

The Regulation of Body and Wing Disk Growth in *Manduca sexta*

by

Alexandra Tobler

Department of Biology  
Duke University

Date: \_\_\_\_\_

Approved:

\_\_\_\_\_  
H. Frederik Nijhout, Supervisor

\_\_\_\_\_  
François Lutzoni

\_\_\_\_\_  
Steve Haase

\_\_\_\_\_  
Kathleen K. Smith

\_\_\_\_\_  
Gregory Wray

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

2009

ABSTRACT

The Regulation of Body and Wing Disk Growth in *Manduca sexta*

by

Alexandra Tobler

Department of Biology  
Duke University

Date: \_\_\_\_\_

Approved:

\_\_\_\_\_  
H. Frederik Nijhout, Supervisor

\_\_\_\_\_  
François Lutzoni

\_\_\_\_\_  
Steve Haase

\_\_\_\_\_  
Kathleen K. Smith

\_\_\_\_\_  
Gregory Wray

An abstract of a dissertation submitted in partial  
fulfillment of the requirements for the degree  
of Doctor of Philosophy in the Department of  
Biology in the Graduate School  
of Duke University

2009

Copyright by  
Alexandra Tobler  
2009

## Abstract

A key question in developmental biology is how organisms attain a final size. Deviations in growth patterns can produce different/new phenotypes and these changes can play fundamental roles in ecology and evolution. The size of an organism and of its constitutive organs is determined by the growth rate and the duration of the growing period. In insects, peptide hormones such as insulin-like growth factors have been shown to be involved in determining the growth rates by coordinating metabolism, cell proliferation, and cell size. In contrast, steroid hormones, such as ecdysone, are involved in determining life stage transitions, and thus the termination of the growing period. Although it is clear that insulin and steroid hormones are both involved in the regulation of growth, the ways in which these two regulators interact is yet to be determined. Furthermore, it is not clear how organs and body growth are coordinated during development to arrive to their correct proportions. In this study, using the tobacco hornworm *Manduca sexta* and its wings as a model system, I examine the developmental mechanisms involved in the regulation of organ growth and how developmental processes can drive morphological evolution. First, I examine how the hormonal events that take place during the termination of the body growth period affect wing disk growth. Second, by using gene expression assays and *in vitro* cultures, I examine the interaction between bombyxin, the Lepidopteran insulin-like growth factor, and ecdysone, the molting hormone, and their contributions to wing imaginal disk growth. Finally, by using three different size strains of *M. sexta*, I examine the developmental basis of the allometric relationship between the wings and the body. My results show that during the final instar of *M. sexta* larval development, wing imaginal disks are sensitive to the hormonal events that terminate the growth period. Furthermore, I show that the

bombyxin requirement for wing disk growth is restricted to the early days of the final instar unlike the constitutive effects seen in other species. After the larva has passed a particular critical weight, bombyxin is not necessary for wing disk growth, although its absence does decrease the growth rate. In contrast, ecdysone is required for promoting the growth of wing imaginal disks primarily through its stimulation of cell proliferation. Finally, I show how selection on body size has unpredictable consequence for the response of wing size. These results demonstrate how specific allometries have a developmental basis in the cross-talk of the various signals that regulate growth itself. Therefore, direct selection on allometric relationships may not need to be strong in order to hold scaling relationships constant, at least over short evolutionary periods.

## Dedication

*a mis padres*

# Contents

Abstract .....	iv
List of Tables.....	ix
List of Figures .....	x
Acknowledgements.....	xii
Introduction .....	1
Chapter I.....	10
Development of the wing imaginal disks in <i>Maduca sexta</i> .....	10
Introduction .....	11
Materials and Methods.....	14
Results.....	17
Discussion .....	32
Chapter II .....	38
Bombyxin and ecdysone in the regulation of wing disk growth.....	38
Introduction .....	39
Materials and Methods.....	41
Results.....	44
Discussion .....	57
Chapter III.....	62
Correlated wing–body allometric response to body size selection in <i>Manduca sexta</i> .	62
Introduction .....	63
Materials and Methods.....	67
Results.....	70
Discussion .....	91

References .....	97
Biography.....	106

## List of Tables

Table 3.1: Body size and wing size for the three <i>M. sexta</i> size strains (mean $\pm$ SD).....	74
Table 3.2: Allometric parameters for the wing-body relationship for individuals from the three size strains of <i>M. sexta</i> .....	76
Table 3.3: Wing disk and larval body growth rates for the three size strains.....	81
Table 3.4: Wing disk mass and cell doubling time for the three size strains (mean $\pm$ SE) .....	82
Table 3.5: Effect of food deprivation on the wing-body size allometry for the three size strains.....	85
Table 3.6: Effect of wing disk removal on the allometric relationship for females of the three size strains.....	88
Table 3.7: Correlation between wing area and wing mass for the three size strains.....	90

## List of Figures

Figure 1.1: Body and wing disk growth for <i>M. sexta</i> during the final instar .....	18
Figure 1.2: Fore- and hindwing growth curves for <i>M. sexta</i> during the last larval instar .....	20
Figure 1.3: Forewing imaginal disk growth curve for <i>M. sexta</i> during the last larval instar .....	21
Figure 1.4: Wing disk cell size during the last larval instar.....	22
Figure 1.5: Endocrinology and wing growth rate during the last instar of <i>M. sexta</i> .....	23
Figure 1.6: Effect of starvation on wing imaginal disk growth.....	25
Figure 1.7: Comparison of the size change between fed and starved larvae after 48h for day 4 and 5 of the final instar.....	26
Figure 1.8: Effect of starvation before and after critical weight on wing imaginal disk growth .....	27
Figure 1.9: Quantification of cells undergoing mitosis in the forewing imaginal disk ....	28
Figure 1.10: Effect of the brain and fat body on wing imaginal disk growth .....	30
Figure 1.11: Effect of removing brain and fat body factors on fore- and hindwing imaginal disk growth .....	31
Figure 2.1: Hemolymph glucose and trehalose concentration for fed fifth instar <i>M. sexta</i> larvae .....	46
Figure 2.2: Effect of food deprivation on hemolymph glucose and trehalose concentration depending on larval age at the initiation of starvation.....	47
Figure 2.3: <i>Bbx</i> and <i>InR</i> mRNA levels during the last instar of <i>M. sexta</i> larvae .....	49
Figure 2.4: The effect of food deprivation on <i>Bbx</i> and <i>InR</i> mRNA levels .....	50
Figure 2.5: Effect of <i>Bbx</i> and 20E on <i>in vitro</i> wing disk growth.....	53
Figure 2.6: Effect of 20E on wing disk growth from wandering larvae.....	54
Figure 2.7: Dose response to 20E of wing imaginal disks.....	55
Figure 2.8: Effect of 20E on <i>InR</i> mRNA transcript levels in wing imaginal disks.....	56
Figure 3.1: Body size distribution as a function of strain and sex .....	72
Figure 3.2: Wing size distribution as a function of strain and sex.....	73

Figure 3.3: Allometric relationships between wing and body size for the three size strains of <i>M. sexta</i> adults .....	75
Figure 3.4: Larval body growth rate for the final instar in the three size strains .....	79
Figure 3.5: Wing disk growth rate for the three size strains .....	80
Figure 3.6: Effect of food deprivation on wing-body relationship in the three size strains of <i>M. sexta</i> .....	84
Figure 3.7: Effect of wing disk removal on the allometric relationship of the three size strains .....	87
Figure 3.8: Correlation between wing area and wing mass for the three size strains .....	89
Figure 3.9: Larval body growth rate for the Wt strain during the fifth instar .....	96

## Acknowledgements

I would like to thank:

My advisor Fred Nijhout, for his support, guidance and generosity during the past years. His knowledge, dedication and enthusiasm are always inspiring.

My committee members, Kathleen Smith, Greg Wray, Steve Haase, and François Lutzoni, for their guidance and support.

My past and former lab members, Lou D'Amico, Julia Bowsher, Vivian Callier, Rick Dilling, Inder Jalli, Kevin Preuss and Yui Suzuki for providing intellectually stimulating discussions.

Chris Shrive, for rearing thousands of *Manduca sexta*.

Laura Grunert, for all her help and also for making the lab a fun place.

My English writing "teachers", Nathalie Nagalingum, Julia Bowsher, Vivian Callier, and many others, for making my writing look so much better.

Nathalie Nagalingum, for being an exceptional friend.

Daniel Runcie, for his support, patience and love.

My friends and family, for keeping my life in perspective.

I believe that no project in life is the sole achievement of one person. Therefore, my gratitude goes to all the people that directly or indirectly contributed to the completion of this project.

*Gedanken ohne Inhalt sind leer, Anschauungen ohne Begriffe sind blind*

-Kant

# Introduction

“It is hardly an exaggeration to state that the problem of growth is a focusing point where the most fundamental problems of biology, from the biophysical bases of the phenomena of life up to the questions of human behavior converge and intersect.”

Bertalanffy (1960, p 252)

An organism's size is one of its most apparent characteristics with important implications in different biological aspects, such as ecology, evolution and physiology (reviewed in LaBarbera, 1989; Peters, 1983). Individuals with larger body sizes tend to produce more offspring, have higher mating success, and a higher probability of survival relative to smaller individuals (Kingsolver & Pfennig, 2004). However bigger individuals also tend to have longer developmental periods, larger food demands, and be more vulnerable to environmental crises increasing their probability of going extinct (McKinney, 1997). Thus there are different selection pressures that act upon an individual's size that result in the extraordinary diversity in organismal size.

Size differences account for most of the diversity observed in the animal kingdom and the range of sizes exhibited is extraordinary. Large animals, such as a giraffe or an elephant, attract everybody's attention; in contrast, smaller animals, like ants or fleas, usually pass un-noticed. Remarkable size differences do not only apply to organisms from different species. Within the same species, size difference can also be dramatic; a close example to us is dogs. Different breeds of dogs can exhibit striking differences in size. A Chihuahua usually weighs between 1-3 kg and has an average height of 20 cm. This is incredibly small when compared to its relative the Great Dane, which can reach a size of one meter and can have a weight of 60 kg, accounting for a 60 fold size difference between these two breeds. Although animal size can display

dramatic differences, organisms from the same population tend to arrive to a predictable size range. All giraffes will arrive to an adult size within its population size range and will not arrive to the size of a mouse.

An important aspect of an individuals' size is the proportionality between the overall body size and the structures and organs that compose that body. A larger individual will bear relatively larger organs that are proportional to its body size. As an example, larger giraffes have relatively longer necks in comparisons with smaller sized giraffes (Simmons & Scheepers, 1996). However, larger giraffes have a larger neck relative to its body size when compared to a smaller sized giraffe. Thus a larger giraffe is not a proportionally sized version of a smaller giraffe, and the proportionality between organs and body size within individuals is not constant. However, the size of the neck and the body follow an allometric relationship (Simmons & Scheepers, 1996).

Allometries refer to the relationship between the size of a trait relative to other traits or with the overall body size (Huxley, 1932). In the case of the giraffe, neck size displays a positive allometry in relationship to its body, and is a result of the differential growth rate between the neck and body size.

