Males (light grey line) passed their threshold size (small dashed horizontal line) in seven instars. Females (dark grey lines) also passed their threshold size (long dashed horizontal lines) in seven instars. Inset is same graph on logarithmic scale. Error bars represent ±1 standard deviation.

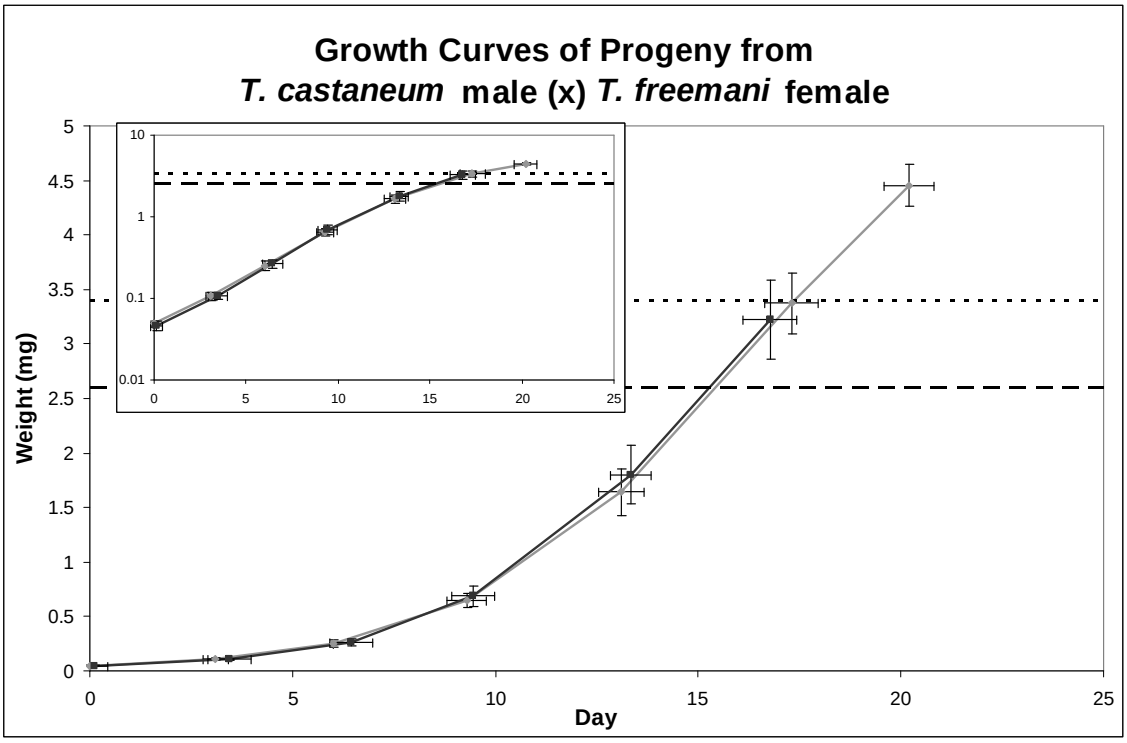

Figure 14: Average Growth Curves of Progeny from *T. castaneum* male and *T. freemani* female interspecies cross. Points depict the final weight of an instar with the exception of the final instar, for which weight at the time of gut purge (maximal weight) was used. Males (light grey line) passed their threshold size (small dashed horizontal line) in seven instars while females (dark grey lines) passed their threshold size (long dashed horizontal lines) in six instars. Inset is same graph on logarithmic scale. Error bars represent ±1 standard deviation.

2.3 Discussion

My results are the first to demonstrate that variation in threshold size can account for body size variation within and between insect species. Genetic manipulations of *Drosophila melanogaster* altered body size by changing growth rate and/or duration of larval instars (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005). Studies using size selected strains and environmental variations in *M. sexta* have also shown that changes in the growth rate and/or duration of larval instars can affect body size (D'Amico et al., 2001; Nijhout et al., 2006). Although threshold sizes have been reported in *M. sexta* (Nijhout, 1975; Kingsolver, 2007) and in genetically modified *D. melanogaster* (Zhou et al., 2004), variation in threshold size for *M. sexta* or *D. melanogaster* has not yet been reported. Using *T. castaneum* and *T. freemani*, sister species that differ in body size, as well the *Giant* strain of *T. castaneum*, I have shown that threshold size can vary within and between insect species. My analysis of the hybrid offspring from reciprocal *T. castaneum* and *T. freemani* interspecies crosses could only consistently correlate threshold size with the phenotypic differences in body size of the progeny and not initial weight, growth rate, duration of larval instars, or number of larval instars. These data indicate that threshold size is an important factor in the regulation and evolution of body size in insects.

2.3.1 X-linked Effect on Threhold Size

My results show that the additive X-linked effect on body size in *T. castaneum* and *T. freemani* cross is correlated with differences in threshold size and not other growth parameters such as growth rate, number and duration of instars, and initial size. Although these other physiological mechanism may influence the resulting hybrid progeny phenotypes, only threshold size showed an additive X-linked inheritance pattern consistent with the phenotypic results of the interspecies crosses.

Perhaps the most interesting results of this study were from the hybrid females from the reciprocal interspecies crosses (Figure 15). Although the hybrid females from the reciprocal interspecies crosses were genetically similar, having equally inherited autosomes and X chromosomes from both parental species, their maternal parent determined the start weight of the hybrid females. Hybrid females born from *T. freemani* mothers were nearly twice the size of those hybrid females born of *T. castaneum* mothers. Despite this difference in initial weight, both groups of hybrid females had similar growth rates for non-final instars (instars 2 though 5) and arrived to the same size at the time of gut purge. The difference in the number of instars of the two genetically similar groups of hybrid females indicates that the number of instars is not genetically determined. Furthermore, by comparing the hybrid females to their hybrid male counterparts as well as to the parental species, I can discount a purely maternal effect for

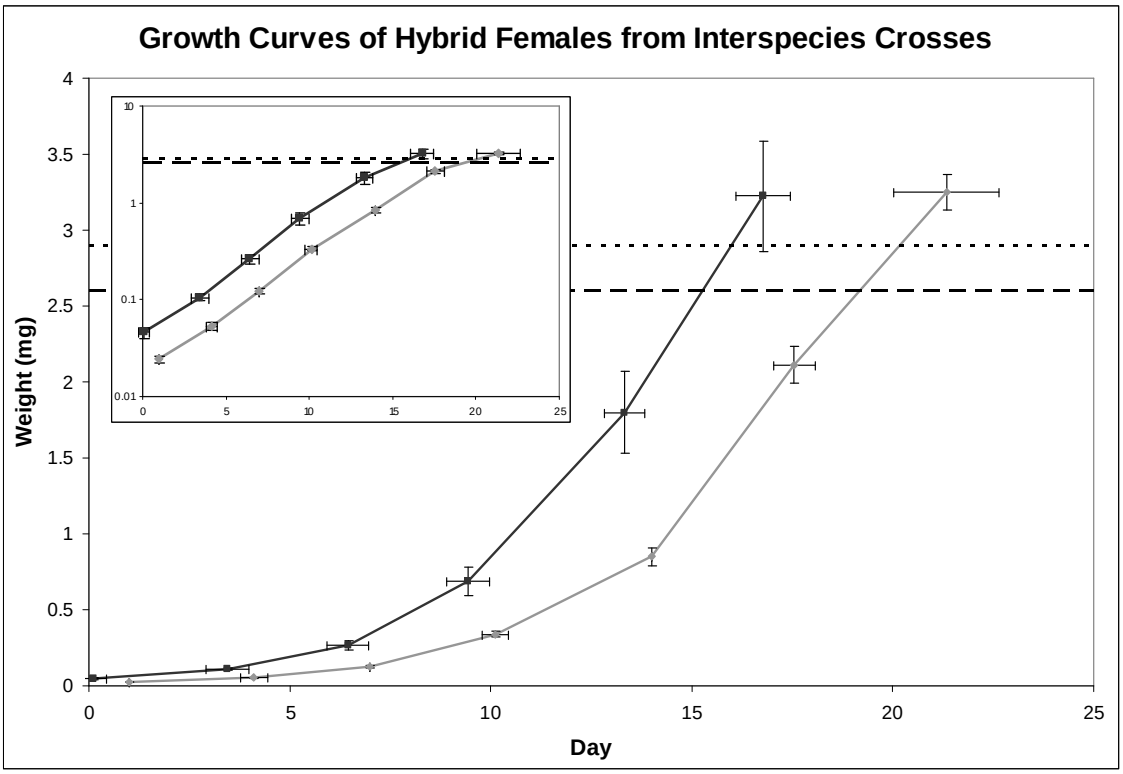

Figure 15: Average Growth Curves of Female Progeny from *T. castaneum* and *T. freemani* interspecies crosses. Points depict the final weight of an instar with the exception of the final instar, for which weight at the time of gut purge (maximal weight) was used. Hybrid female progeny from the *T. freemani* male and *T. castaneum* female cross (light grey line) passed their threshold size (small dashed horizontal line) in seven instars while hybrid female progeny from the *T. castaneum* male and *T. freemani* female cross (dark grey lines) passed their threshold size (long dashed horizontal lines) in six instars. Inset is same graph on logarithmic scale. Error bars represent ±1 standard deviation.

instar number. These data suggest that the similarity in threshold size for the hybrid females explains the phenotypic similarity in size at the time of gut purge.

2.3.2 Threshold Size Evolution

Variation in threshold size suggests that evolution of body size could be mediated by threshold size evolution. I found that variation in threshold size exists within differently sized strains of *T. castaneum*. The *Giant* strain, a naturally occurring isolate, has a larger threshold size than the *TIW1* standard laboratory strain. Although this is not an extensive sampling of the many different strains of *T. castaneum*, it does show that threshold size variation in the wild can and does exist. Results from my hybrid cross show that threshold size is a heritable trait. Therefore, threshold size has a foundation on which selection could act, implicating threshold size as an evolutionary target for altering body size in insects.

2.4 Conclusion

Threshold size is an important, but poorly understood, regulator of body size in insects. This study shows that differences in threshold size can account for phenotypic differences in body size within and between two *Tribolium* species. The findings presented here suggest that the evolution of body size in *T. castaneum* and *T. freemani* is due, at least in part, to the evolution of threshold size. Because threshold sizes have been reported in other insect species, variation in threshold size may be an important aspect of body size regulation in many insect species. Unfortunately, the mechanism by

which threshold size acts is completely unknown. Understanding the threshold size mechanism will provide insight into how body size is regulated and it can evolve.

3. Insects Measure Threshold Size by Dilution of Juvenile Hormone

Although many species have a general species-specific body size, how organisms sense that they have achieved that body size is unknown. Holometabolous insects provide an ideal system for studying the regulation of body size because much of the physiology, genetics, and development that underlie growth are well-understood. Holometabolous insects grow throughout their larval life phase. To accommodate this growth within their exoskeleton, insects must molt periodically to produce a larger exoskeleton and continue growing. The stage between molts is known as an instar. For many insects the number of molts, and thus the number of instars, can be quite variable (Esperk et al. 2007). The larval growth phase concludes with the initiation of metamorphosis. Proper timing is critical for the initiation of metamorphosis because larval growth is roughly exponential, and therefore differences in timing the initiation of metamorphosis can cause large differences body size. Larvae sense they have achieved the appropriate size to initiate metamorphosis via the physiological mechanism known as threshold size. In *Manduca sexta*, the instar that reaches or exceeds threshold size is the final larval instar (Nijhout, 1975). In *M. sexta*, threshold size must be surpassed by the start of the final instar, but other species, such as *Psacothea hilaris* and *Tribolium* species, threshold size can be attained during the final instar (Munyiri et al., 2004; previous chapter). Although many of the processes involved in metamorphosis have

been well characterized, little is known about how the larva senses it has attained the appropriate size to initiate metamorphosis.