Despite the relevance of an organisms' size, how an organism and its organs attain a particular size is still an unanswered question. From a cellular perspective, the size of an organism or an organ is determined by the number and size of the cells it contains and, therefore, size is determined by cell growth, cell proliferation and cell death. However, from a physiological or more organismal perspective, final size is determined by the growth rate and the duration of the growing period. The attainment of a particular size must then be controlled by mechanisms that coordinate both cellular and physiological processes. Therefore, to elucidate the mechanisms of growth and size we need to understand how hormones and cellular processes interact.

This dissertation is designed to integrate the cellular process of size regulation with physiology of growth to contribute to an understanding of the regulation of growth and how final size is attained. I will use the tobacco hornworm *Manduca sexta* and its wings as a model system for studying growth. Chapter 1 examines how hormonal events that are involved in the termination of the growth period can affect the growth trajectory of the wing disks. Chapter 2 examines the interaction between the molting hormone, ecdysone, and the insulin-like growth factor, bombyxin, and its contribution to the regulation of wing disk growth. Finally, Chapter 3 examines the relationship between wings and body growth and the evolution of wing body allometries in three size strains. This study will be used to provide insight into understanding how hormonal events interact with cellular processes to regulate body and organ size.

*Study system: Growth regulation in the Tobacco Hornworm Manduca sexta*

*M. sexta*, the tobacco hornworm, offers an exceptional system for the study of growth regulation because it is one of the few organisms for which the physiological mechanisms that regulate final body size are well understood (Nijhout *et al.*, 2006). During its five larval instars, size increases at an almost exponential rate so that most of the larval growth occurs during the final instar. The feeding period of the final instar usually lasts 5 days and the larva grows from about 1.2g to 12g. When the larva reaches its maximum weight it stops feeding, clears the gut, enters the wandering phase and pupates six days later.

The final size that an adult *Manduca* will reach is largely determined by the interaction between nutrition and hormonal events that occur throughout the final instar (Baker *et al.*, 1987). The cascade of events that induce the termination of the growing period are triggered by the larva attaining a critical weight (Nijhout & Williams, 1974b).

When the larva reaches the critical weight it triggers the clearance of juvenile hormone (JH) from the hemolymph. The clearance of JH is caused by a decrease in JH synthesis and by its degradation by JH esterase. The absence of JH allows for the secretion of the prothoracicotrophic hormone, which in turn stimulates the release of the molting hormone, ecdysone. It is the presence of ecdysone that leads to the cessation of feeding, the cessation of larval growth, and the initiation of the pre-pupal wandering behavior (Nijhout, 1994). Thus, larval growth stops when the sequence of endocrine and physiological events initiated by the critical weight culminate in the secretion of ecdysone. Critical weight, *per se*, does not cause the metamorphic molt, but it is the first step required in the events that lead to metamorphosis.

Critical weight is usually about 55% of the peak larval mass (Davidowitz *et al.*, 2003) and is operationally defined as the minimum size at which further feeding and growth is not required for a normal time course to metamorphosis and pupation (Nijhout *et al.*, 2006; Nijhout & Williams, 1974b). Nijhout *et al.* (2006) demonstrated that critical weight is a function of the larval weight at the beginning of the final instar and is the weight at which the larvae would molt if there would be another instar. These results suggest that the larva possess a mechanism that assesses the difference between the mass at critical weight and the mass at the beginning of the final instar. It is possible that Lepidopterans have a mechanism similar to that identified in Hemipterans (true bugs). This group of insects possess stretch receptors in the abdomen that when expanded trigger the events that will terminate the growth period and lead to metamorphosis. (Nijhout, 1979; Nijhout, 1984). However, the stretch receptors or any mechanism that is able to assess critical weight has yet been identified for *M. sexta*. Therefore, it is unclear how larvae “know” when they have attained the critical weight.

## *Wing disk growth*

Wing imaginal disks have been used extensively as an experimental system to dissect the mechanisms involved in the control of organ growth. Wing disks are particularly suited to this purpose because they are relatively big and therefore easy to manipulate and measure, and can be dissociated into individual cells allowing cell size and cell number to be monitored throughout development. In addition, disks can be cultured *in-vitro* for many days, which makes it possible to precisely control different growing conditions. Experiments with wing disks have provided a wealth of knowledge about the processes of spatial patterning and also about the molecular processes involved in growth regulation (Crickmore & Mann, 2008). Although it is clear that wing disks offer an attractive system to study the regulation of organ growth, surprisingly little is known about their growth trajectory during development and about how extrinsic factors, such as nutrition, can alter this trajectory.

The wings of *M. sexta* develop as imaginal disks within the growing larvae. Wing imaginal disks develop from independent cell lineages that are established early in larval development and are separate from the larval epidermal cells. At metamorphosis the wing disks evaginate from the larval body and differentiate into the adult wings (Knutze, 1935). Wing imaginal discs grow by cell proliferation throughout larval development. Because growth is exponential, most of the increase in mass occurs during the final instar and early pupal stage.

In *M. sexta* wing disks and the body grow at different times during development. Most of the wing disk growth takes place after the larva has stopped feeding and body growth has ceased. While the larva is preparing to pupate, wing disks continue to grow exponentially. Thus, the events that terminate the larval growth period do not cause the immediate cessation of wing disk growth, but must affect the wing disk growth

trajectory. In *M. sexta* there is little knowledge about how the different hormonal titers affect wing disk growth

### *Bombyxin and ecdysone and the regulation of growth*

Hormones regulate many aspects of insect development including growth. In insects, two hormones have been shown to play a major role in the regulation of growth: insulin-like peptides and ecdysone. Insulin-like peptides have been shown to be involved in coordinating cell growth and cell proliferation with nutrition and metabolism. In contrast, ecdysone, the molting hormone, is involved in regulating the termination of the growth period.

In *Drosophila melanogaster*, insulin-like peptides have been shown to have a direct effect on final adult size (Bohni *et al.*, 1999; Britton & Edgar, 1998; Brogiolo *et al.*, 2001; Ikeya *et al.*, 2002; Nijhout & Grunert, 2002; Oldham *et al.*, 2002; Weinkove *et al.*, 1999). Manipulation of the expression of components of the insulin pathway has generated flies with strikingly different sizes (Brogiolo *et al.*, 2001; Ikeya *et al.*, 2002). Furthermore, the inactivation of the single Dilp receptor (InR) results in smaller flies (Brogiolo *et al.*, 2001). Finally, when Dilp producing cells are ablated, adults show an undergrowth phenotype (Rulifson *et al.*, 2002).

In contrast to *Drosophila*, in Lepidoptera little is known about the requirements for insulin-like peptides and how they might affect growth. Bombyxin, the Lepidoptera insulin-like peptide, has been shown to act as a growth factor (Nijhout & Grunert, 2002). However, in cultured wing disks of the butterfly *Precis coenia*, bombyxin alone could not stimulate normal growth unless ecdysone was present (Nijhout & Grunert, 2002). Apart from triggering metamorphosis, ecdysone has also been shown to stimulate cell proliferation in different tissues by turning on and off cell-cycle progression (Champlin

*et al.*, 1999; Champlin & Truman, 1998a; Champlin & Truman, 1998b; Kirschenbaum *et al.*, 1995; Koyama *et al.*, 2004). It is unknown how ecdysone and bombyxin interact to promote growth. In this thesis, I will investigate the effects of these two hormones during *Manduca* development. I will show how the specificity of bombyxin and ecdysone changes throughout out the larval life, and thus the complexity of the interactions between bombyxin and ecdysone in regulating wing disk growth.

#### *Allometries and the evolution of body size*

Changes in allometric relationships – scaling relationships between body parts or organs with overall body size – can have important functional, adaptive, and ecological consequences and therefore, are of great evolutionary importance. A large part of morphological diversity in the animal kingdom has arisen from changes in the relative sizes of morphological features such as wings and legs - relative both to one another, and to overall body size. Therefore, changes in allometric relationships are a major source of diversity.

However, interpreting allometric change in an evolutionary context can be difficult because the various tissues of an organism do not develop independently. Each organ necessarily must share much of the same developmental conditions and experience much of the same signaling environments and nutritional states as other organs. Therefore, a change in an allometric relationship may not be due to selection on that feature at all, but may be due entirely to pleiotropy – changes in the shared developmental pathways caused by selection for a change in the size of one of its components. Disentangling these relationships is necessary to understand how differently proportioned species can evolve.

A key trend in butterfly evolution is the differential enlargement between fore- and hindwings and body size, making this group and its wings suitable to study how developmental variation influences phenotypic variation. However, there is little knowledge about the developmental bases of allometries. Using three size strains of *M. sexta*, I will explore if changes in growth rates are responsible for changes in allometries, and responsible for new morphologies.

## Chapter I

### Development of the wing imaginal disks in *Maduca sexta*

## Introduction

Wing imaginal disks have been used extensively as an experimental system to dissect the mechanisms involved in the control of organ growth. Wing disks are particularly suited to this purpose because they are relatively big and therefore easy to manipulate and measure, and can be dissociated into individual cells allowing cell size and cell number to be monitored throughout development. In addition, disks can be cultured *in-vitro* for many days, which makes it possible to precisely control different growing conditions. Experiments with wing disks have provided a wealth of knowledge about the processes of spatial patterning and also about the molecular processes involved in growth regulation (reviewed in Affolter & Basler, 2007; Crickmore & Mann, 2008; Day, 2000; Hufnagel *et al.*, 2007; Weinkove & Leever, 2000). Although it is clear that wing disks offer an attractive system to study the regulation of organ growth, surprisingly little is known about their growth trajectory during development and about how extrinsic factors, such as nutrition, can alter this trajectory.

In Lepidoptera, adult wings develop as imaginal disks within the growing larvae. Wing imaginal disks develop from independent cell lineages that are established early in larval development and are separate from the larval epidermal cells. Each wing imaginal disk represents an autonomous unit; thus, extirpation of a single wing disk during the larval instar results in the absence of that particular wing in the adult but not of those of other wing disks. At metamorphosis the wing disks evaginate from the larval body and differentiate into the adult wings (Knutze, 1935). Wing imaginal discs grow by cell proliferation throughout larval development. Because growth is exponential, most of the increase in mass occurs during the final instar and early pupal stage.

The larval stage of insects is specialized for feeding and growth. During the larval stage most of the incoming nutrients are utilized for growth. Therefore, nutrient availability must be one of the major environmental signals influencing growth. It is known that nutrients do not act directly on the growing organs. Instead, nutrients act by stimulating the production of growth hormones and growth factors that stimulate cell growth and cell division in target tissues. One of the principal growth factors in animals are the insulin-like peptides. In insects, many of these peptides are produced by the central nervous system as neurosecretory factors.

The brain in insects is the principal neuroendocrine organ. It contains several clusters of neurosecretory cells that are responsible for the secretion of a variety of hormones such as the prothoracicotrophic hormone (PTTH, which stimulates the secretion of ecdysone) and the insulin-like peptides. Insulin-like peptides are expressed in medial neurosecretory cells in the brain (Ikeya *et al.*, 2002; Iwami *et al.*, 1996; Rulifson *et al.*, 2002) and have been shown to be the primary nutrient-responsive growth regulators (Britton *et al.*, 2002; Ikeya *et al.*, 2002). Insulin-like peptides are synthesized in response to nutrient availability and released into the hemolymph where they travel to their target tissue (Ikeya *et al.*, 2002; Masumura *et al.*, 2000). The brain is therefore the primary organ involved in coordinating growth with nutrition.

In addition to the brain, the fat body also plays a significant role in growth regulation (Britton & Edgar, 1998; Colombani *et al.*, 2003). The fat body is the insect's equivalent of the liver, and is the major tissue for metabolism and storage of nutrients, and the source of most of the proteins that circulate in the hemolymph (Haunerland & Shirk, 1995). The fat body is a diffuse collection of cells distributed throughout the larval body, but concentrated around the animals' gut (Dean *et al.*, 1985). Besides its metabolic function, the fat body also serves as an endocrine organ. The fat body appears to produce a factor that stimulates cell proliferation and growth (Britton & Edgar, 1998;

Colombani *et al.*, 2005). Although the brain and the fat body are both capable of acting as sensors for nutritional conditions, their individual contributions to growth regulation are poorly understood, as are the mechanisms by which the signals they produce might interact to coordinate growth.

Adult body size in insects is determined by the growth rate and by the duration of the growing period (Nijhout, 2003a). *M. sexta* is the only insect for which the hormonal events that cause the cessation of body growth are well understood (Nijhout *et al.*, 2006). In *M. sexta* the growing period is terminated by a series of hormonal events that begin with the attainment of the critical weight and culminate in metamorphosis. The critical weight is the size at which larvae become irreversibly committed to pupation. When the larva attains critical weight, juvenile hormone (JH) secretion stops and its degradation by JH-esterase increases. JH inhibits the secretion of PTTH and ecdysone (Nijhout & Williams, 1974a; Rountree & Bollenbacher, 1984), and after JH is cleared from the hemolymph, the secretion of ecdysone can occur. The surge of ecdysone then triggers the cessation of feeding and the transition to the wandering phase, thus terminating larval growth (Nijhout, 1981).

In holometabolous insects –insects that undergo complete metamorphosis– wing disk and body growth occur asynchronously. Most of the wing disk growth takes place after the larva has stopped feeding and body growth has ceased. Therefore, most of the wing disk growth happens in a closed system where there is no exchange of matter with the environment. While larvae are preparing to pupate, wing disks continue to grow exponentially. Thus, the events that terminate larval growth do not stop wing disk growth. Although the hormonal events that terminate the growing period are well understood, little is known about how these hormonal events affect wing disk growth. Furthermore, it is not clear how the wings maintain proportionality with the body despite the fact that they do most of their growth after body growth has stopped.

In this chapter, I examine how wing disk growth responds to nutrition and to endocrine factors coming from the brain and the fat body. I show that wing imaginal disks are sensitive to hormone levels that mark the larval transition into the pupal stage. Furthermore, my results indicate that factors from the fat body, but not from the brain are necessary for wing disk growth during the wandering phase of *Manduca* larval development.

## **Materials and Methods**

### *Experimental animal*

Larvae of the tobacco hornworm *Manduca sexta* were reared on a standard laboratory diet in individual cups at 26°C under long-day conditions (16 light: 8 dark) (Bell & Joachim, 1976). During the feeding phase, larvae were fed *ad libitum* except in the starvation experiments. After they initiated wandering, larvae were placed in holes bored in wooden blocks for pupation. Age during the fifth (last) larval instar was measured as time since ecdysis: the day of ecdysis to the last larval instar was designated as day 1 of the feeding phase. On day 6 most of the larvae had transitioned from the feeding phase to the wandering phase, characterized by the appearance of the dorsal vessel. The wandering phase lasted from day 6 to day 10, after which most animals pupated. All experiments were performed 2-4 hours after lights-on.

### *Starvation*

Fifth instar larvae were starved at different ages during the feeding phase and kept without food for 48h. To prevent larvae from desiccating, their containers were

sprayed with water twice a day. After 48h of starvation, larvae were anesthetized with CO<sub>2</sub> for 5 min and their wings were dissected. The effect of starvation on wing disk growth was analyzed by measuring the fold-increase in size of the wing disks during 48h of food deprivation.

#### *Brain removal*

Removal of the brain was performed on CO<sub>2</sub> anesthetized larvae through a small triangular incision in the front of the head capsule on the first day of the wandering phase (day 6). The brain was gently drawn out of the incision and removed with micro-scissors. The incision was sealed by applying a small drop of wax around the triangular flap of cuticle. The effect of the treatment was measured by comparing the difference in wing disk growth between brainless and sham-operated larvae after 24h.

#### *Ligations*

Ligations were performed by placing a tight cotton thread around the larval body at the first day of the wandering phase (day 6). Abdominal ligations were placed between the thorax and the abdomen. Thoracic ligations were placed between the second and third thoracic segment. Larvae were anesthetized with CO<sub>2</sub> before placing the ligations. The effect of this treatment was measured by comparing the differences in wing disk growth between ligated and non-ligated larvae after 24h.

### *Wing size measurement*

Wing disks were dissected out after anesthetizing the larvae for 5 min in CO<sub>2</sub>. Wing disk dry weight was determined by rinsing dissected wing disks in water, placing them on a small tared disc of aluminum foil, and drying them at 60°C for 48h. Wing disk weight was determined to the nearest 1μg on a Cahn-25 Electrobalance. The average of the two fore- or the two hindwings was used as an estimate of wing size for the individual. Cell number estimates were obtained by dissociating wing disks in 0.35M citric acid and counting the cells in a hemocytometer (Martin, 1982). Cells were counted in duplicate samples in a total volume of 0.1μl.

### *Cell proliferation*

To obtain an estimate of wing disk cell proliferation I counted the number of cells that were in M phase in an area of 0.0875mm<sup>2</sup>. Three different positions of the wing disk were counted and the average of the three areas was used as an estimate of the wing cell proliferation. The three positions were maintained relatively constant throughout all treatments. Cells in M phase were identified with a 1:2000 dilution of anti-phosphorylated histone H3 antibody (PH3), conjugated to Alexa Flour® 488 (Cell Signaling Technology, Inc. # 9708). Wing disks were fixed for 1h in 3.7% formaldehyde in phosphate-buffered saline (PBS; 130mM NaCl, 7mM Na<sub>2</sub>HPO<sub>4</sub>, 3mM NaH<sub>2</sub>HPO<sub>4</sub>, pH 7.2) for 1h at room temperature, followed by rinses in PBS and 1% Triton-X.

### *Statistical analysis*

Growth rates for wing disk mass, wing disk cells and fore- and hindwings were analyzed using an analysis of covariance (ANCOVA) with age as a covariate. Differences in cell proliferation were examined using a mixed model nested analysis of variance (ANOVA). The effect of age on cell size was examined using ANOVA. If ANOVA detected significant effects of age, I used Tukey's HSD ( $\alpha=0.05$ ) to conduct pair-wise comparisons of the effects of individual ages to test if they were different. The effect of starvation, brain removal and abdomen ligatures on wing disk growth was analyzed using a one-tailed T-test. The effect of thoracic ligations on fore- and hindwing growth was examined by ANCOVA with forewing as a covariate. All statistical analyses were conducted using JMP® 7.0.2 (SAS Institute Inc., Cary, NC).

### **Results**

Under normal feeding conditions fifth instar larvae feed for 5 days and then enter the wandering phase (a non feeding phase) in preparation for pupation. Larval body increases in mass at an approximately exponential rate during the feeding phase, but after the larva enters the wandering phase, body size decreases due to the purging of the gut contents. The cessation of larval growth is controlled by endocrine changes that take place during the final instar. Wing imaginal disks, in contrast, continue to grow throughout the feeding and wandering stage, and do not stop growing until several days into the pupal stage (Fig. 1.1).

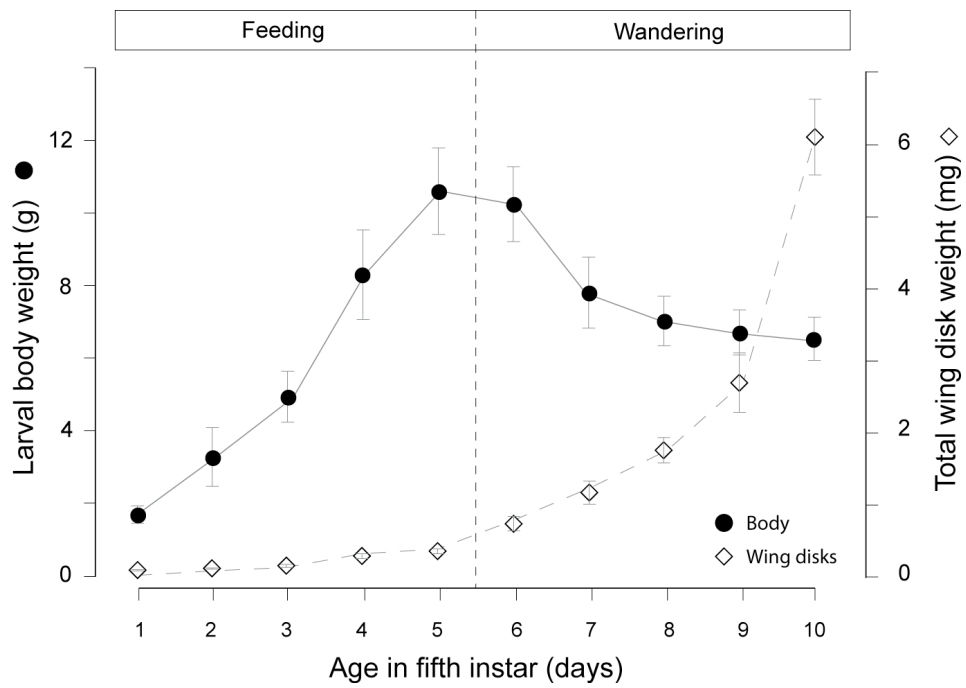


Figure 1.1. Body and wing disk growth for *M. sexta* during the final instar. The dotted line between days 5-6 represents the transition from the feeding to the wandering phase. Each circle represents the mean of the body weight of at least 50 individuals. Diamonds represent the mean of total wing weight (fore- and hindwing) for 20-22 individuals. Error bars represent SD.

### *Wing disk growth during the 5<sup>th</sup> larval instar*

During the final larval instar, *Manduca* wing imaginal disks grow at an exponential rate (Fig. 1.2). Fore- and hindwings are dramatically different in size ( $P < 0.001$ ), but they follow the same overall growth trajectory during the final instar (Fig. 1.2). This is expected since fore- and hindwings are growing in the same environment and respond to the same growth factors circulating in the hemolymph. Average cell doubling time for the forewing occurs every  $30 \pm 3$  hours, and wing mass doubling occurs every  $32 \pm 4$  hours. Cell proliferation thus happens at a slightly, but significantly higher rate than the increase in mass ( $P = 0.0017$ ) (Fig. 1.3). Early in the instar, wing disk cell size remains almost constant. However, when the larva enters the wandering stage wing disk cell size decreases significantly ( $P < 0.05$ ) (Fig. 1.4).

Although wings grow continuously, growth does not seem to occur at a constant rate. Results for wing cell number and fore- and hindwing mass all indicate that the wing disk undergoes periods of higher and lower growth rates (Fig. 1.5). Higher than average, growth rates occur on days 3-4, days 5-6, and days 9-10.