3.1 Hormonal Control of Metamorphosis

Two key hormones control the initiation of metamorphosis: ecdysone and juvenile hormone. At high concentrations, juvenile hormone is known to inhibit pupation (Parthasarathy & Palli, 2009). Precocious pupation can be induced by removal of the gland that produces juvenile hormone, the corpora allata, as well as by knockdowns of juvenile hormone receptors or biosynthetic enzymes (Fukuda, 1944; Konopova & Jindra 2007; Minakuchi et al. 2008). The precise timing of that pupation, however, is under the control of ecdysone. Large peaks of ecdysone induce the molting events during the larval development and during the larval-pupal transition whereas lower levels commit larval tissue to a pupal state (Koyama et al. 2004). In this way, juvenile hormone controls whether the molt will be larval-larval or larval-pupal, and ecdysone controls when the molt will occur. What is currently unknown is how these hormones, especially juvenile hormone, are controlled to initiate metamorphosis at the proper size.

The red flour beetle, *Tribolium castaneum*, is an ideal system in which to study how insects sense that they have attained the appropriate size to initiate metamorphosis. *T. castaneum* has a highly plastic larval developmental period, which can vary from 5 instars in approximately 18.5 days to 11 instars in about 104.6 days (Sokoloff, 1974).

Even inbred populations have individuals that vary in the number of larval instars (Sokoloff, 1974; personal observation). This variability shows that *T. castaneum* larvae are not measuring time or number of instar as a determinant for the initiation of metamorphosis. I took advantage of this plasticity to examine why individuals from the same strain in controlled environmental conditions would initiate metamorphosis in different instars. I found that final body size is a result of the complex interaction of ecdysone signaling and the attainment of threshold size. Threshold size functions as a set-point for the minimal size of pupation for a given genotype in a particular environmental condition. I propose a simple molecular mechanism by which threshold size could be sensed and show that this is a broad phenomenon within holometabola that is not specific to *T. castaneum*.

3.2 Materials and Methods

3.2.1 Insect Strains

TIW1 is a common laboratory wild type *T. castaneum* strain obtained from Dr. Beeman of GMPRC, USDA-ARS. All *T. castaneum* experiments were conducted at 30°C and 40% relative humidity. The *black M. sexta* strain is a common mutant strain used in insect physiology maintained in the laboratory of Dr. Nijhout.

3.2.2 Daily Growth Curves

T. castaneum eggs were collected for 24 hours. Larvae were collected on the day of hatching (larval day 0), and reared separately in 1.5 ml microcentrifuge tubes with

four holes poked in the lid for ventilation. Larvae were reared on standard medium (95% flour, 5% brewers yeast by weight). Individual larvae were weighed daily and observed for molting until pupation. Data from each individual was pooled according to the number of instars the larva had undergone. Growth rates were calculated for each instar by fitting a standard exponential growth curve (weight = initial weight (x) $e^{(\text{growth rate (x) time})}$) using least squared fit.

3.2.3 Threshold Size Determination in *M. sexta*

Threshold size for *M. sexta* was determined as previously described (Nijhout, 1975; Kingsolver, 2007). Briefly, larvae were fed limited amounts of standard *Manduca* diet during their third and fourth instars to obtain larvae of differing weights at the end of the fourth instar. Weights at head capsule slippage in the fourth instar were recorded and larvae were transferred to a larger container and fed standard diet ad libitum. They were observed and recorded for pupation or molting to another larval instar. The percent pupating was calculated using a sliding average for the size of eight individuals. A logistic trend line for percent pupating was fitted to the data using SigmaPlot 8.0 (Systat Softwar Inc.).

3.2.4 Exponential Decay Rate Calculation

To determine the rate of exponential decay for *juvenile hormone acid O-methyl-transferase* (*jhamt*) in *T. castaneum*, an exponential decay curve was fitted using the least squared method to the expression data from Minakuchi et al. 2008.

3.2.5 Overall Exponential Growth Rates of *T. castaneum* with Seven Instars

The overall exponential growth rates were calculated by fitting an exponential curve to the weights and times of hatching and gut purge using the least squared method.

3.3 Results

I found that larvae of the *T. castaneum TIW1* strain had either 6 or 7 total larval instars under standard conditions. The number of total larval instars did not correlate with sex, indicating that both males and females can go through 6 or 7 larval instars. To determine what factors may be influencing whether a larva underwent 6 or 7 instars, I grouped the larvae by number of total larval instars and examined various aspects of their larval phase.

3.3.1 Ecdysone Events under Standard Conditions

Ecdysone is known to control the timing of molts, both larval and pupal, as well as the gut purge before the wandering stage in insects. Figure 16 shows that the timing of events controlled by ecdysone pulses does not differ between *T. castaneum* larvae that undergo 6 or 7 instars, although the type of event (larval molt, gut purge, or pupal molt) does differ.

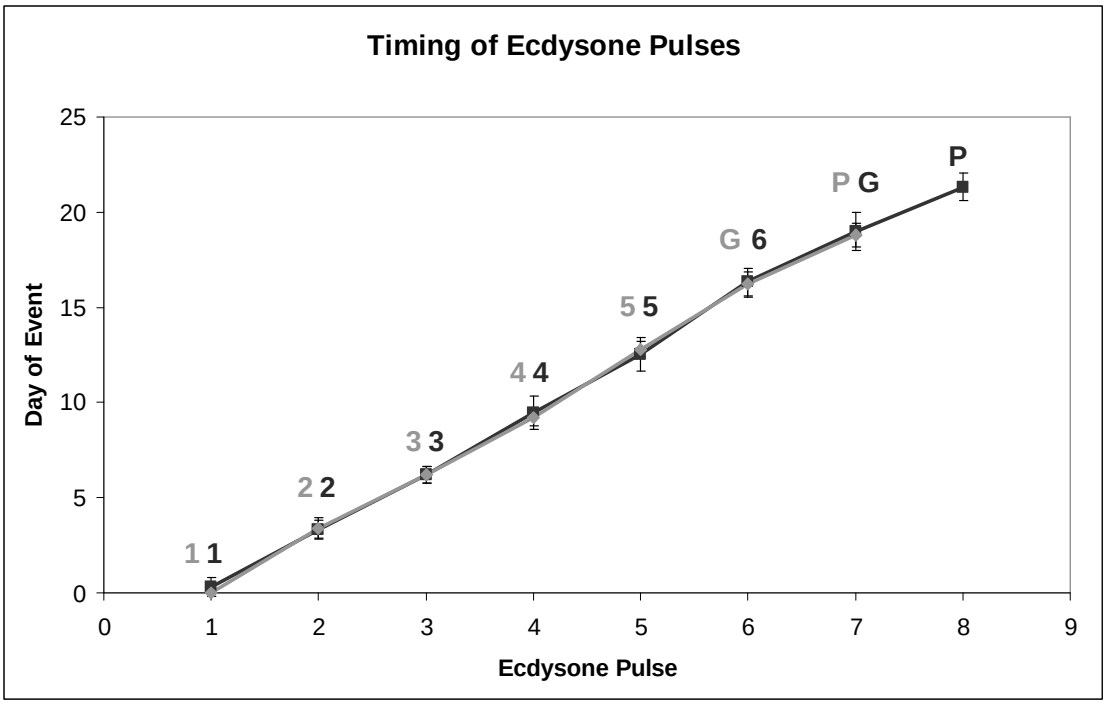

Figure 16: Timing of Ecdysone Events. This graph shows that the timing of events controlled by ecdysone pulses does not differ between *T. castaneum* larvae that undergo 6 or 7 instars, although the type of event (larval molt, gut purge, or pupal molt) does differ. The larval group undergoing 6 instars is depicted in light grey; the group undergoing 7 instars is in dark grey. Numbers indicate the molt at the end of the respective instar, G represents gut purge, and P represents the pupal molt for the respectively colored groups. Error bars represent ±1 standard deviation.

3.3.2 Growth Rates under Standard Conditions

The growth rate exponents of *T. castaneum* were calculated for each instar by fitting the growth curves of each instar to a standard exponential growth curve (weight = initial weight * $e^{(\text{growth rate} * \text{time})}$). These data show that growth rates change between instars through the entire larval phase (Figure 17). However, I found no statistical difference in growth rate in equivalent instars between larvae that underwent 6 or 7 instars.

3.3.3 Instar Weights in Standard Conditions

I compared the average weight of the larvae undergoing 6 larval instars with those undergoing 7 larval instars at the end of each instar and at the time of gut purge. I found that the larvae undergoing 7 instars weighed consistently less than those larvae undergoing 6 instars at equivalent instars (Figure 18). These data indicated that weight may influence the total number of larval instars.

3.3.4 Threshold size for black Manduca strain

I also determined threshold size for the *black M. sexta* strain. The *black M. sexta* strain has a lower juvenile hormone titer than the standard laboratory strain (Safranek & Riddiford, 1975). Figure 19 shows that the *black M. sexta* strain also had an 11.6% lower threshold size (0.53 g) than the standard *M. sexta* strain (0.6 g) (Kingsolver, 2007).

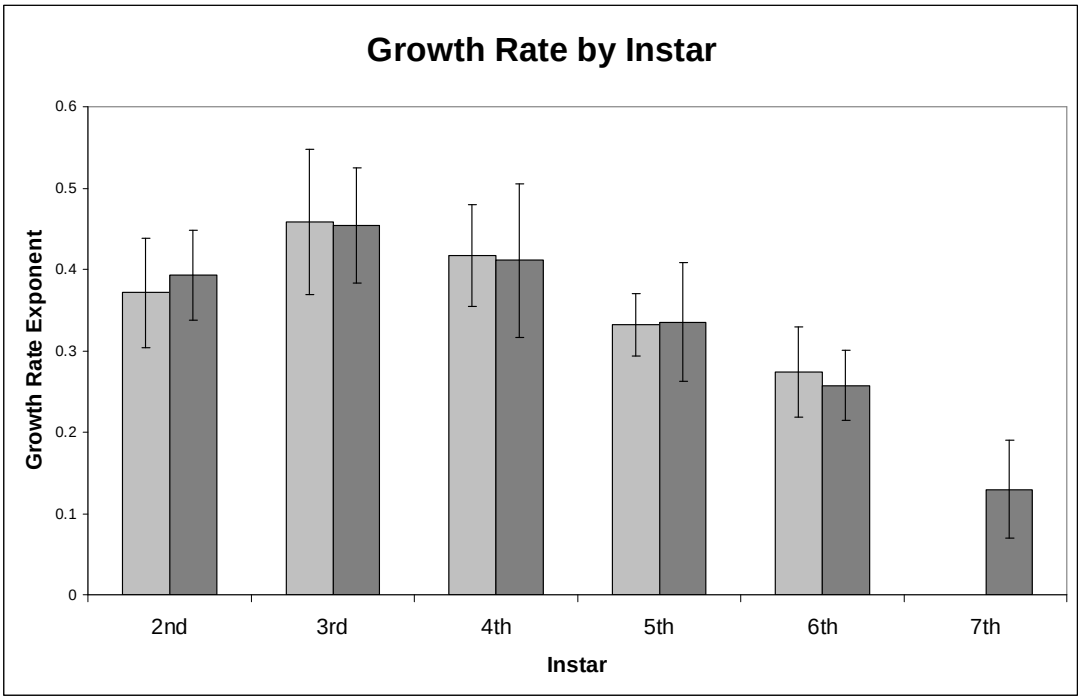

Figure 17: Growth Rate by Instar. Bars represent the average growth rate exponent of *T. castaneum* larvae undergoing 6 (light grey) or 7 (dark grey) instars. Error bars represent ±1 standard deviation.