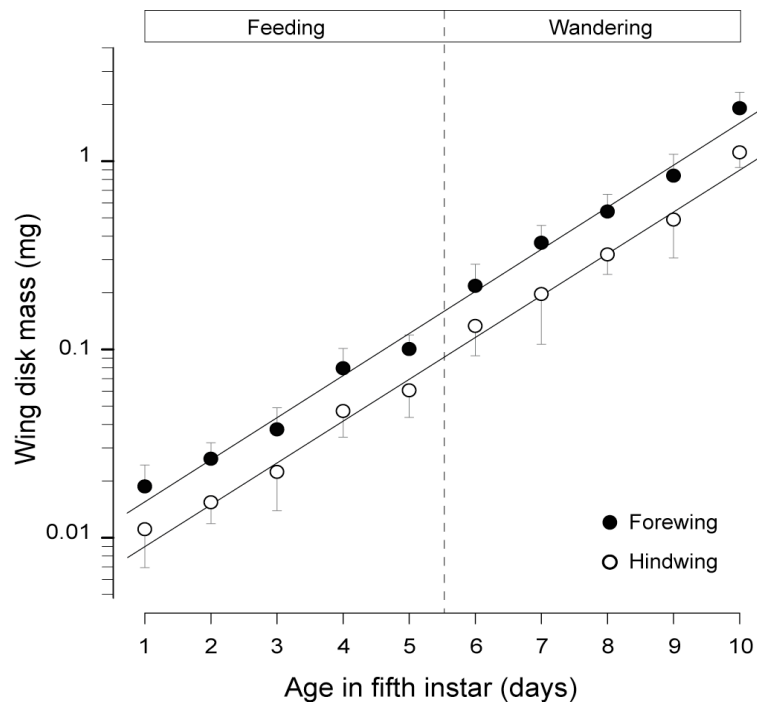


Figure 1.2. Fore- and hindwing growth curves for *M. sexta* during the last larval instar. Each point represents the mean for 20-22 individuals. Exponential regression on wing disk mass were determined by the least squares method. Best-fit equations were: Forewing disk weight= $0.0093 \exp(0.514 \cdot \text{Age})$ ,  $r^2=0.98$ ; Hindwing disk weight= $0.0055 \exp(0.512 \cdot \text{Age})$ ,  $r^2=0.98$ . Growth rates for fore- and hindwing are not statistically different (ANCOVA  $P=0.78$ ). The dotted line between days 5-6 represents the transition from the feeding to the wandering phase. Each point represents the mean for 20-22 individuals. Error bars represent SE.

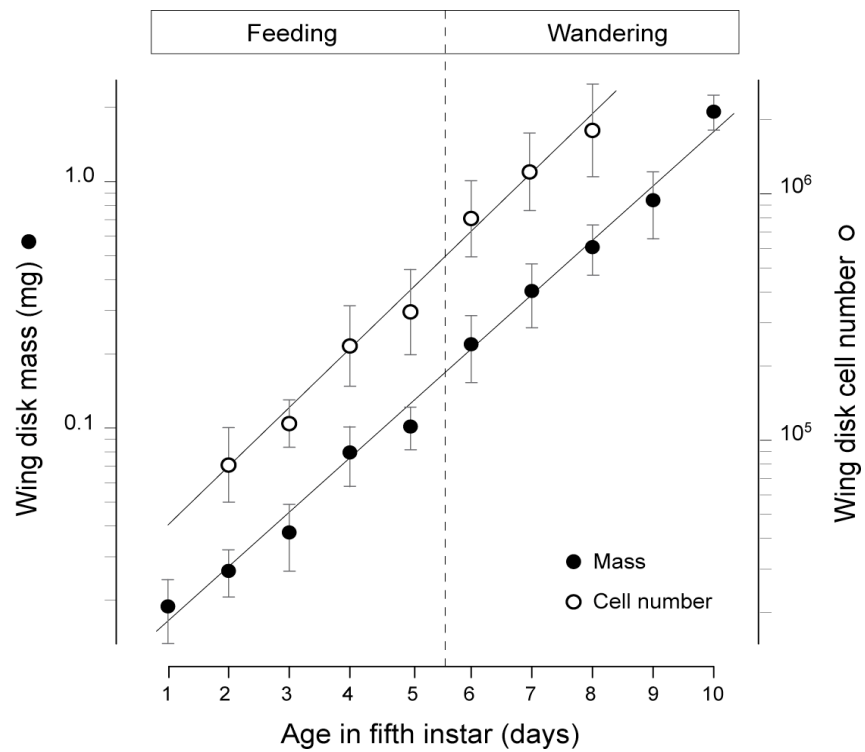


Figure 1.3. Forewing imaginal disk growth curve for *M. sexta* during the last larval instar. Exponential regressions on wing disk mass were determined by the least square method. Best-fit equations were: Wing disk weight= $0.0093 \exp(0.514 \cdot \text{Age})$ ,  $r^2=0.98$ ; Wing cell number= $26152 \exp(0.549 \cdot \text{Age})$ ,  $r^2=0.96$ . Growth rate and cell proliferation rate for wing disks are statistically different (ANCOVA,  $P=0.0017$ ). Each filled circle represents the mean of the forewing dry weight for 20-22 individuals. The dotted line between days 5-6 represents the transition from the feeding to the wandering phase. Each open circle represents the mean of the forewing cell number for 12 individuals. Error bars indicate SD.

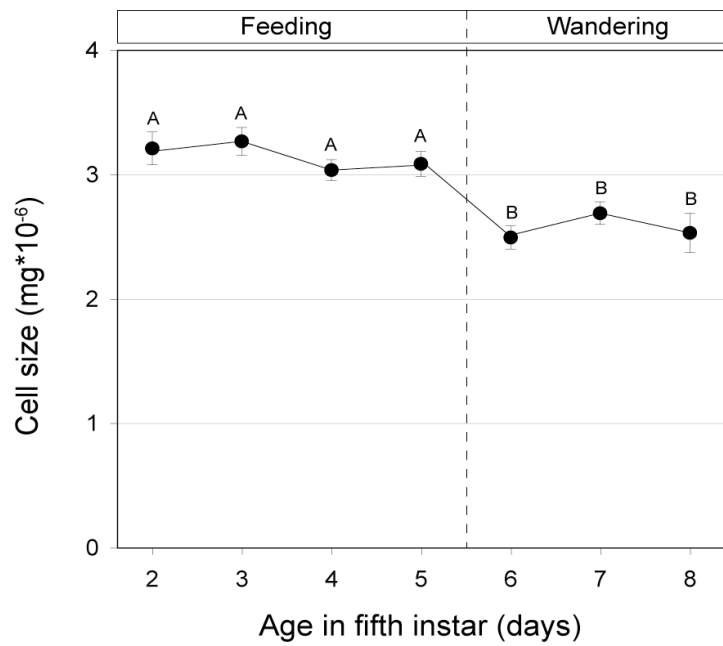


Figure 1.4. Wing disk cell size during the last larval instar. Mean cell mass was calculated by dividing wing disk mass by the number of cells in the disk. Means labeled with the same letter were not statistically different at  $p < 0.05$  by ANOVA and a post-hoc comparison of the means, with Tukey HSD ( $\alpha = 0.05$ ). The dotted line between days 5-6 represents the transition from the feeding to the wandering phase. Each circle represents the mean of forewing cell number for 12 individuals. Error bars indicate SE.

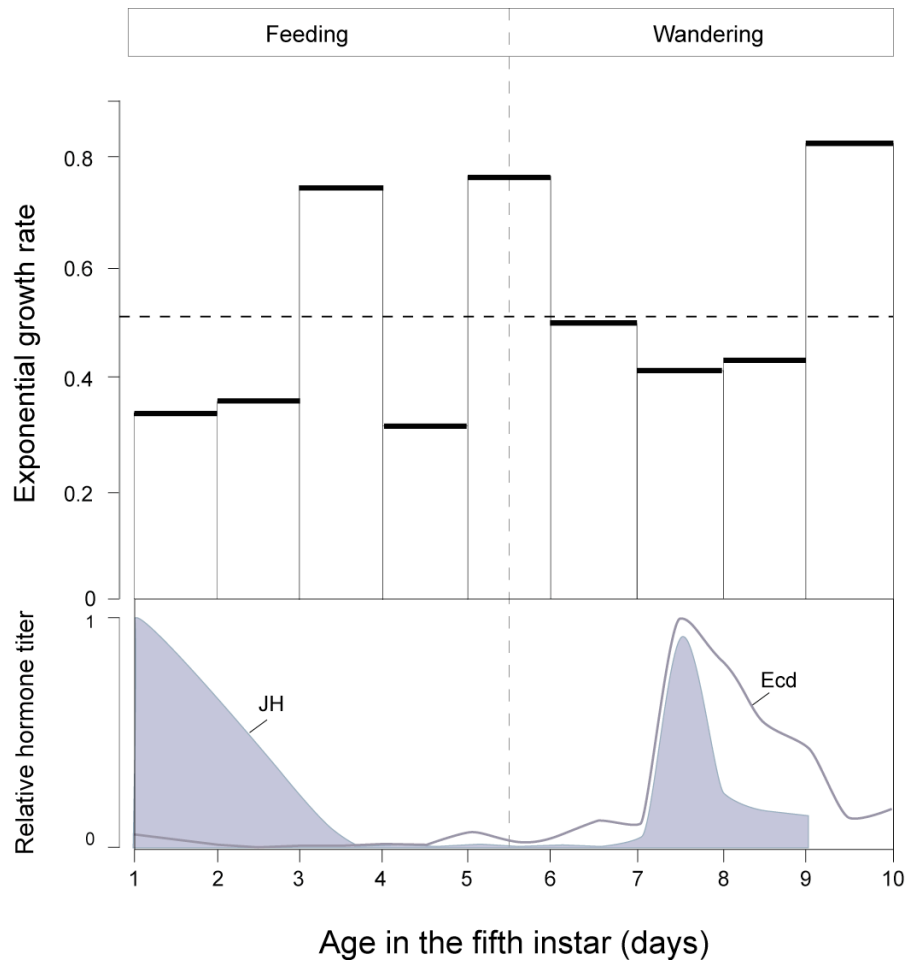


Figure 1.5. Endocrinology and wing growth rate during the last instar of *M. sexta*. Dotted horizontal line indicates the average wing disk growth rate during the final instar. Horizontal bars show the estimated growth rates for the periods between consecutive days. Hormonal titers for ecdysone (Ecd, black line) and juvenile hormone (JH, shaded area under the curve) are plotted on the same time scale. Ecdysone titers from (Baker *et al.*, 1987; Bollenbacher *et al.*, 1981) JH titers (Baker *et al.*, 1987; Fain & Riddiford, 1975). The dotted vertical line between days 5-6 represents the transition from the feeding to the wandering phase.

### *Influence of nutrition on wing growth*

Food deprivation has two different effects on wing disk growth. When food deprivation occurs early in the instar it causes an arrest in both body and wing disk growth. In contrast, when food deprivation happens later in the instar, body growth stops but wing disks continue to grow (Fig. 1.6). Wing disks from larvae starved later in the instar showed a significant increase in size in 48h ( $P < 0.0001$ ), however their growth rate was significantly lower than that of disks from fed larvae ( $P < 0.0001$ ) (Fig. 1.7).

The differential effect of starvation depended on larval weight and not on age. Larvae of the same age that differed in having or not attained critical weight were food deprived. Critical weight is the size at which food is not necessary for a normal course of development and is usually attained midway through the feeding phase of the final instar (Nijhout & Williams, 1974b). In our lab population *M. sexta* attains critical weight at about 5.5g (Davidowitz *et al.*, 2003). Starvation caused wing disk growth arrest on larvae that had not attained critical weight. Wing disk of starved larvae did not show a significant increase in size during 48h ( $P = 0.052$ ). In contrast, wing disk from larvae that had passed critical weight continued growing, despite being food deprived for 48h ( $P < 0.0001$ ) (Fig. 1.8).

Although wing disks from larvae that had passed critical weight continued growing, the number of cells undergoing mitosis decreased significantly compared to that of fed larvae ( $P = 0.021$ ) (Fig. 1.9). The reduced cell proliferation can account for the reduction in growth rate observed in wing disks from starved larvae when compared to wing disks from fed larvae (Fig. 1.7). Larvae that were starved after the critical weight reached the wandering and pupal stages at the same time as fed larvae and produced small but normally-looking adult wings. In contrast, larvae that were starved before

critical weight had a significant extension of the developmental time and never emerged as adults (data not shown).

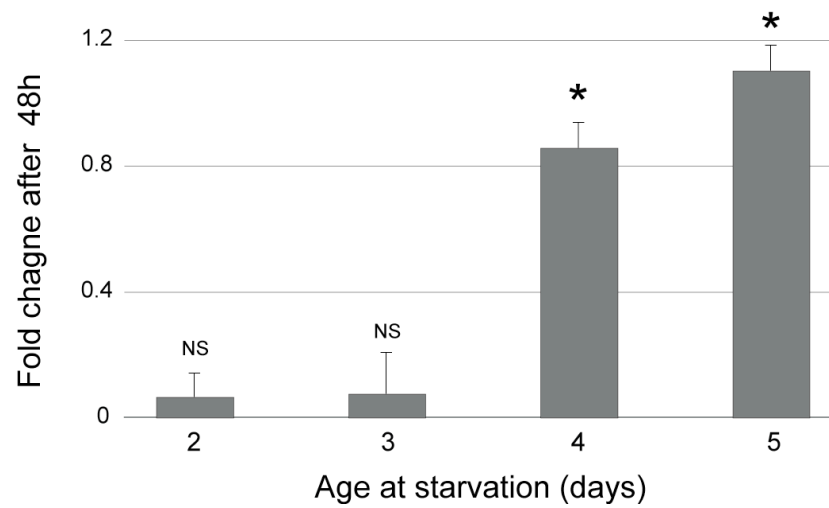


Figure 1.6. Effect of starvation on wing imaginal disk growth. Each bar represents the fold change in forewing disk size after 48h of starvation at different ages in the fifth instar of *Manduca* larvae (N=18). Error bars indicate SE. \* P<0.0001.

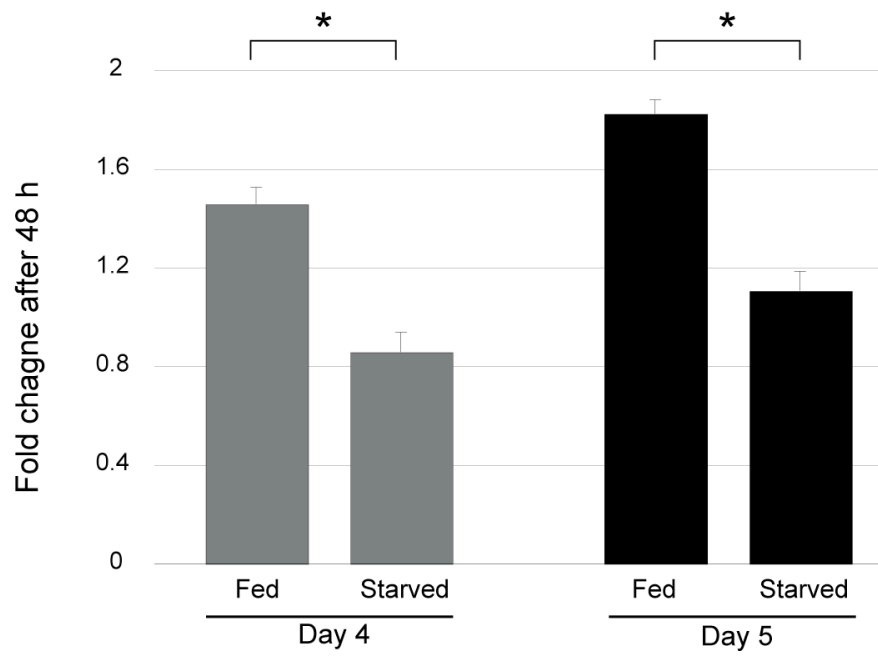


Figure 1.7. Comparison of the size change between fed and starved larvae after 48h for day 4 and 5 of the final instar. Each bar represents the fold change in forewing disk size after 48h (N=18). Error bars indicate SE. \*P<0.0001.

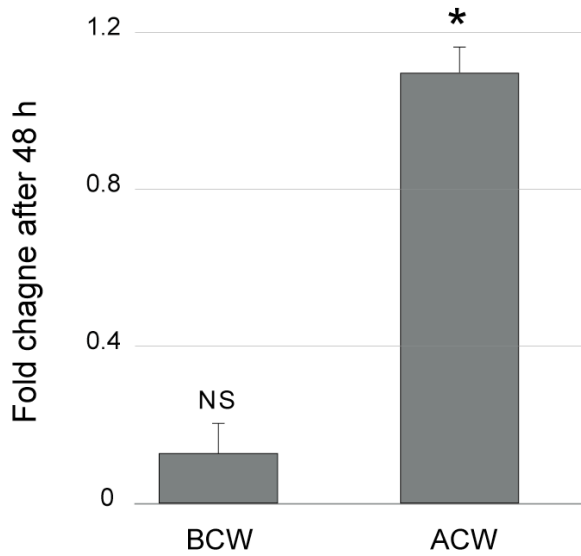


Figure 1.8. Effect of starvation before and after critical weight on wing imaginal disk growth. Fold change in forewing disk size after 48h of starvation for *Manduca* larvae of the same age that have not yet (BCW) or have already attained (ACW) the critical weight of 6g. Each bar represents the mean of 16 disks. Error bars indicate SE. \*P<0.0001.

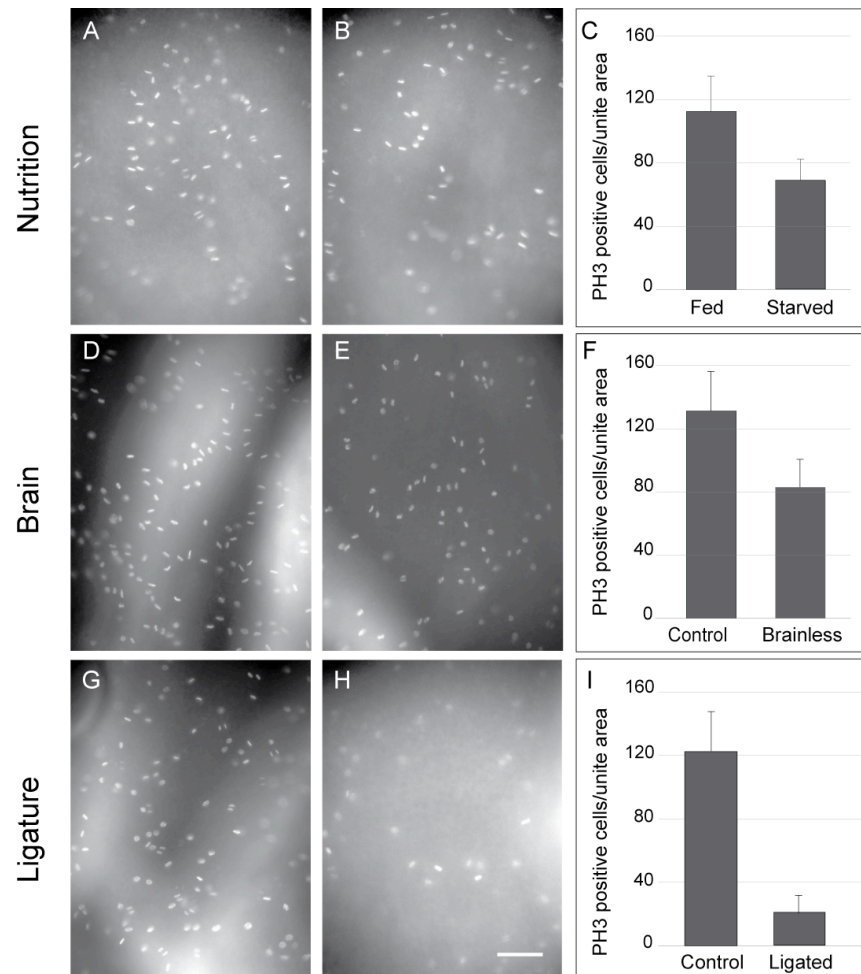


Figure 1.9. Quantification of cells undergoing mitosis in the forewing imaginal disk. Cell proliferation was measured as the average number of PH3 positive cells in an area of  $0.0875 \text{ mm}^2$  of the wing imaginal disk (Bar =  $50 \mu\text{m}$ ). (A)-(C) Effect of nutrition. Larvae were starved on day 4 of the feeding phase and PH3 cells in the wing disk were counted 48 h later. (A) Fed, and (B) food deprived larvae. (C) Differences in cell proliferation between disks from control and food deprived larvae ( $P=0.021$ ). (D)-(E) Effect of brain removal. The brain was removed the first day of the wandering phase (day 6) and PH3 cells were counted 24 h later. (D) Control and (E) brainless larvae. (F) Differences in cell proliferation between disks from control and brainless larvae ( $P<0.001$ ). (G)-(H) Effect of the ligature. A ligature was placed between the thorax and the abdomen on the first day of the wandering phase (day6) larvae and PH3 cells were counted 24 h later. (G) Control and (H) ligated larvae. (I) Differences in cell proliferation between disks from control and ligated larvae ( $P<0.001$ ). Each bar represents the mean of 10 disks. Error bars indicate SE.

### *Influence of factors in the hemolymph on wing disk growth*

I used *in vivo* brain extirpation and *in vivo* body ligations to test the interaction between factors coming from the brain and the fat body on disk growth. Brain removal prevented the wings from responding to any growth factors produced in the brain. Brain extirpation did not cause wing disks to stop growing (Fig. 1.10). However, the disks from brainless larvae showed a slower growth rate and a significant decrease in cell proliferation when compared to disks from control, sham-operated, larvae ( $P < 0.001$ ) (Fig. 1.10). Brainless larvae whose wings were not dissected continued to grow and eventually pupated, however, they never eclosed.