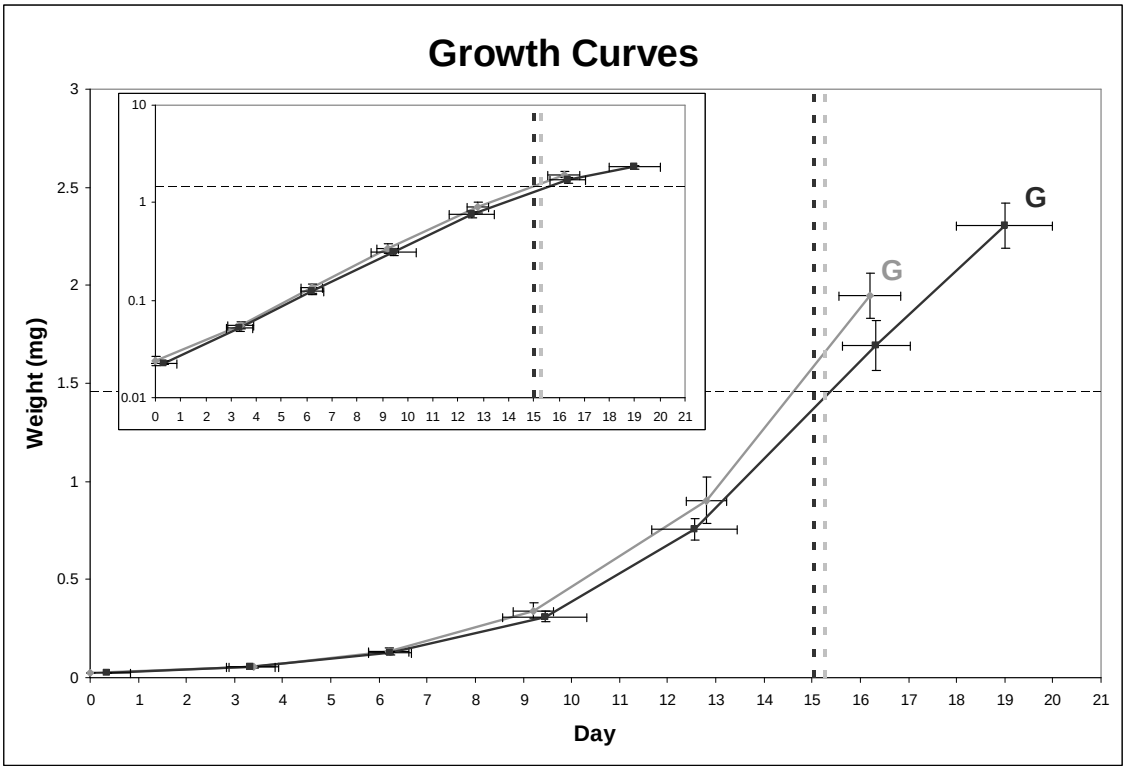

Figure 18: Average Growth Curves of *T. castaneum TIW1* strain. This graph shows the average growth curve for those larvae undergoing 6 (light grey line) or 7 (dark grey line) instars until gut purge (G), the earliest sign of metamorphosis. The threshold size of the *TIW1* strain is 1.46 mg (horizontal dashed line). The approximate time of the ecdysone peak in the sixth instar, 60 hours after exdysis to sixth instar, for each is depicted as a respectively colored vertical dashed line. Larvae undergoing only 6 instars (light grey) are above threshold size at the time of the ecdysone peak in the sixth instar, whereas those that will undergo 7 instars (dark grey) are still below threshold size and molt to another larval instar. Once in the seventh instar, larvae are well above threshold size and will pupate in response to the ecdysone peak in the seventh instar. Inset is same graph on logarithmic scale. Error bars represent ±1 standard deviation.

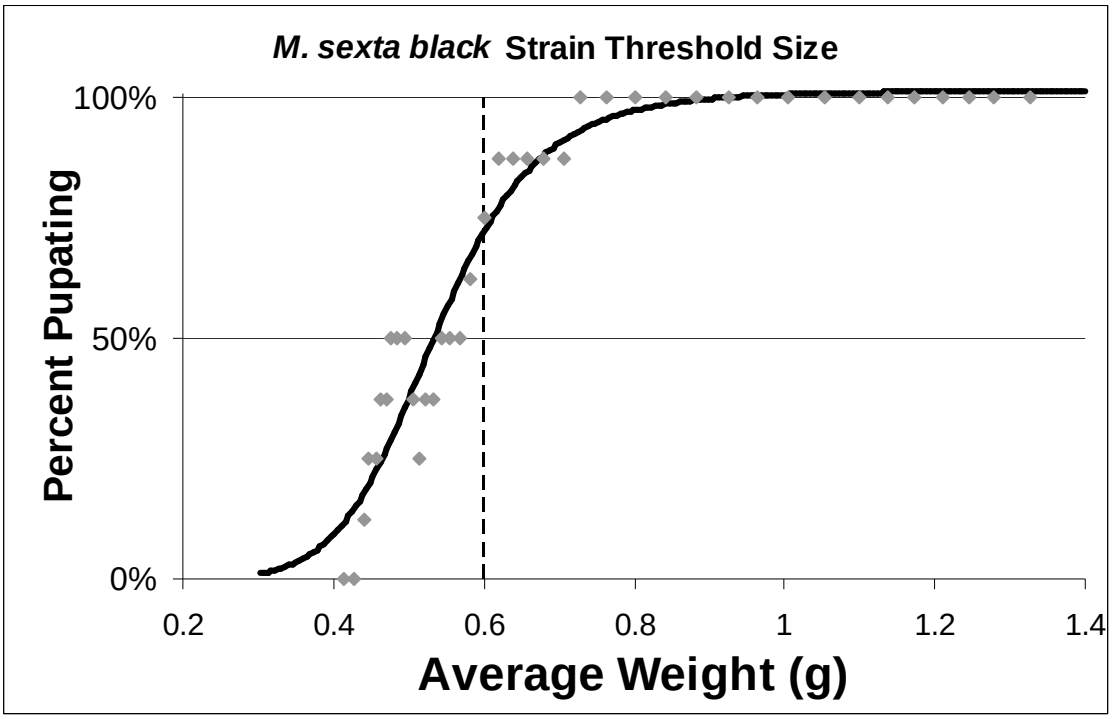

Figure 19: ***M. sexta black*** **Strain Threshold Size. Dark grey line shows that the percent pupating for the black strain of** ***M. sexta*** **reaches 50% at a lower weight than the threshold size for wild type M. sexta (vertical dashed line), indicating threshold size is lower for the black strain.**

3.4 Discussion

3.4.1 Theshold Size and Number of Larval Instars

To determine how larvae sensed they were large enough to initiate metamorphosis, I examined the relationships of the larval growth curves, ecdysone events, and threshold size from hatching until the time of gut purge for *T. castaneum* larvae undergoing 6 and 7 instars (Figure 18). Parthasarathy and Palli found that ectopic application of exogenous juvenile hormone analogs induced supernumerary larval molts in *T. castaneum* up to 60 hours after larval ecdysis (2009). It has also been shown that at 60 hours after larval ecdysis, ecdysone levels in *T. castaneum* larvae begin to rise (Parthasarathy et al. 2008). Together, these results suggest that the additional juvenile hormone signaling of the exogenous juvenile hormone analog caused the rise in ecdysone levels to induce molts to additional larval instars whereas lower juvenile hormone signaling of larvae without applied exogenous juvenile hormone analog allowed pupation to occur.

I have previously shown that threshold size, the size at which the final instar is determined, for the *TIW1* strain is 1.46 mg (see Chapter 2). In my experiments, I found that the weights of the average growth curves for larvae with 6 total larval instars were above threshold size at 60 hours after ecdysis to their sixth instar (Figure 18, intersect of light grey curve with light grey vertical dashed line), whereas larvae with 7 total larval

instars were still below threshold size at 60 hours after their larval ecdysis to the sixth instar (Figure 18, intersect of dark grey curve with dark grey vertical dashed line). This suggests that at 60 hours after ecdysis to the sixth larval instar, larvae above threshold size had juvenile hormone levels were low enough to allow pupation, and thus only had 6 total instars. In contrast, larvae below threshold size at 60 hours after ecdysis to the sixth larval instar had juvenile hormone levels too high to allow pupation to occur. These larvae below threshold size instead induced a supernumerary molt to a seventh larval instar. These results indicated that threshold size may be mediated by juvenile hormone levels.

3.4.2 Correlation of Threshold Size and Juvenile Hormone Titers

To further test the association of threshold size and juvenile hormone levels, I determined threshold size for the *black* strain of *M. sexta*. The *black* strain of *M. sexta* has lower juvenile hormone titers than wild type *M. sexta* (Safranek & Riddiford, 1975; Kramer & Kalish, 1984). I found that threshold size for *M. sexta* of the *black* strain (0.53 g) was lower by 11.6% than that of the wild type strain (0.6 g) (Kingsolver, 2007). Therefore, insects with lower juvenile hormone levels also have lower threshold sizes. Taken together, these results and those in the previous paragraph suggest that threshold size is correlated with juvenile hormone titers such that juvenile hormone levels are too high at weights below threshold size to allow pupation. What remained unclear was the mechanism by which size influenced juvenile hormone titer.

3.4.3 Juvenile Hormone Titer and Larval Body Size

Juvenile hormone titers fall as larvae increase in size. Although hemolymph juvenile hormone levels fall throughout the fourth and fifth (final) instar of the silkworm *Bombyx mori* in vivo, in vitro assays of the juvenile hormone production from the corpora allata did not show similar decreases (Sakurai & Niimi, 1997). Sakurai and Niimi found it necessary to adjust their in vitro findings with differences in larval weight in order to recapitulate the in vivo titer levels (1997). These results indicate that declining juvenile hormone levels were not due to decreased juvenile hormone production.

Koch found in *Cerura vinula* and *Araschnia levana* that juvenile hormone levels were high in early instars and decreased in later instars (1994). The decline in *C. vinula* and *A. levana* was roughly similar to an exponential decay. These results are consistent with juvenile hormone levels decreasing as a function of growth because larvae grow in a roughly exponential fashion, therefore juvenile hormone titers would be expected to fall in a roughly exponential decay in response to larval growth. The melanization in the *black* strain of *M. sexta* also indicates juvenile hormone levels drop throughout the larval phase. Although the *black* strain are known for their dark black color during in the fourth and fifth instars, larvae from the black strain are green at hatching and become increasingly more black at every instar (personal observation). The melanic black color of the *black* strain of *M. sexta* is known to be inhibited by application of juvenile hormone

or juvenile hormone analogs (Safranek & Riddiford, 1975), suggesting that the small early larval instars of the *black* strain larvae have sufficiently high juvenile hormone titers to inhibit melanization. Taken together, these data indicate that juvenile hormone levels decline throughout the larval phase specifically in response to the increase in size of the larva.

3.4.4 Juvenile Hormone Titer Regulation by Body Growth

The effect of body size on juvenile hormone is mediated, at least in part, by the dilutive effect of growth of the body. In general, it is believed that juvenile hormone titers are largely controlled by the rate of juvenile hormone production from the corpora allata (deKort & Granger, 1996). Threshold size in *B. mori* is similar to threshold size in *M. sexta* in that it must be passed by the start of the final instar (Asano et al., 1987). However, in vitro juvenile hormone production of the corpora allata in *B. mori* do not show decreases in juvenile hormone production capability at the time threshold size is achieved (Sakurai & Niimi, 1997). Expression levels of *juvenile hormone acid O-methyl-transferase* (*jhamt*), a necessary enzyme for juvenile hormone production, relative to *Ribosomal protein 49* (*Rp49*) remain high from the third instar through the first day of the final instar in the corpora allata, well after threshold size has been passed (Shinoda & Itoyama, 2003). These data indicate that the correlation of juvenile hormone with threshold size is not being mediated by a decrease in juvenile hormone production by the corpora allata.

Although the small size of *T. castaneum* larvae has made direct measurements of juvenile hormone difficult at this time, the developmental expression profiles of *jhamt* are known. Minakuchi et al. have shown that knockdowns of *jhamt*, which is specifically expressed within the corpora allata during the larval phase, induced precocious pupation in *T. castaneum*, directly linking *jhamt* expression level with the ability to inhibit pupation (2008). However, the whole body expression levels of *jhamt* exponentially decay throughout the larval phase relative to *Rp49*, which is expressed throughout the body (Minakuchi et al. 2008). Because the *T. castaneum* study used whole body for the expression assay, whereas the *B. mori* expression study used only the corpora allata, the *jhamt* expression results between *T. castaneum* and *B. mori* are not at odds. The results of the two studies are consistent because *jhamt* is specifically expressed the corpora allata, whereas *Rp49* is expressed throughout the body. Therefore, the exponential decay seen in the *T. castaneum* study may be due to the difference in relative growth of the larval body with the corpora allata. Using the published *jhamt* expression data of *T. castaneum* with seven instars (Minakuchi et al. 2008), I have found the rate of exponential decay (exponent is approximately = -0.1985 to -0.187) is consistent with the overall exponential growth rates of *T. castaneum* with seven instars in my study (average exponent = 0.2029; standard deviation = 0.0072). These data suggest that the expression of *jhamt* in the body of *T. castaneum* is decreasing in response to growth of the body relative to the corpora allata. Because *jhamt* is a key

enzyme in the biosynthetic pathway of juvenile hormone the change in the relative amounts could influence the relative juvenile hormone concentrations in the body. Even if the corpora allata was producing the same amount of juvenile hormone, as the *B. mori* data suggest it does, that constant amount of juvenile hormone produced would not produce the same concentrations in a large larva as it would in a small larva. These data indicate that the regulation of juvenile hormone titers via production may be due to the differential growth of the corpora allata and the rest of the larval body.