A ligation applied between the third thoracic segment and the abdomen isolated wing disks from signals coming from the abdomen and fat body. Wing disks from larvae with an abdominal ligation stopped growing soon after the ligation was placed. Wing disks dissected before and 24h after ligation were not significantly different in size ( $P = 0.39$ ) (Fig. 1.10). Furthermore, wing disks from ligated larvae showed very little cell proliferation, significantly lower than in un-ligated larvae ( $P < 0.0001$ ) (Fig. 1.9).

Because fore- and hindwings are located on the second and third thoracic segments respectively, a ligation placed between the second and third thoracic segments isolates the forewing from factors coming from the fat body, and simultaneously isolates the hindwing from factors produced in the brain. After 24 hours of the thoracic ligation the ratio between hindwing and forewing mass had significantly increased compared to wing disks from non-ligated larvae ( $P < 0.001$ ) (Fig. 1.11). Hindwing that had access to factors coming from the fat body showed a significant increase in size, while forewings that had access to brain factors, but deprived from fat body factors, showed reduced increase in size compared to hindwings. This result suggests that fat body factors are more effective at promoting growth than factors from the brain.

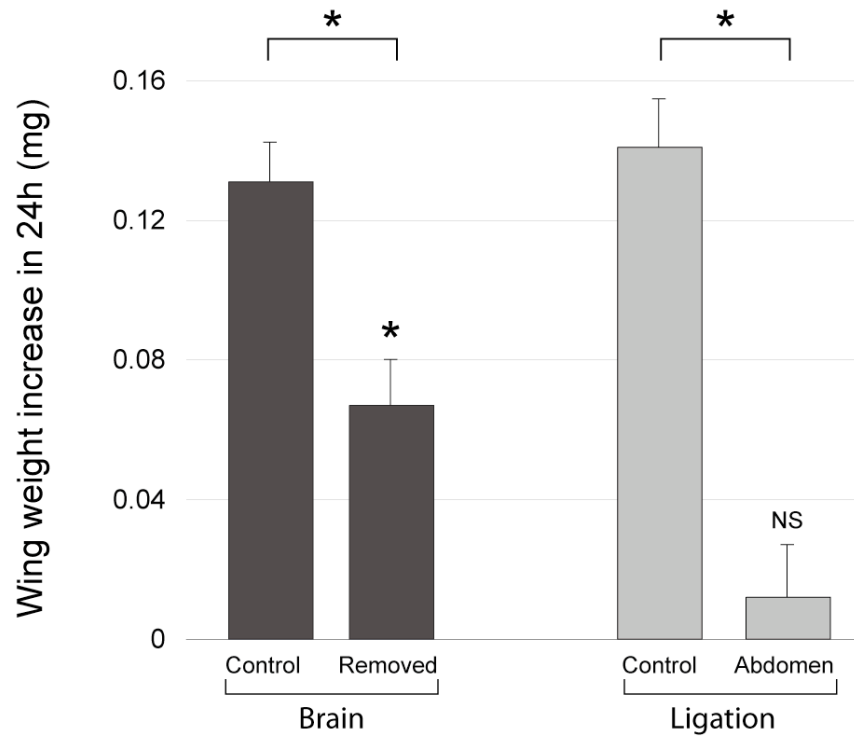


Figure 1.10. Effect of the brain and fat body on wing imaginal disk growth. Dry weight gain after 24h of brain removal or abdominal ligation for the forewing disk of first day wandering phase (day 6) of *Manduca* larvae. Each bar represents the mean in forewing disk size of 18-20 disks. Error bars indicate SE. \*P<0.001.

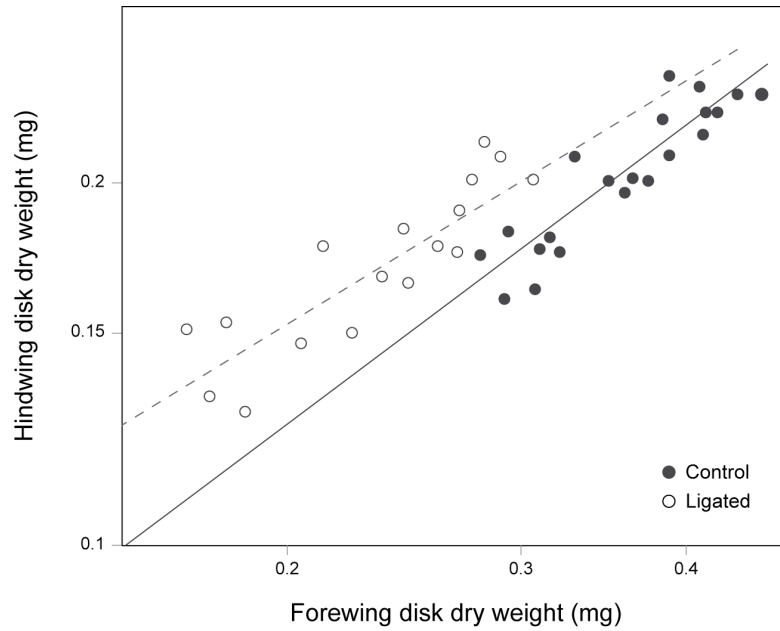


Figure 1.11. Effect of removing brain and fat body factors on fore- and hindwing imaginal disk growth. A ligature was placed between the second and third thoracic segment preventing the forewing from receiving most fat body factors and the hindwing from receiving brain factors. The lines for control and ligated larvae for fore- vs. hindwing disk sizes are statistically different ( $P < 0.001$ ).

## Discussion

During the final (fifth) instar of *Manduca sexta* larval development, changes in the titers of several developmental hormones determine the transition from the larval to the pupal stage (Nijhout, 1994). These events cause the larva to stop feeding, which stops somatic growth, and enter the wandering phase in preparation for pupation. Wing disks, in contrast, grow continuously and almost exponentially during the feeding and wandering phases and only stop growing during the first week of the pupal stage. Because most of the wing disk growth occurs in the closed environment of the wandering phase and pupa, wing disk growth should be influenced only by events that take place inside the larval body. Since no feeding occurs during the majority of the period of wing disk growth, most of the disk growth must rely on the nutrients that were stored during the larval life.

### *Wing disk growth rate*

During the final instar wing disks grow continuously, however, the rate at which they grow is not constant. Changes in growth rate are correlated with developmental changes that take place in the larva. The growth curves for fore- and *hindwing* disk mass and for cell proliferation rate all point to three periods when growth rate is increased. These periods occur between days 3-4 of the feeding phase, between days 5-6 (the transition to the wandering phase), and between days 9-10, just before the larva enters the pupal stage (Fig. 1.5). In *M. sexta* wing imaginal disk growth is observed to be largely due to an increase in cell proliferation, and not necessary in cell size (Fig. 1.3 and 1.4). As wing disk grow, there is a concomitant increase in cell number and a decrease in cell

size. Therefore, the factors that are responsible for the change in growth rate must affect the processes of cell growth and proliferation.

The first point at which a significant increase in growth occurs is between days 3-4. The rise in growth rate during this time point might be in response to the clearance of juvenile hormone from the hemolymph, which occurs at this time (Baker *et al.*, 1987). At the beginning of the final instar there is a precipitous drop in juvenile hormone (JH) levels, leading to a nearly complete removal of JH from the hemolymph a few days into this instar (Nijhout & Williams, 1974a). JH is cleared due to the combined effect of turning off the gland that produces the hormone, the corpora allata, and increased expression of JH-esterase, the enzyme that degrades this hormone. Several studies investigating the control of metamorphosis have shown that JH can act as a growth suppressor (Kremen & Nijhout, 1998; Miner *et al.*, 2000; Truman *et al.*, 2006). Removing a growth suppressor from the hemolymph can stimulate wing disk cell proliferation and, thus, result in an increase in wing disk size. The clearance of JH can, therefore, account for the observed increase in wing disk growth rate that occurs between days 3-4.

The second point when growth rate increases sharply occurs between days 5-6, which is the transition from the feeding to the wandering phase. Just before entering the wandering phase there is a small pulse of ecdysone. This pulse is responsible for the cessation of feeding and the purging of the gut (Truman *et al.*, 1974). Besides its function as the molting hormone, ecdysone has also been shown to stimulate cell proliferation in various tissues (for some examples Champlin & Truman, 1998a; Champlin & Truman, 1998b; Franco *et al.*, 2007; Koyama *et al.*, 2004). Furthermore, in the Greater Wax Moth *Galleria mellonella* injection of ecdysone into larval abdomen was observed to stimulate cell proliferation in wing imaginal disks (Meyer *et al.*, 1980). Therefore, the pulse of ecdysone prior to entering the wandering phase might stimulate cell proliferation in the wing disks, leading to the observed rise in wing disk growth rate.

The third point of growth rate increase is observed one day before the transition to the pupal stage. At this point the cuticle around the wing disks thickens and hardens and thus might account for the increase in mass.

#### *The effect of nutrients on wing disk growth*

During the feeding phase, body and wing disk growth are directly regulated by the amount of food intake. If larvae are food-deprived, body growth will arrest. As soon as food is available, body growth resumes. Starved larvae drop their metabolic rate to a minimum soon after starvation and maintain it at a low level until nutrients are available (ref). Wing disks, in contrast, show a different response to starvation. During the early days of the fifth instar (days 1-3), nutrient withdrawal causes an arrest in wing disk growth. If nutrients are withdrawn later in the instar (days 4-5), wing disk growth continues despite the lack of nutrients. Food withdrawal, however, results in a decrease in growth rate (Fig. 1.7)

Although it appears that age at which the larvae is starved produces the differential response of wing disk growth, the determinant factor is actually weight (Fig. 1.8). The differential response coincides with the attainment of the critical weight. Critical weight represents the point at which the developmental switch to pupation takes place and is marked by the clearance of JH. Therefore, it is possible that the attainment of critical weight also represents a change in the mechanism of growth regulation.

Food deprivation early in the fifth instar causes the JH titers to remain at a high level (Cymborowski *et al.*, 1982; Nijhout & Williams, 1974a), and prevents the clearance of JH from the hemolymph. Recently, Truman *et al.* (Truman *et al.*, 2006) have shown that the increase in JH caused by starvation is responsible for preventing further wing

disk growth. Elevated JH titers early in the instar should, therefore, explain the arrest in wing disk growth observed during starvation. When larvae have attained critical weight, the gland that secretes JH has been “turned off” (Baker *et al.*, 1987; Nijhout & Williams, 1974a). Therefore, it is likely that after the attainment of critical weight starvation can no longer simulate the secretion of JH. The absence of JH in the hemolymph cannot prevent wing disk growth and accounts for the growth observed despite the lack of nutrients. This explains why disks continue growing, but it does not explain the decrease in growth rate and cell proliferation observed in wing disks from starved larvae.

In insects, the response of growing tissues to nutrients is in part mediated by insulin-like growth factors (Britton *et al.*, 2002; Ikeya *et al.*, 2002; Rulifson *et al.*, 2002). In *Drosophila* there is compelling evidence that components of the insulin pathway stimulate cell proliferation in response to nutrients (Bohni *et al.*, 1999; Brogiolo *et al.*, 2001; Weinkove *et al.*, 1999; Zhang *et al.*, 2000). Bombyxin (Bbx), the insulin-like growth factor present in *M. sexta* and other Lepidoptera has been shown to be sensitive to nutritional levels as well (Masumura *et al.*, 2000; Nijhout & Grunert, 2002; Satake *et al.*, 1997)(and see Chapter 2). Food deprivation in *M. sexta* can lead to a drop in Bbx levels which in turn result in the observed decrease in cell proliferation. Lower levels of Bbx could thus account for the decrease in growth rate observed in wing disks from starved larvae.

#### *Factors from the brain and the fat body*

Organ growth is primarily determined by the interaction of different endocrine factors that regulate cell proliferation and growth. In insects, the brain and the fat body

are two major organs that serve as the source for growth promoting signals. Therefore, any alteration in one of these organs should have an effect on wing disk growth.

In *M. sexta*, as in other insects, the brain is the source of many hormones such as bombyxin and the prothoracicotropic hormone (the hormone that stimulates the synthesis of ecdysone) that are necessary for normal development and that exert an effect on growth (Nijhout 1994). Therefore, in larvae without a brain we expected to see an arrest in wing disk growth. In contrast to our expectation, wing disks from brainless larvae continued growing, suggesting that the brain is not necessary for wing disk growth. However, wing disk from brainless larvae did not grow at a normal rate and showed reduced cell proliferation when compared to disk from fed animals. This suggests that the brain exerts an effect on wing disk growth but does not provide the factors that are primarily controlling growth. Brainless larvae pupated with normal-looking and well differentiated wing disks. Although this is surprising, it has been previously shown in the gypsy moth *Limantria dispar* that brain extirpation does not prevent the animal from further growth and pupal development (Kopec, 1922). Taken together, these results suggest that the factors that exert the primary function in growth regulation are originated in an organ different than the brain.

In insects, the fat body is mostly located in the abdominal region surrounding the gut and serves in the storage and metabolism of nutrients. Besides this function, it has also been shown to serve as an endocrine organ (Nijhout, 1994). Isolating the wing primordia from factors coming from the fat body prevented the wing disks from growing. The small amount of growth we observed after placing the abdominal ligature is likely to be stimulated by the small amount of fat body tissue remaining on the anterior side of the ligature, or remnants factors circulating in the hemolymph. The growth arrest observed in abdomen ligated larvae suggest that during the final instar, factors from the fat body are necessary for wing disk growth. The thoracic ligature

allowed the forewings to be in contact with factors from the brain and not the fat body and allowed that hindwings to be in the opposite situation (Fig. 1.11). Forewings that were in contact with the brain grew smaller relative to hindwings that were in contact with the fat body. Thus, these results further corroborate that the fat body is producing a factor that is necessary for wing disks to grow.

*In vitro* cultures of wing imaginal disks have shown that fat body extracts are necessary for growth and development. In *Drosophila* as well as in lepidopterans, wing disk growth proceeded normally only when fat body extracts were added to the culture media (Davis & Shearn, 1977; Smagghe *et al.*, 2001). Furthermore, experiments performed with the Indianmeal moth, *Plodia interpunctella*, suggested that that a factor from the fat body was necessary in order for wing disks to grow (Dutkowski & Oberlander, 1974). Furthermore, in the past years three independent groups working with *Drosophila* (Britton & Edgar, 1998; Colombani *et al.*, 2003; Zhang *et al.*, 2000) have demonstrated the importance of the fat body in the coordination of nutrition and growth, suggesting a primary of the fat body in the control of growth.

In this study we showed how developmental events such as the attainment of critical weight and entering into the wandering phase, have an impact on the growth trajectory of the wing disk and may account for the changes in wing disk growth rate. Furthermore, we have shown that the critical weight not only marks a developmental transition in relation to metamorphosis but also a transition in the mechanism that coordinates nutrition and organ growth. Finally, we show that during the wandering phase, the fat body, but not the brain is necessary for wing disk growth. It is important to understand the physiological events during development to dissect how organ growth is regulated.

## Chapter II

### **Bombyxin and ecdysone in the regulation of wing disk growth**

Portions of this chapter were previously published as:

Nijhout, H.F., W.A. Smith, I. Schachar, S. Subramanian, **A. Tobler** and L.W. Grunert. 2007. The control of growth and differentiation of the wing imaginal disks of *Manduca sexta*. *Developmental Biology*, 302 (2): 569-576.

## Introduction

From insects to mammals, growth regulation involves very similar genes and pathways. In flies and humans, determinants of growth such as cell size, metabolism, and final body size are mediated by the insulin-signaling pathway (reviewed in Nijhout, 2003b). Similarly, steroid hormone-nuclear receptors that control life stage transitions are regulators of growth and are also conserved across these two groups (reviewed in Edgar, 2006). Although it is clear that insulin and steroid hormones are both involved in the regulation of growth in humans and flies, the way in which these two regulators interact is yet to be determined. Insofar as humans and flies are distantly related, it is assumed that these mechanisms are phylogenetically widespread, but this remains to be established.

In insects, as in other organisms, nutritional input is necessary for normal growth; however, nutrition does not act directly on tissue and organ growth but exerts its effect via hormonal signals that circulate in the insect hemolymph. In the past years, insulin-like peptides have been elucidated as the primary mediators between nutrition and growth (Bohni *et al.*, 1999; Britton *et al.*, 2002; Ikeya *et al.*, 2002). Nutrition, via circulating sugar levels, promotes the release of insulin from specialized cells from the brain into the hemolymph, and then acts on peripheral tissues to stimulate protein synthesis and cellular growth (Ikeya *et al.*, 2002; Masumura *et al.*, 2000).

Insulin-like peptides have been identified in a variety of insects (for some examples Dai *et al.*, 1994; Iwami *et al.*, 1996; Kimura-Kawakami *et al.*, 1992; Nagasawa *et al.*, 1986), but their function has been studied primarily in *Drosophila melanogaster* (reviewed in Garofalo, 2002). In *Drosophila*, ablation of the insulin producing cells in the brain leads to a reduction in animal growth rate and eventually to small adults (Rulifson *et al.*, 2002). Over-expression of the insulin-like peptides by the insulin producing cells

during larval development results in bigger adult flies (Brogiolo *et al.*, 2001). In contrast, mutations in members of the insulin signaling pathway such as the insulin receptor or the insulin receptor substrate results in growth deficiency with a reduction in organ and body size (Bohni *et al.*, 1999).

The insulin-like peptides identified in Lepidoptera are called bombyxins (Bbx). Bbx were first identified in the silkworm *Bombyx mori* (Nagasawa *et al.*, 1986) and since then have been identified in other lepidoptera, including the tobacco hornworm *Manduca sexta* (Dai *et al.*, 1994; Nijhout & Grunert, 2002). *Manduca* Bbx has been shown to function as a growth factor for wing imaginal disks (Nijhout & Grunert, 2002; Nijhout *et al.*, 2007). Interestingly, in wing imaginal disks Bbx by itself is not able to stimulate growth, but also requires the action of ecdysone. It appears that these two hormones work synergistically to promote growth in the wing imaginal disks.

Ecdysone, the insect molting hormone, is a steroid hormone that controls the timing of molting and metamorphosis. Ecdysone is produced by the prothoracic glands as an inactive pro-hormone that is converted into its active form 20-hydroxyecdysone (20E) in the fat body and epidermis. Studies have shown that 20E can promote cell proliferation in various insect tissues such as the epidermis (Truman *et al.*, 1974), muscle cells (Champlin *et al.*, 1999; Luedeman & Levine, 1996), and neural precursor cells in the eye primordia (Champlin & Truman, 1998a; Champlin & Truman, 1998b), and wing imaginal disks (Koyama *et al.*, 2004; Nijhout & Grunert, 2002). However, 20E also appears to negatively regulate growth (Caldwell *et al.*, 2005; Colombani *et al.*, 2005; Mirth *et al.*, 2005). Increasing the levels of ecdysone can cause a decrease in larval growth rate, and, conversely, inhibiting ecdysone signaling induces a general growth increase. Furthermore, 20E can also oppose the action of insulin signaling and inhibit growth (Mirth *et al.*, 2005). These contradictory effects of ecdysone and insulin “highlight” the

complexity of hormone stimulation and interaction. It is evident that hormones function can be tissue and time specific.

In the present study I examine the individual effects of Bbx and 20E and their interaction on the regulation of wing imaginal disk growth in the final instar of the tobacco hornworm *Manduca sexta*. Using an *in vitro* culture system I show how ecdysone, but not Bbx is relevant in stimulating wing disks growth during the wandering phase. Furthermore, I show that early in the final larval instar (before the attainment of critical weight) hemolymph carbohydrate concentrations and insulin secretion are sensitive to nutrition, but this sensitivity is lost when the larva attains critical weight and is committed to pupation.

## **Materials and Methods**

### *Experimental animals*

*Manduca sexta* larvae were reared as described in chapter 1.

### *Hemolymph glucose and trehalose concentration*

Glucose and trehalose (a disaccharide of glucose) are the two major forms of carbohydrates circulating the *Manduca* hemolymph. Hemolymph was collected in micro-centrifuge tubes by making an incision in the proleg of a CO<sub>2</sub> anesthetized larva and gently pressing the abdomen to expel several drops of hemolymph. The concentration of sugars was determined by using the FreeStyle<sup>®</sup> Blood Glucose Monitoring System (Abbott). The limit of detectability for glucose is 0.2mg/ml, so any concentration below was recorded as 0. Trehalose concentration was determined by incubating 100µl of hemolymph with 0.01 units of trehalase (Sigma) and measuring the amount of glucose

liberated. To obtain the actual trehalose concentration the initial amount of glucose was subtracted from the final amount and divided by two. Glucose and trehalose titers of starved larvae were determined at 6-12h intervals for the next 48h. To prevent starved larvae from desiccating cups were sprayed with water twice a day.

#### *RNA extraction*

Total RNA was extracted using the RNeasy Mini Kit (Qiagen). Larvae were anesthetized for 5min in CO<sub>2</sub> and tissues were dissected in cold sterile insect saline. Samples from larvae at different developmental times during the final instar were obtained. For a given determination the RNA was extracted from 6 brains or 3 wing disks. RNA concentration was measured using a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific) at 260nm.

#### *Expression of Bbx and InR*

mRNA levels of Bombyxin (Bbx) levels in brain tissue and InR levels in the wing disks were determined using real-time quantitative PCR (qPCR). First stranded cDNA was generated using oligo-dT priming with the Superscript® II kit (Invitrogen). Q-PCR was performed using an iCycler (Bio-Rad) with iQ™ SYBR® Green Supermix (Bio-Rad). Q-PCR reactions were performed according to manufactures instructions. Primers were designed based on cDNA sequences for *Manduca sexta* retrieved from genbank: *Bbx* (DQ080209), *InR* (FJ169464) and *actin* (L13764). Primers are: Bbx-Fw, AGTGCGCAGTGGTGTGTGT and Bbx-Rv, ATAGTTCGTCCAGCGTGCAG; InR-Fw, GGGATTTCGGCATGACCAGAGATATT and InR-Rv, TCGTTCGACAGGCCCTGATATGG; Act-Fw, AAGGACCTGTACGCCAACAC and

Act-Rv, ACATCTGCTGGAAGGTGGAC. Relative expression levels of *Bbx* and *InR* were calculated based on the  $\Delta\Delta C_t$  method (Livak & Schmittgen, 2001) and normalized with actin levels. Each sample was run in triplicate and 3-5 biological samples were run for each of the experimental conditions.