3.4.5 Threshold Size Corresponds to Juvenile Hormone Titer Dilution

I hypothesize that threshold size corresponds to the point were body size become large enough to dilute the juvenile hormone titer below the threshold needed to inhibit protein-protein interactions, such as *methoprene tolerant* (*met*) dimerization. Studies have shown that juvenile hormone or its mimics can bind and inhibit dimerization of *met* with itself or its paralogs in a dose dependent fashion (Godlewski, et al., 2006). Thus, increases in body size would cause juvenile hormone titers to decrease until insufficient to inhibit *met* dimerization (Figure 20). The dimerization of met would effectively switch the transcriptional milieu upon which subsequent ecdysone signals would act. The dose dependent interaction of juvenile hormone on protein-protein interactions linked with the reduction in juvenile hormone titers via dilution can explain why threshold size is so tightly linked to body size.

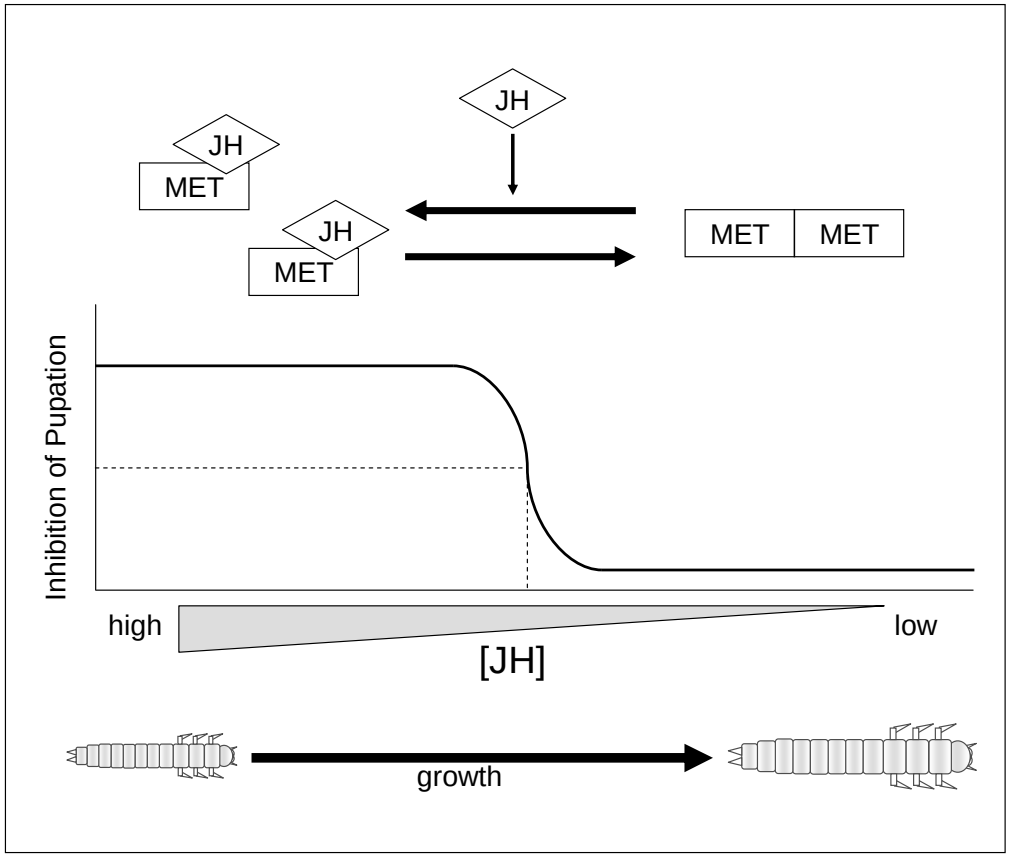

Figure 20: Contol of Juvenile Hormone Titers by Body Growth. As the larva grows larger (bottom), the titer amount of juvenile hormone drops. At a certain threshold (middle) the concentration of juvenile hormone is no longer sufficient to inhibit dimerization of *methoprene tolerant* (top).

3.4.6 Species Specific Considerations for Threshold Size

Threshold size can occur at different times in different species. It has been shown that the wings of Lepidoptera become committed to produce pupal characteristics within 24 hours of entering the final instar (Nijhout & Kremen, 1998). In *T. castaneum*, wings do not even begin to grow until the last half of the final instar (Tomoyasu Y, personal communication; personal observation). It is likely that species specific differences in the development of certain organs, such as the wings, may affect how late within an instar threshold size can be achieved to determine the final instar. A better understanding for the mechanistic basis of threshold size may yield clues as to how these species specific differences arose in evolution of holometabolous insects.

3.4.7 Threshold Size Regulation of Metamorphosis and Body Size

Threshold size regulates the size at which insects sense they are large enough to initiate metamorphosis. I have shown that threshold size determines when the larva becomes competent to metamorphose in *T. castaneum*. Threshold size, however, does not dictate the exact timing of initiation. The exact timing for the initiation of metamorphosis is determined by a pulse of the molting hormone, ecdysone, but only after threshold size has been reached. These results indicate that metamorphosis initiation is controlled by two factors: (1) attainment of threshold size, at which the larva becomes competent to initiate metamorphosis and (2) the timing of an ecdysone pulses after attaining threshold size. Because juvenile hormone also controls competence for

metamorphosis, I examined the link between juvenile hormone titers and threshold size in *M. sexta*. My results show that the *black* strain of *M. sexta*, known to have lower juvenile hormone titers than wild type *M. sexta*, also has a lower threshold size that wild type *M. sexta*. I have proposed a hypothesis linking threshold size and juvenile hormone based the difference in relative growth of the larval body and the corpora allata, which secretes juvenile hormone.

I hypothesize that the difference in relative growth of the larval body and the corpora allata causes juvenile hormone concentrations to gradually become diluted as the body gets larger. When juvenile hormone concentrations fall below a threshold, changes in protein-protein binding occur that can cause changes in signaling networks and ultimately gene expression. These changes make the larva competent for metamorphosis. Because changes in regulation of the competence for metamorphosis could have a dramatic impact on the overall development of a species, it may be interesting to examine threshold size in an evolutionary context. It is possible that a better understanding of threshold size could yield a better understanding of the many diverse metamorphosis types seen in holometabolous species.

4. Effect of Physiology on Phenotype

Among insects, the evolution of complete metamorphosis (holometaboly) enabled a diverse and successful radiation of forms. Holometabolous insects occupy many and diverse niches in terrestrial environments. In holometabolous development, individuals progress via molting through larval stages, known as larval instars, to the pupal stage before molting to become an adult. The interval between molting is referred to as an instar. Although the holometabolic metamorphosis of Endopterygota is of a monophyletic origin, metamorphic development is quite diverse, ranging from 3 larval instars in less than a week for *Drosophila melanogaster* (Ashburner & Thompson, 1978) to 31 instars in two and a half years for *Prionoxystus robiniae* (Solomon, 1973).

Among extant holometabola this diversity has been partitioned into two polyphyletic groups based on when the wing imaginal discs become distinguishable from other tissues, such as the epidermis, which can occur either (1) before or (2) during the last larval instar (Truman & Riddiford, 1999). In their analysis of the evolution of complete metamorphosis from the incomplete metamorphosis of hemimetabola, Truman and Riddiford (1999, 2002) concluded that in the ancestral condition for holometabolous metamorphosis, wing development began during the final instar. Therefore, insects with wings imaginal discs that become distinguishable in the last larval instar show the primitive character state. Although the analysis of Truman and Riddiford (1999, 2002) provided a strong conceptual framework about how

holometabola were derived from hemimetabola, there is still much to explore within the diversity of holometabolous groups. It is now possible to examine the diversity of metamorphic forms that exist today by comparing primitive holometabolous insects with those that have a more derived holometabolous metamorphosis.

In this paper, I will be identifying some general trends in the evolution of metamorphosis types, focusing on the most broadly studied imaginal tissue, the wing. I will discuss differences among metamorphosis types at both the morphological and physiological level. I will show that the basis for features found in derived conditions exists in the primitive condition and that the multiple convergent features of the derived metamorphosis types are likely due to similar heterochronic shifts in developmental processes. Finally, I will use a simple model to examine the tradeoffs associated with different metamorphic types and provide a rationale for both the continued existence of the primitive condition and the many instances of derived metamorphosis types.

4.1 Morphological Changes

In the development of holometabolous insects, certain groups of cells which I will refer to as imaginal tissue, develop into adult structures not present in the larva. For some time it was believed that a dichotomy existed between larval and imaginal cells and that the two were fundamentally different from each other. This was largely a result of using *D. melanogaster*, a highly derived model system as the representative species for all of holometabolous development. In recent years, the utilization of other

species to study development has led to a more nuanced view of what are larval and imaginal tissues (see Svácha, 1992). Given the homology of wings within all insects, it should be possible to compare developmental phases of the primitive condition to that of the derived states. Using the widely studied wing as my focal tissue is also advantageous as larvae do not have external wings which should reduce functional larval adaptations not related to development (i.e. locomotion, camouflage, or shielding). I will compare wing development in one primitive metamorphosis type and three independently evolved derived metamorphosis types.

I will break up the development of the wing during the period before the prepupal stage into five phases: (1) thickening of the imaginal tissue, (2) apolysis and invagination, (3) competence for metamorphosis, (4) metamorphic development, and (5) commitment to pupal cell fates. The specific signals that direct the processes in each developmental phase are poorly understood, and informative comparative studies at the molecular level are still lacking. It is for this reason that I will keep the discussion focused at the level of comparative morphology and, where possible, comparative physiology.

4.1.1 Thickening of the Imaginal Tissue

Because the cells that will eventually give rise to the wing are of ectodermal origin, the thickening of a patch of the larval epidermis, forming a placode, is usually the earliest morphological indication of imaginal cell fate in most insects. Although

specification of the imaginal cells as different from the surrounding cells must have been completed by this time, there is no indication of when imaginal specification began. The stage in development at which epidermal thickening occurs varies widely. In some species the thickening phase occurs in the last instar, whereas in others it is completed during embryonic development before the larva hatches from the egg.

4.1.2 Apolysis and Invagination of the Imaginal Tissue

Because the epidermis of insects is associated with the non-living cuticular exoskeleton, disassociation of the cells from the cuticle must precede any changes in epidermal morphology. Apolysis from the cuticle and invagination of the imaginal tissue and surrounding peripodial membrane is a critical step that sets the stage for future morphological changes and development. Apolysis and invagination occur at different times in development in different species. Progression through the different phases of wing development is often arrested after apolysis and invagination in many species with "classic imaginal discs". This does not mean that proliferation of cells within the imaginal tissue is arrested, but merely that the tissue is not progressing to the next morphological phase. In fact, by discontinuing their association with the overlying cuticle, imaginal tissue after apolysis and invagination are freed from the constraint of producing a functional larval exoskeletal cuticle. Without that constraint, invaginated imaginal tissue can either cease proliferation or, as typically happens, proliferate faster relative to other epidermal tissues that remain attached to the exoskeleton. The ability to

proliferate faster relative to other tissue is believed to be one of the major reasons for earlier invagination among the species with a more derived metamorphosis type.

4.1.3 Imaginal Tissue Competence for Metamorphosis

Once invaginated, wing imaginal tissue must become ready, or competent, to develop into a wing. Larvae sense they have achieved the appropriate size to become competent for metamorphosis via the physiological mechanism known as threshold size. In Lepidopterans like *Manduca sexta* and *Bombyx mori*, threshold size must be surpassed by the start of the final instar, but in *Tribolium* species and the longicorn beetle, *Psacothea hilaris*, threshold size can be attained during the final instar (Nijhout, 1975; Asano et al., 1987; Munyiri et al., 2003). However, for all the aforementioned species, threshold size determines if the larva will be able to pupate. Threshold size is an indicator that juvenile hormone titers have fallen below the level which inhibits metamorphic development. Threshold size thus regulates competence for, but not initiation of, metamorphosis. Threshold size is usually associated with lower juvenile hormone titers. Although it is common to conceptually lump invagination and competence for metamorphosis together, experimental investigations using ectopic hormones, transplantation assays, and in vitro studies have made it clear that not all invaginated wing imaginal tissues are capable of producing a wing (Riddiford & Ashburner, 1991; Nijhout & Kremen, 1998).

4.1.4 Metamorphic Development of Imaginal Tissue

Metamorphic development differs from mere proliferation because metamorphic development is the phase when developmental processes to become a wing, and not just a larger larval stage imaginal structure, begin to occur. Because metamorphic development is inhibited by juvenile hormone, metamorphic development can only occur after threshold size has been achieved, when juvenile hormone titers are below inhibitory levels. Metamorphic development is initiated by ecdysone, although often at much lower levels that the ecdysone peaks required for molting. Although metamorphic development is the beginning of the developmental processes to form the prepupal wing, application of ectopic juvenile hormone can inhibit and, in some cases, reverse metamorphic development until the time of commitment to pupal cell fate (Quennedey & Quennedey, 1990; 1999).

4.1.5 Commitment of Imaginal Tissue to Pupal Cell Fate

Commitment to pupal cell fate is the point at which the developmental processes begun during metamorphic development have reached an irreversible point in development. After commitment to pupal cell fate, the imaginal wing tissue will produce some pupal features and is unable to correctly make morphologically larval versions of the tissue. Commitment is in no way the completion of development for the wing however. Commitment to pupal cell fate means that failure to complete development will produce forms incompatible with survival. Ectopic juvenile hormone

application after commitment can lead to pupal-larval mosaic or intermediate forms of the tissues which are usually detrimental, even in laboratory conditions. It is clear from the literature that commitment to pupal cell fate occurs at different developmental times for different tissues, even within a single organism (Quennedey & Quennedey, 1999; Nijhout & Kremen 1998). Although I will be focusing on only wing development here, coordination of commitment to pupal cell fate between tissues would be important for proper metamorphosis at the whole organism level.

Although each of the metamorphosis types I examine has the five developmental phases discussed above, the relative timing of each phase through larval development is often quite different. In some species the wings progress rapidly through all the phases, in other species the wing imaginal tissues remain in a particular phase for an extended period of larval development. To compare how these phases relate in different species, I will examine four metamorphosis types that give a glimpse of the diversity found in holometabolous metamorphosis (Figure 21). I will use the metamorphosis of Tenebrionid beetles as an example of the hypothesized primitive metamorphic type (Truman & Riddiford, 1999). I will then examine three derived metamorphosis types to highlight the differences and convergence among these three independent lineages of insects with early imaginal disc formation.

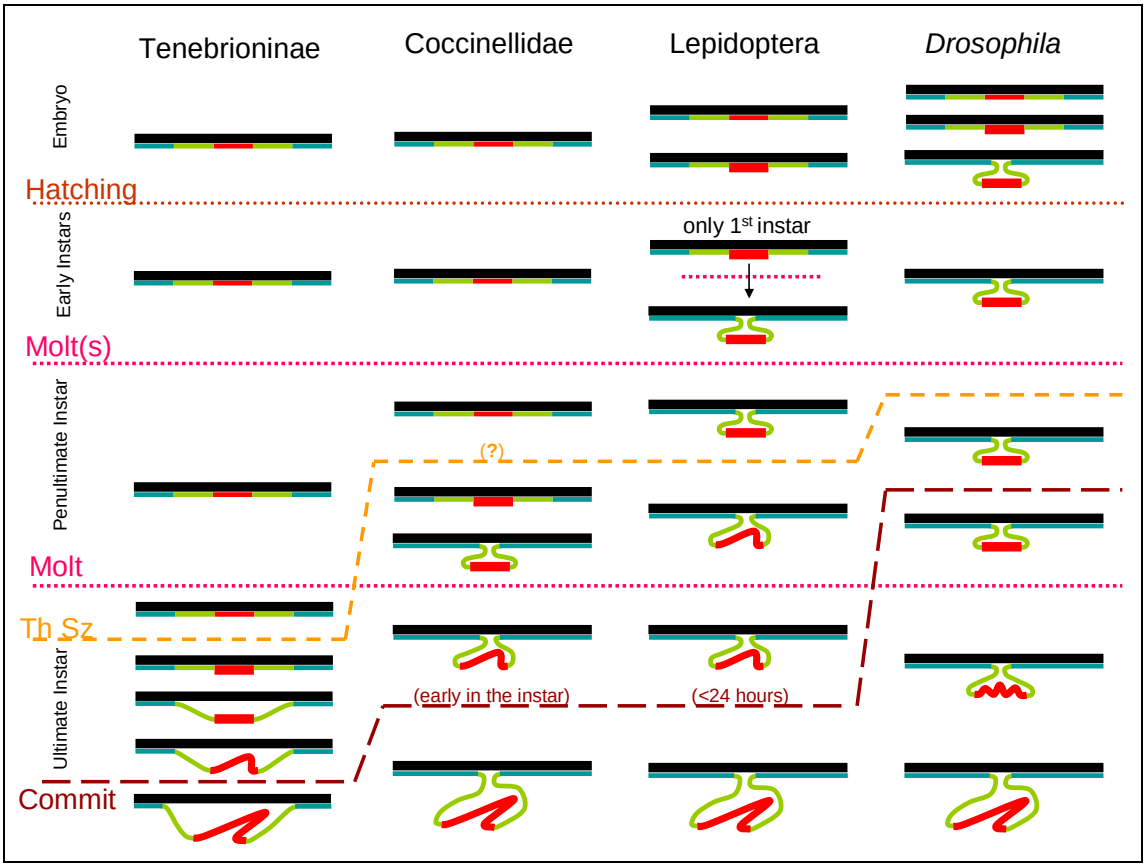

Figure 21: Generalized Diagrammatic Representation of Wing Development in Different Metamorphosis Types. Wing Imaginal (red), Peripodial Tissue (green), Epidermis (aqua), and Cuticle (black) are shown throughout development of four metamorphic types. Molts (pink dashes) and Hatching (brown dots) are indicated. Competence for metamorphosis occurs at threshold size (orange, Th Sz) as is commitment for pupal cell fate (brown dashes, Commit). Please see text for descriptions. These representations are intentionally diagrammatic to make them more generalized for ease of comparison for both species mentioned here and for future investigation. For exact histological representations, please see references.

4.2 Metamorphic Types

4.2.1 Primitive Metamorphic Condition

In their paper on the origin of holometabolous metamorphosis, Truman and Riddiford (1999) argued that extant Tenebrionid beetles display primitive characteristics hypothesized to have existed in the ancestral condition. Although they are by no means the only group to have primitive characteristics, Tenebrionid beetles offer a reasonable baseline for comparison to more obviously derived metamorphic types. Although these species are most notable as pests of dry foodstuffs, they are found in many different habitats. In particular, species of the genus *Tribolium* have been found under tree bark, in the nests or burrows of hymenopterans and vertebrate, as well as feeding on fungi, indicating their larvae can utilize a wide variety of resources (Sokoloff, 1974).

In the subfamily Tenebrioninae, the tissues that will eventually give rise to the wings are indistinguishable from the surrounding epidermis throughout most of larval life. My work has shown that *Tribolium* species pass threshold size during their final instar. It is only after threshold size has been surpassed in the final instar that the wings will become distinguishable from the epidermis. Approximately midway through the final instar the future wing tissue begins to thicken. The imaginal wing and some of the surrounding epidermal tissue that is functional similar to peripodial tissue apolyse and begin to invaginate to become a shallow depression in which the developing wing will

form (Quennedey & Quennedey, 1990; 1999). As the depression forms, metamorphic development begins and the thickened wing imaginal tissue evaginate within the shallow invagination to form the future wing.

Although the early stages of wing development have begun, the wings are still not committed to pupal development. Quennedey and Quennedy (1990; 1999) have shown that in both *Tenebrio molitor* and *Zophobas atratus*, wing development can be reversed by application of a juvenile hormone mimic. Using the *pu11* line of *T. castaneum*, which expresses GFP in the developing wing, I have also observed that early wing development can be reversed by the application of the juvenile hormone mimic, pyriproxifen, indicating that commitment to pupal cell fate has not yet occurred (Tomoyasu Y, personal communication; personal observation). However, for all three species there comes a point in development when the pupal wing fate can no longer be fully inhibited by juvenile hormone; this is referred to as commitment to pupal cell fate. After commitment, the wing is no longer able to fully reverse its development and any ectopic juvenile hormone applications produce larval-pupal intermediates with stunted wings (Quennedey & Quennedey, 1999; Parthasarathy & Palli, 2009).

Perhaps one of the most striking aspects of this primitive metamorphosis type is how very late in larval development it takes place. Indeed, authors have used "late" and "early" to distinguish between primitive and derived forms (Truman & Riddiford, 1999). Although groupings based on developmental timing alone may not be sufficient

to unambiguously distinguish all derived forms from the primitive forms, such information is readily available in the literature and should provide a good first approximation. Another feature of the primitive metamorphic condition is that most of the phases appear to be tied to the initiation of metamorphic development, after attainment of threshold size. It is unclear if the signal for metamorphic development, ecdysone, is a common signal for all of these processes to occur or if the signals controlling all of these process merely happen to occur at nearly the same time. Further studies are needed to better understand the mechanisms behind these developmental processes.

4.2.2 Lepidoptera

Lepidopterans are among the best understood physiological model organisms. All of Lepidoptera seem to have a common derived metamorphosis types. Indeed, detailed accounts of early development in moths and butterflies are almost identical (Mercer 1900, Köhler 1931). It is unclear if this is merely a sampling bias or due to an underlying biological reason, but for the time being I will treat Lepidoptera as one group with a common metamorphosis type.

In Lepidoptera, the presumptive wing imaginal tissue has already become thickened at the time of hatching (Mercer 1900, Köhler 1931). The presumptive wing imaginal tissue remains as a thickened placode just underneath the cuticle for the entire first instar. Just after the apolysis and molting to the second instar, the wing imaginal

tissue forms a small invagination (Mercer 1900, Köhler 1931). This invagination enlarges during the third instar and takes on a "classic imaginal disc" appearance in which the thickened imaginal tissues form a flat disk, connected to the epidermis by peripodial membranes. Although invaginated, some of the wing imaginal tissue and surrounding peripodial membranes do still produce a small, thin bit of cuticle (Mercer 1900, Köhler 1931, Svacha 1992).

The rest of the developmental phases occur in the final two instars. During the penultimate instar, typically the fourth instar, Lepidopterans surpass threshold size (Nijhout 1975, other). Larvae that pass threshold size molt to their final instar whereas those that are below threshold size molt to another non-final instar. During the penultimate (usually fourth) instar, the wing imaginal tissues begin to change their shape (Mercer 1900, Köhler 1931). The flat disc-like placode evaginates to form a wing bud in which the wing imaginal tissue is folded back upon itself and is connected to the epidermis by peripodial membrane tissues. Usually after the first day of the final (usually fifth) instar, the larval wings will become committed to pupal cell fate (Nijhout & Kremen, 1998). After commitment to pupal cell fate the wings cannot be induced to remain larval.

4.2.3 Drosophila

One of the major reasons for the rise of *D. melanogaster* to be one of the most widely studied genetic model systems is its short life cycle and rapid development.

Many *Drosophila* species specialize on ephemeral habitats, such as rotting fruit, so rapid development is an important adaptation. Although its rapid development has made *D. melanogaster* an extraordinary genetic model system, its highly derived mode of metamorphosis has been an obstacle to the development of a deep understanding of its developmental physiology.

One of the most derived features of *D. melanogaster* is the rapid progression of the wing imaginal tissue through the developmental phases. By the start of the first instar, *D. melanogaster* has already invaginated its wing imaginal tissue (Madhavan & Schneiderman, 1977). Although invaginated, the *D. melanogaster* wing imaginal tissue is not yet fully competent to begin metamorphic development. Bownes and Roberts showed that 44% transplanted wing discs from larvae at the first to second instar molt made thoracic tissue and 47% made wing tissue (1979). These data indicate that not all wing discs are competent to metamorphose, but percentages in the results suggest that threshold size, and therefore competence for metamorphosis, does occur near the time that larvae molt from the first to second instar. Bownes and Roberts also showed that in wing discs taken just 17 hours after larvae molt from the first to second instar, 92% made thoracic tissue and 100% made wing tissue, indicating threshold size had been passed in nearly all D. melanogaster larvae by this time during the second larval instar (1979). Later work by Zhou et al. (2004) confirmed the existence of a threshold size in the second instar using genetically modified *D. melanogaster*. The threshold size in the

second instar indicates that *D. melanogaster* imaginal wing tissue become competent to metamorphose during the second instar.

Commitment to pupal cell fate is also very early in *D. melanogaster* larval development. Riddiford and Ashburner (1990) used juvenile hormone mimics to inhibit pupation in *D. melanogaster* larvae at different developmental stages. They found that *D. melanogaster* imaginal wing tissue had become insensitive to juvenile hormone, and thus were committed to pupal cell fate, by the late second instar (Riddiford & Ashburner, 1990). Their results indicate that wing imaginal tissue in *D. melanogaster* larvae had committed to becoming a pupa before the start of the third (ultimate) instar. After the molt to the third instar that the imaginal discs continue developing and take on a convoluted appearance as proliferation rapidly expands the wing disc. Eventually, the wing imaginal tissue will extend and elongated to form the pupal wing.

4.2.4 Coccinellidae

Ladybird beetles (Coccinellidae) show some remarkable similarities to both the primitive condition of the beetles in the Tenebrioninae subfamily, and some of the derived metamorphosis types, such as the Lepidopteran metamorphoses. The Coccinellidae demonstrate the diversity and continuum of metamorphic types, and illustrate the fact that there is no sharp dichotomy between wing imaginal tissue of the primitive condition and the wing discs of *Drosophila* and Lepidoptera. It may be easy to view the Coccinellidae metamorphosis type as an intermediate form but it is important

to realize that the Coccinellidae metamorphosis type has evolved independently of other derived metamorphic forms and any similarities must be the result of convergence.

Although threshold size has not been specifically examined in ladybird beetles, there are some indications in the literature from which it is possible to deduce approximately when it occurs. Ladybird beetles usually have four larval instars, but five larval instars can occur in *Adalia bipunctata* and *Harmonia axyridis* (Dimetry, 1974; Labrie, et al., 2006). Changing feeding conditions in only the final (fourth) instar did not induce additional instars in *Coleomegilla maculata*, *H. axyridis*, and *Hippodamia convergens* (Phoofolo, et al., 2009). These data suggest that threshold size likely occurs in the penultimate instar, which is also when the first morphological indications of metamorphosis become visible (Tower, 1903; Lommen, et al., 2009). Like the primitive metamorphosis type, the wing imaginal tissues are indistinguishable from the epidermis throughout much of larval life. In the late penultimate (third) instar, the wing imaginal tissue begins to thicken and becomes recognizable (Tower, 1903; Lommen, et al., 2009). The thickened imaginal wing then apolyses and invaginates to form a pocket of tissue into which the future wing will evaginate and develop inside during the final instar. Feeding studies have shown that within approximately 24 hours of entering the last instar, Coccinelid beetles become committed to pupation in a manner similar to that of Lepidoptera (Phoofolo, et al., 2009; Nijhout & Kremen, 1998).

4.3 Evolutionary Trends

Although these four metamorphic types seem quite different, they share a common developmental basis inherited from the ancestor of all holometabola. The major differences between the metamorphic groups are the timing of developmental events. Relative to the primitive condition, heterochronic shifts have occurred in the timing of thickening of the imaginal tissue, apolysis and invagination, competence for metamorphosis, metamorphic development, and commitment to pupal cell fate. This means that the independent instances of derived (early) metamorphosis types found orders like Lepidoptera and *Drosophila* are not convergent novel structures, but instead parallel heterochronic shifts of developmental events. This view may help us correctly interpret the independent origins of different derived metamorphosis types described by Truman and Riddiford (1999), including those presented above, as well as the many apparent similarities of independently evolved derived metamorphosis types.

4.4 Adaptive Significance of "Early" and "Late" Metamorphosis

The reason for such diversity of morphology and developmental timing of physiological events among different species during holometabolous metamorphosis, as exemplified above, is unknown. It is possible that morphological and physiological differences have potential advantages or disadvantages for different niches. Until now, there was only sufficient information to hypothesize about the morphological influence on development. Recent advances in physiology now allow us to begin to explore the

physiological influence on development as well. Here I will touch on the potential morphological affects on development and use a simple model to address the potential physiological influence on metamorphosis.

4.4.1 Morphological Hypotheses

Hypotheses about the influence of different morphologies on metamorphosis have focused on the advantage of having early invaginated imaginal discs. Simply put, early invaginated discs would no longer be constrained by the need to produce larval cuticle in proportion to the rest of the larval body. The early invaginated imaginal tissue would be free to proliferate, grow, and develop faster for a longer period of time than would be possible if the cells remained attached to the overlying cuticle (Truman & Riddiford, 1999). This explanation is definitely reasonable for explaining the numerous independent origins of invaginated imaginal wing tissue. However, the morphological explanation alone seems insufficient to address the persistence and success of the many holometabolous insects with primitive metamorphosis types.

The evolution of derived metamorphosis types with early invagination of wing imaginal tissue has occurred no less than six independent times in holometabolous insects (Truman & Riddiford, 1999). Early invagination of wing imaginal tissue has even evolved three times independently within Coleoptera (Truman & Riddiford, 1999). Given that derived metamorphosis types with early invaginated wings have independently evolved several times, it seems odd to suggest the only reason so many

species retain the primitive metamorphosis type is simply that they cannot, or just have not, evolved a more advantageous derived metamorphosis type. It seems far more likely that there may be an underlying tradeoff that is not apparent in the morphology alone. For this reason I sought to develop a different rationale that might help explain why such diversity exists.

4.4.2 Functional Consequence of Different Physiologies

To better understand how diversity in metamorphosis types might be maintained, I created a mathematical model to examine the functional consequences of differences in physiology and their interaction with the ecological pressures faced by different groups. I chose to specifically examine the effect physiology had on body size. Increased adult body size produces benefits in many species. The benefits of large body size include greater fecundity in females, access to mates in males, and resource sequestration advantages (Honěk, 1993; Lighton et al., 1994; Parker & Simmons, 1994; Rivero & West 2002). Because increased body size often has many benefits for the adult, larvae with larger final body sizes at pupation would reap those benefits as adults. The goal of the model is to gain a qualitative understanding of how the basic physiology of growth and metamorphosis affects the final body size of larvae. I hope that the qualitative results of this model will provide some insight into why species specific factors may have evolved and pave the way for more quantitative models to be developed.

Larvae do not pupate until their wings have fully developed and are ready for pupation. Therefore, the timing and duration of the developmental phases in the wing can affect when the larva will pupate. Because larvae grow in a roughly exponential manner, changes in the duration of the larval phase can dramatically affect the size of the larva at pupation. In natural environments, resource availability can fluctuate by locale, season, and can even be influenced by recent weather patterns. The growth rates of larvae would be naturally affected by differences in such environmental variables as temperature and resource availability.

How differences in growth rate interact with different physiologies can be examined by means of a simple model. My goal was to understand the effect of physiological differences on an important phenotypic characteristic, body size. To model body size, I use a simple exponential model to simulate the growth of larvae until they reach their highest weight, just before the larva purges its gut contents in preparation for pupation:

$$Body\ Size = Initial\ Weight * e^{(Growth\ Rate * Time)}$$

Although there are many different growth equations that could be used, the exponential growth equation has fewer variables and does not require the choice of an arbitrary upper limit, as would a Gompertz or Logistic equation. The choice of growth model is supported by the fact that insect growth is roughly exponential during most of larval life. Furthermore, for the phylogenetic range over which I want to utilize this

model, it would reduce generality to include what may be species specific factors such as number or timing of molts, and mechanisms that affect growth, or diapause.

4.4.3 Two Phase Model

4.4.3.1 First Phase

I have designed a model to specifically examine how heterochronic shifts of metamorphic competence (threshold size) to earlier times in development might affect the phenotypic landscape of final body size when growth rate varies (Figure 22). For the model, I have simplified larval growth into two phases, similar to what was proposed by Robertson for *D. melanogaster* (1963). The first phase represents growth to threshold size. Larvae that have not achieved threshold size continue molting to additional larval stages until threshold size is achieved. Because larvae that have not achieved threshold size cannot begin metamorphosis, this first phase in my model is variable in its duration.

4.4.3.2 Second Phase

The second phase in my model represents the amount of time needed to complete metamorphic development and is not variable in duration. In some insects, like Tenebrionid beetles, the duration of the second phase is short relatively to the

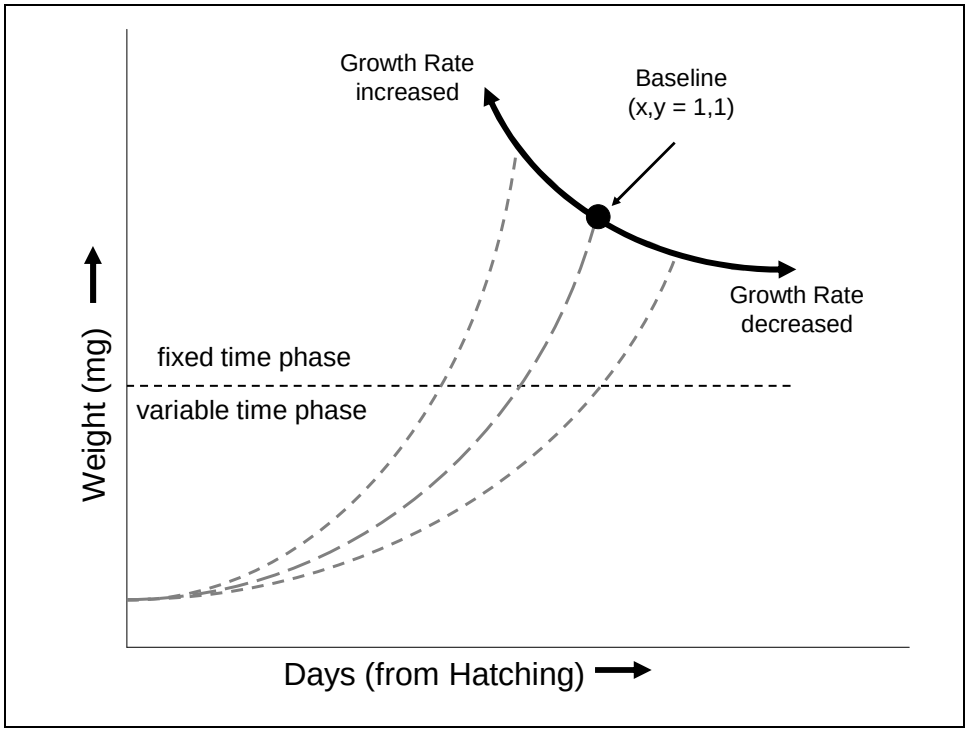

Figure 22: Two Stage Model. From the hypothetical baseline individual (long grey dashes), growth rate variation (small grey dashes) causes the phenotypic landscape (black line with arrowheads) to change. See text for details of model.

overall developmental time (see above). In others, like *D. melanogaster*, the second phase would comprise most of the overall developmental time (see above). My model terminates at the high weight of the larva, often referred to as the weight at the time of gut purge. This endpoint is just before entry into the prepupal stage.

4.4.3.3 Generic Baseline Individual

To make the model results comparable between different physiological setups, a common generic baseline individual needs to be established. The baseline individual represents growth under moderate conditions. The baseline individuals will have an identical final body size and total development time in the different physiologies of the model. The baseline individual will therefore act as a common point of reference between physiologies. Once the parameters for the baseline individual are established, how the growth rate variation interacts with the baseline physiology to affect final body size and total development time can be assessed.

For an example, I will use an initial weight of 0.01, a final body size of 1.0, and a total development time of 1.0 for the generic baseline individuals. From those parameters, one can solve the exponential equation to determine the growth rate for the baseline individual, which is equal to the natural logarithm of the fold increase in final body size from initial weight (i.e. ln(100 fold) = 4.6017).

$$\textit{Exponential Equation: body size} = \textit{initial weight} * e^{(\textit{growth rate} * \textit{time})}$$

$$\textit{Baseline Individual: final body size} = \textit{initial weight} * e^{(\textit{growth rate} * \textit{total developmental time})}$$

$$1.0 = 0.01 * e^{(4.6017 * 1.0)}$$

Because I have set the baseline final body size to 1.0, threshold size of the different physiological setups can vary between 0 and 1. To obtain the fixed time for metamorphosis completion for a given physiology, the time at which threshold size is obtained is subtracted from the total development time, in this case, 1.

Time to Threshold Size = (ln (threshold size / initial weight)) / growth rate

Baselines: time to threshold size = (ln (threshold size / 0.01)) / 4.6017

Time for metamorphosis completion = (total development time) - (time to threshold size)

Baselines: time for metamorphosis completion = 1 - time to threshold size

With the baseline parameters set, the model can now address how growth rate variation would affect total development time and final body size in three steps.

Time to Threshold Size=(ln(threshold size of physiological setup/0.01))/growth rate

Total development time =

(time to threshold size) + (baseline's time for metamorphosis completion)

$$final\ body\ size = 0.01 * e^{(growth\ rate * total\ developmental\ time)}$$

I can then graph the results of growth rate variation within each physiology to show the phenotypic landscapes the different physiologies would produce when growth rate varies (Figure 23).

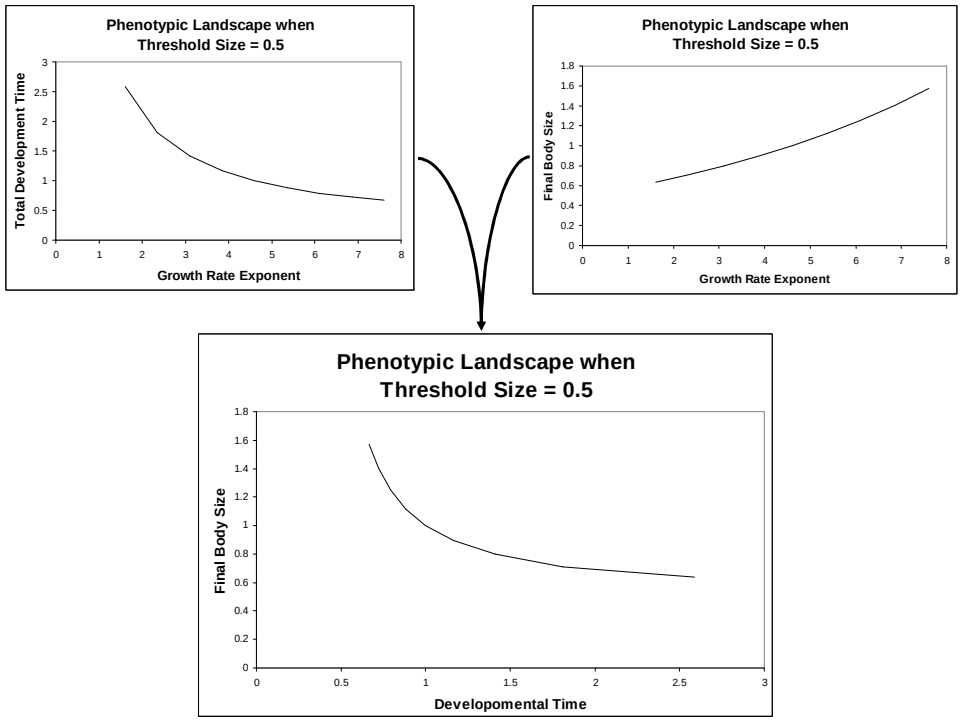

Figure 23: Example Physiology with a Threshold Size of 0.5. To determine the phenotypic landscape (bottom), the affect of growth rate variation on total development time and final body size are intergrated.

4.4.4 Model Results

The model results show that physiology can affect the phenotypic landscape of final body size and total development time (Figure 24). Because threshold size in different species occurs at different times in larval development, when a larva achieves competence for metamorphosis, as well as the time required to complete metamorphic development and commit to pupal cell fate, also differs between species. The differences in physiology interact with variation in growth rate to produce a phenotypic landscape for that physiology. The model shows that as growth rate increases, final body size increases and total development time decreases for all physiologies. In contrast, as growth rate decreases, final body size decreases and total development time increases for all physiologies. However, the physiology affected the magnitude of the change in final body size or total development time caused by growth rate variation. For my analysis below (Figures 24 and 25), I varied the exponential growth rate up to +/- 3 of the baseline's exponential growth rate (between 1.60517 and 7.60517).

4.4.4.1 "Early/Low" Threshold Size

Growth rate variation in "early/low" physiologies, with threshold sizes less than 0.3 of the baseline final body size, caused greater range in variation for final body size than total development time (Figure 24). The amount by which changes in growth rate increased or decreased final body size increased for as threshold size was decreased. Conversely, total development time was less affected the lower the threshold size was

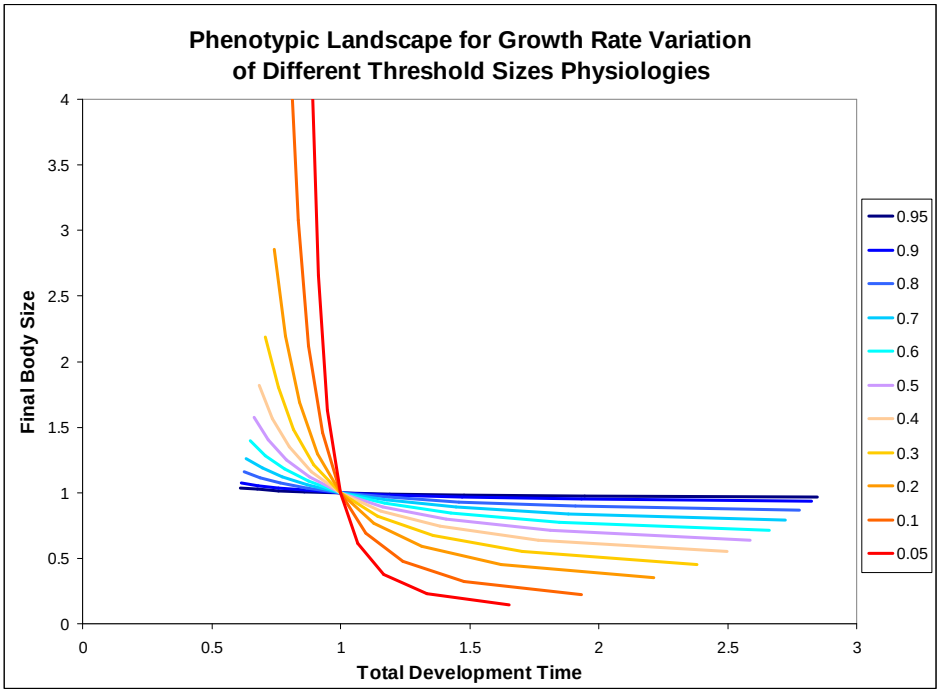

Figure 24: Phenotypic Landscapes for a Set Range of Growth Rate Variation on Different Threshold Size Physiologies. Each line represents a physiology with a threshold as indicated in the ledged. For each physiology, the same range of growth rate variation was used to determine the shape of the phenotypic landscape. Later/Higher threshold sizes (i.e. 0.95) are nearly flat while earlier/lower threshold sizes have steeper slopes for this range of growth rate variation.

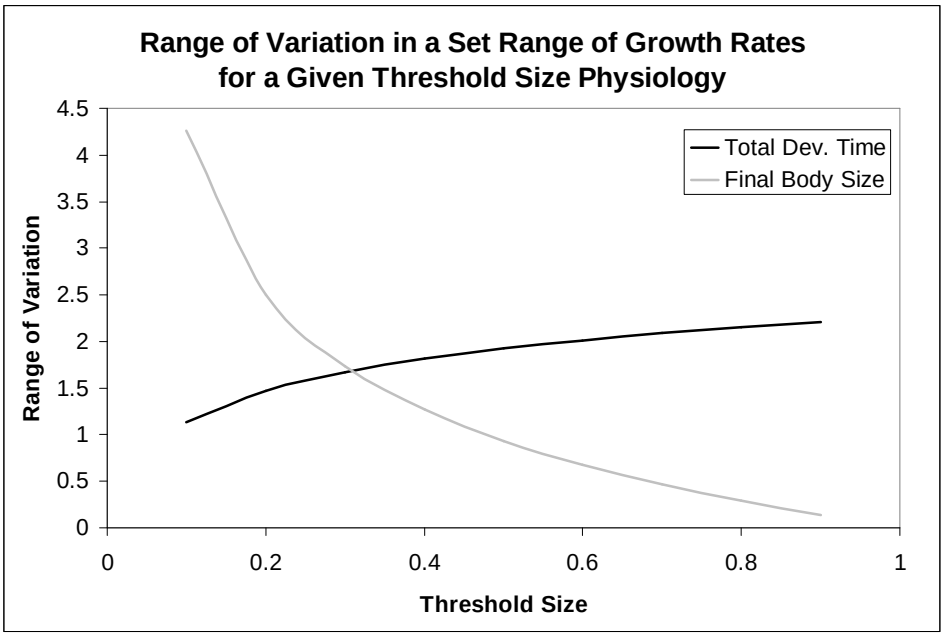

Figure 25: Effect of Threhold Size on Total Development Time (black line) and Final Body Size (grey line) for a Set Range of Growth Rate Variation. Early/Low Threshold sizes vary more in final body size while late/high threshold sizes vary more in total development time.

relative to the baseline final body size. Therefore, lower threshold sizes are more advantageous for developing a large body size as long as growth rates increase above the baseline growth rate. When growth rate falls below that of the baseline growth rate, lower threshold sizes are more disadvantageous for large body sizes. For this reason, early/low threshold sizes are likely for species that lay eggs near optimal or reliable resources for the hatching larvae. The larvae would then be in an environment in which high growth rates would lead to increased body sizes.

4.4.4.2 "Late/High" Threshold Size

Growth rate variation in "late/high" physiologies, with threshold sizes greater than 0.3 of the baseline final body size, caused greater range in variation for total development time than final body size (Figure 25). As threshold size increased, the range in which final body size varied decreased and the range for total development time variation increased in response to growth rate changes. This meant that the latest/highest threshold sizes were very advantageous for larger body size when growth rates decreased. Because threshold size acts as a minimum size for metamorphosis, it counteracts decreased growth rates by increasing development time. Although the larva will take longer to metamorphose, it will be larger when it does so. However, the later/higher threshold sizes are at a disadvantage for final body size when growth rate is increased relative to others. Those larvae with higher/later threshold sizes may develop faster, but will not be as large as larvae with similar growth rates and earlier/lower

threshold sizes. This means that higher/later threshold sizes would be advantageous for larger body size when resources are poor or variable.

4.4.5 Implications for Real Insects

The development and physiology of imaginal tissue development, such as the wing, can influence the overall phenotypic landscape for a species and thus the niches for which a species is suited. In favorable conditions (left of baseline 1,1), physiologies with early/low threshold sizes would be more advantageous for larger final body sizes than those physiologies with late/high threshold sizes. This suggests that early/low threshold sizes would likely be found in species that specialize on a particular resource or environment where growth rates could be optimized.

Of the exemplar metamorphosis types above, there is specialization in groups with early/low threshold sizes (Figure 26). Lepidopterans often specialize on certain plants, such as *B. mori* on mulberry, *M. sexta* on tobacco, and Monarch butterflies on milkweed. *D. melanogaster* is also specialized for a specific resource, rotting fruit. Even Ladybird beetles seek out suitable prey species when laying eggs (Labrie et al. 2006). Such specialization has allowed each of these species to optimize larval life to utilize the respective resource for that species and thus favored early/low threshold sizes.

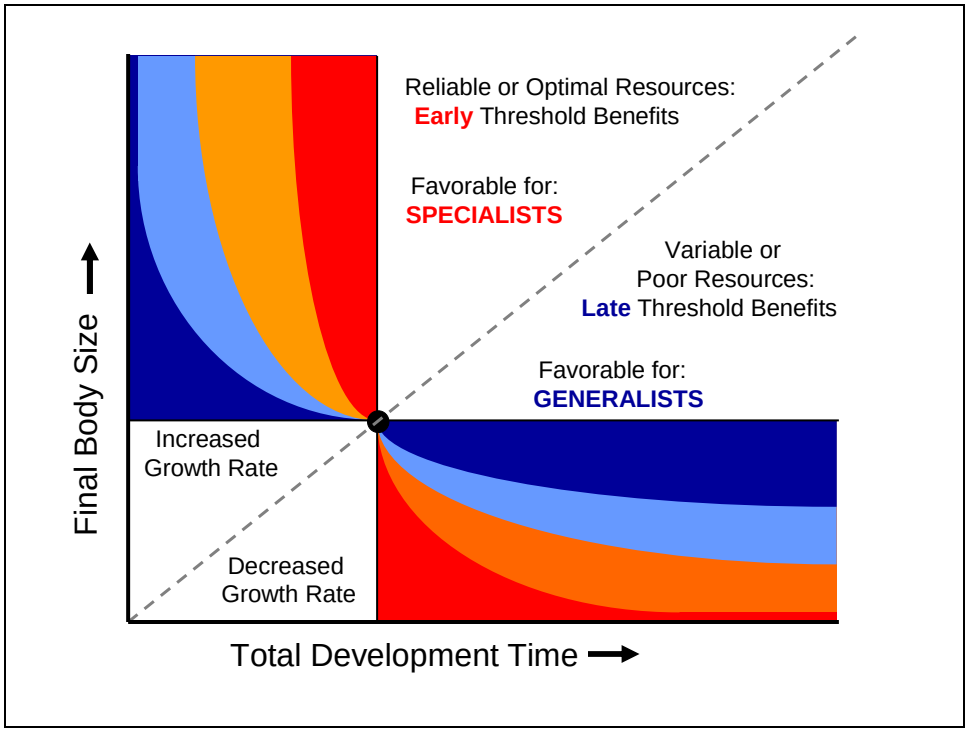

Figure 26: Diagram Representing Which Physiologies are Best Suited for Particular Resouce Environments. Early thresholds are favorable in niches where increased growth is likely, whereas late threshold sizes are favorable for niches where decreased growth rates are likely.

Conversely, when environmental conditions are poor or variable (right of baseline 1,1), physiologies with high/late thresholds would be advantageous for larger final body sizes (Figure 26). Species with late/high threshold sizes would likely be found in generalists where resources for larvae varied among different habitats. Larvae of late/high threshold size species would not likely become optimized for a specific resource as they would need to utilize different resources in different habitats. *T. castaneum* is an excellent example of a generalist as it is known to utilize many different resources and is found in a variety of habitats. *T. castaneum* are known to feed on plant, fungi, and animal matter as larvae (Sokoloff, 1974). They have been found in many other habitats In addition to their presence in human pantries (Sokoloff, 1974). The ability to accommodate such a wide range of resources and environments during larval development has no doubt been advantageous for species with late/high threshold sizes.

4.5 The Effect of Physiology on Phenotype

Using four exemplar metamorphosis types and a simple model, I have shown how changes in physiology can produce different morphologies and different phenotypic landscapes. In the comparison of wing imaginal tissue development in the four metamorphosis types, I have argued that the morphological similarities of wing imaginal tissue often attributed to convergent novel structures may actually be due to parallel heterochronic shifts in the developmental stages of the wing imaginal tissue. I have also shown through use of a simple model that heterochronic changes in at least

one of the developmental stages of the wing imaginal tissue, competence for metamorphosis, can affect final body size and total development time. Through its affect on the phenotypic landscape, competence for metamorphosis also influences what niches a species may be better suited. Species are best suited as specialists when threshold size, which regulates competence for metamorphosis, is at low relative weights or occurs early in larval development. Species with a late/high threshold size, however, would be best suited as generalists. This would mean that primitive metamorphosis types, with late/high threshold sizes, are not just unable to evolve a more derived metamorphosis form, but for generalist niches, primitive types of metamorphosis may actually be beneficial. These results show that the physiology of a species affect both wing developmental morphology and the overall size phenotype of the larva. Therefore, understanding the physiology of a species and the influence physiology has at both the organ and whole animal level is vital for understanding development.

5. Conclusion

One of the main goals of this work has been to develop a broader hypothesis about the control of body size in insects. Through my work on *Tribolium castaneum*, *Tribolium freemani*, and *Manduca sexta*, I have developed a hypothesis that body size is sensed and regulated by the dilution of juvenile hormone as the body grows. I have demonstrated that growth parameters such as growth rate, duration of instars, or number of instars are not correlated with final body size, but threshold size is consistently correlated with body size. Using the black mutant strain of *M. sexta* I have shown that lower juvenile hormone titers correlate with lower threshold sizes. My hypothesis is consistent with the large body of literature indicating the involvement of juvenile hormone and does not require the invoking a novel factor or novel function for an existing growth factor. My hypothesis is a more parsimonious mechanism that can account for the regulation of final body size for a larger range of insect species.

My research has led to a hypothetical mechanism for body size control via the dilutive affects of body size growth on hormonal titers in insects. The dilution mechanism has aspects that can be examined at the genetic level and through environmental manipulation. There is also no reason why the control of hormonal titers by body size growth should be limited to insects or juvenile hormone. It is perfectly possible that other hormones in a wide range of organisms, including vertebrates, could be controlled in this way. Technical advances in the field of physiology would allow us

to test hypotheses and make conceptual advances in understanding the regulation of body size.

5.1 Future Goals

One of the most important goals for the study of body size control in insects will be the development of highly sensitive quantization methods for juvenile hormone. There are currently many different methods for quantifying juvenile hormone levels in an insect, but many require large amounts of starting material. This limits not only which insect species are amenable to juvenile hormone titer quantification, but also limits the quantification of juvenile hormone in specific developmental stages. It is very uncommon for publications to include data of juvenile hormone titers earlier than the penultimate instar. The ability to quantify juvenile hormone from small amounts of starting material will allow for a complete juvenile hormone titer profile for the entire development of a species. Having a complete developmental profile of juvenile hormone titers will no doubt be useful in understanding the role of this hormone in body size control.

Although understanding the relationships of threshold size, juvenile hormone, and body size has been an important step in understanding body size control in insects, there are still many aspects of body size control that are not understood. Specifically, little is understood about the changes in physiology throughout development. It seems as though for some time there has been a general assumption that only the final instar of

any particular species is relevant to metamorphosis. Although this assumption may be true in some instances, I find it to be limiting as one is unlikely to find something where one is not looking. In my research, I have found that although threshold size determines the final instar for insects, it does not necessarily occur at similar times in development. It is my hope that the work presented here will spur others into broadening their perspectives of development to include earlier instars, and not fixate on just the last instar.

Physiology is how the genome interacts with the environment, yet it has been widely overlooked. Most biologists are familiar with the concept that environments can affect different genotypes in different ways, yet the mechanisms behind those responses are largely unknown. A little-understood aspect of growth physiology is the interaction of feeding or nutritional conditions on body size regulation. In the course of my work, I found that feeding *T. castaneum* on a diet of 10% flour, 90% cellulose by weight lowered threshold size (Figure 27). Understanding the complex relationship nutritional signals have with the enzymes controlling production and destruction of juvenile hormone will undoubtedly lead to a better understanding of threshold size and body size control. Future studies may also benefit from the use of a synthetic diet for which the relative amounts of proteins, carbohydrates, and lipids can be more precisely controlled. Understanding the affect of nutrition on the entire network of genes involved the

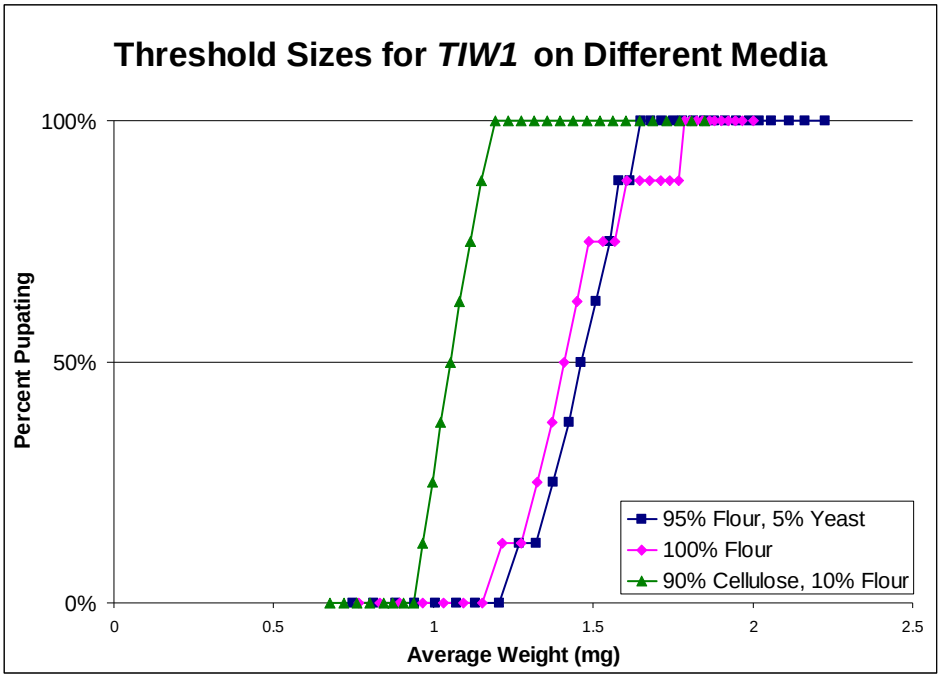

Figure 27: Threshold Sizes of the *TIW1* strain of *T. castaneum* on Different Rearing Media. Under poor conditions (90% Cellulose, 10% Flour), threshold size is shifted to a lower weight relative to controls (95% Flour, 5% Yeast; 100% Flour).

production and destruction of genes will undoubtedly lead to a better understanding of phenotypic responses to changes in nutrition.

Beyond the technical challenges that remain for insect development and physiology, there are conceptual challenges as well. The principle conceptual challenge is the complete reliance on *Drosophila* as the definitive model of insect development. Given the many obviously derived features of *Drosophila* larvae, such as the lack of larval legs and an involuted head, it is surprising that so many researchers use *Drosophila* development to typify insect development. Although *Drosophila* has a wide range of useful tools, the technology is no replacement for the inherent biology of the organism. For many aspects of biology, *Drosophila* is undoubtedly the best model organism to use, but the evolutionary context is severely limited by the lack of information from other insect species. By focusing on *Drosophila* to the exclusion of all other insects, a comprehensive perspective on the evolution, diversity, and development of all insects, including *Drosophila*, is lost.

The differences in body size between the *T. castaneum* mutant and wild type strains and *T. freemani* is well suited for understanding how evolution affects the genes controlling body size. As a generalist, *T. castaneum* has been able to accommodate a wide range of environments with various resources which would also make it useful in understanding how the environment affects the physiology of body size control. Research into physiological genetics and environmental affects in this lowly beetle pest

can potentially inform researchers of other physiological mechanisms which may also be applicable to other species. Understanding how environmental cues are transformed into biological signals, interact with the genome, and produce a biological response has great potential for advancing developmental biology as well as medicine, ecology, and many other fields.

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