#### *In vitro culture of Manduca wing disks*

Larvae were surface sterilized by immersion for 2 min in 95% ethanol and dissected in cold sterile insect saline in a laminar flow chamber. Disks were rinsed in Grace's insect tissue culture medium (Gibco, Invitrogen) that contained 1mg phenylthiourea/ml to inhibit tyrosinase activity. Disks were cultured in 24-well plates (Costar) in 300 $\mu$ l Grace's medium supplemented with 10% fetal calf serum (Atlanta Biologicals) and 10% antibiotic-antimycotic (Gibco, Invitrogen) at 26°C in a 95% O<sub>2</sub>, 5% CO<sub>2</sub> atmosphere. Wing disks were supplemented with different levels of 20-hydroxyecdysone (20E) (Sigma, St Louis, MO) and heat-inactivated hemolymph from fifth instar larvae as the source of bombyxin (Nijhout & Grunert, 2002). A stock solution of 20E was prepared in ethanol and the concentration was measured spectrophotometrically at 240nm ( $E_{240}=12,670$ ). Hemolymph from *Manduca* mid 5<sup>th</sup> instar larva was collected in a cold micro-centrifuge tube containing 10 $\mu$ l of phenylthiourea, immediately immersed in boiling water for 5min and chilled on ice. Coagulated proteins were precipitated by centrifugation for 10min at 4°C. The supernatant was added to the culture medium at different concentrations.

Pairs of wing disks from the same individuals were cultured, using one wing as an untreated control and the other wing as the experimental. Cultured wing disks were rinsed in water and its size was measured as the dry weight as described in chapter 1.

Size change due to the treatment was measured as the difference between control and experimental wing disks from the same individual.

### *Statistical analyses*

Expression data for *Bbx* and *InR* were analyzed using a nested two-tailed t-test. *In vitro* growth culture analysis was performed analysis of variance (ANOVA). If ANOVA detected significant effects of age, I used Tukey's HSD ( $\alpha=0.05$ ) to conduct pair-wise comparisons of the effects of individual treatments to test if they were different. All statistical analyses were conducted using JMP® 7.0.2 (SAS Institute Inc., Cary, NC).

## **Results**

### *Hemolymph sugar concentration in the final instar of Manduca larvae*

In most insects, the primary blood sugar is trehalose, with glucose typically present as a minor component. *Manduca* blood has both trehalose and glucose in significant concentrations. In normally feeding larvae, both glucose and trehalose concentration varied greatly with age in the final instar. Glucose levels start highest and can reach concentrations above 5mM. As the instar progresses glucose levels gradually drop and reach undetectable levels the day when the larva enters the wandering phase. From this day on, glucose concentration remains at undetectable levels. In contrast, trehalose levels start low, about 8mM, and increase as the instar progresses. The concentration of trehalose levels off at the time when larvae enter the wandering phase and are maintained around 14mM through the rest of the instar (Fig. 2.1).

### *Effect of Starvation on hemolymph sugar concentration*

Larvae were starved either before or after passing their critical weight and their glucose and trehalose concentration were measured. Nutrient deprivation has different effects on hemolymph glucose and trehalose concentrations, both in the direction and magnitude of change, and in the period or sensitivity to the nutritional signals.

Glucose levels drops precipitously soon after the animal has been deprived of food. Eight hours after food deprivation glucose concentration falls to about 0.5mM and 24h later glucose in the hemolymph is almost undetectable. This response is consistent regardless of when during the final instar food is removed (Fig. 2.2A). In contrast, the response of trehalose to starvation differs dramatically depending on whether the larvae has attained critical weight or not. In larvae starved before the attainment of critical weight, trehalose levels steadily drop during the first 24 hours of starvation and reached a minimum of 4.5mM after 36hrs. However, when larvae were starved after reaching critical weight, trehalose levels showed a small peak 12h after the beginning of starvation and thereafter trehalose concentration was maintained almost constant at around 12mM (Fig. 2.2B).

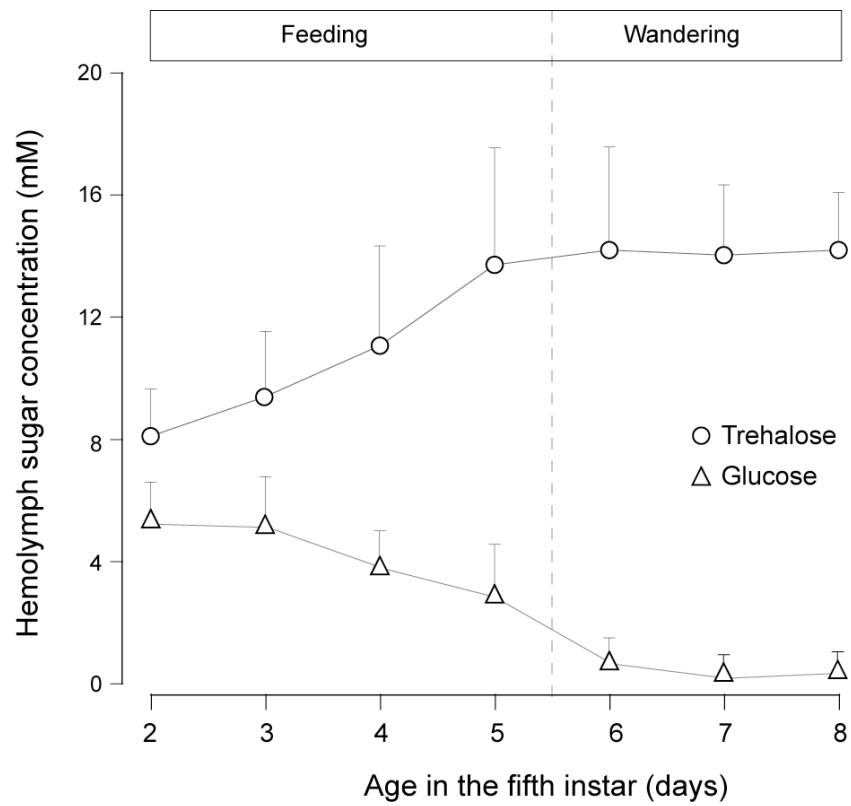


Figure 2.1. Hemolymph glucose and trehalose concentration for fed fifth instar *M. sexta* larvae. Values are means of 10-16 independent samples  $\pm$ SD.

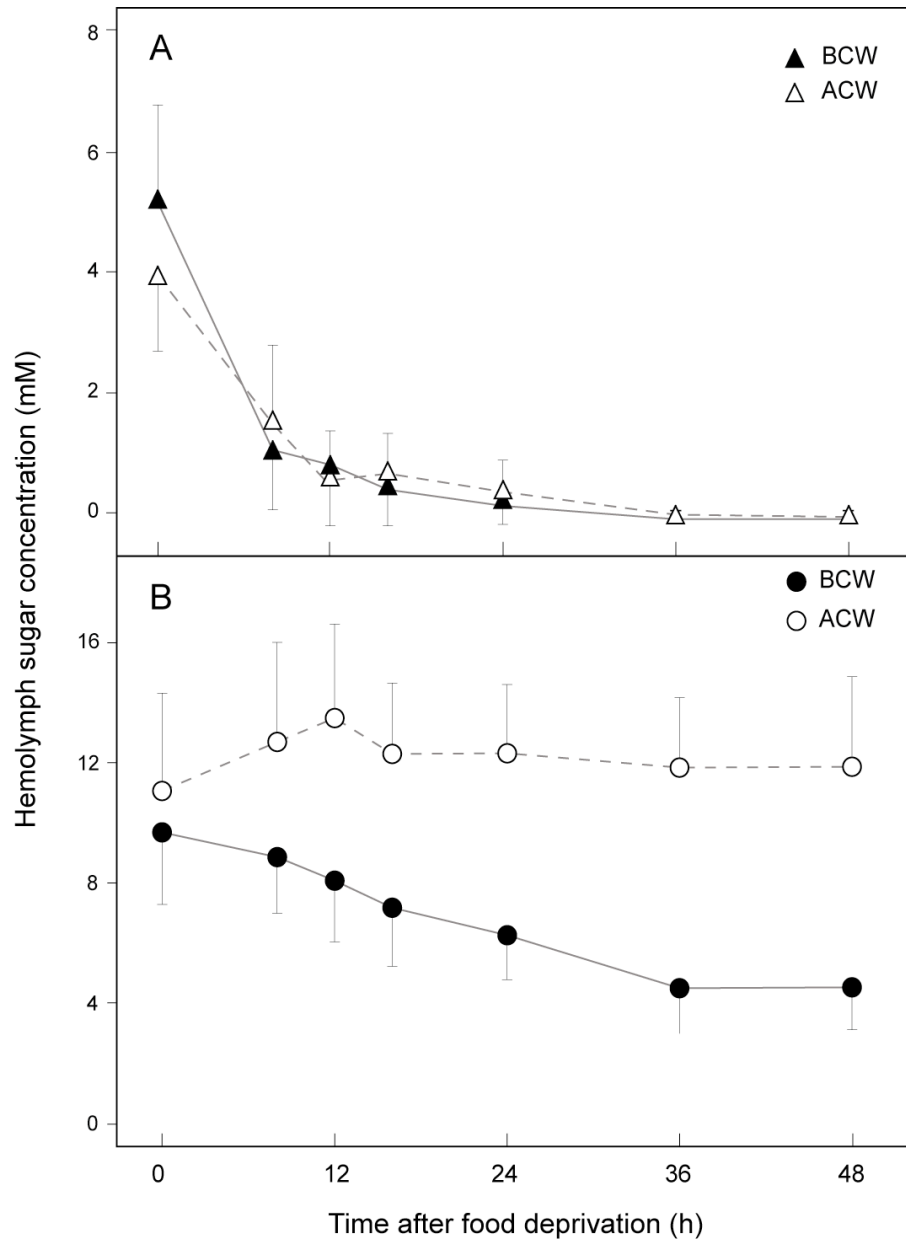


Figure 2.2. Effect of food deprivation on hemolymph glucose and trehalose concentration depending on larval age at the initiation of starvation. (A) Change in hemolymph glucose concentration in starved before (BCW) and after critical weight (ACW). (B) Change in hemolymph trehalose concentration before and after critical weight. Values are means of 8-16 independent samples  $\pm$ SD.

### *Quantification of bombyxin and its receptor in the last larval instar*

To determine the activity of the insulin-signaling pathway we analyzed the expression of the insulin-like peptide *bombyxin* (*Bbx*) in the brain and the *insulin-receptor* (*InR*) in the wing imaginal disks during the final instar of *Manduca sexta* larvae. *Bbx* and *InR* expression levels showed a similar pattern with steadily increasing levels as the larva progresses in development (Fig. 2.3). The results indicate an increase in activity as the larvae is growing and the concomitant levels of *Bbx* and *InR* suggest that this hormone and its receptor are working in concert.

In *Drosophila* it has been shown that insulin signaling is regulated by nutrient availability (Britton *et al.*, 2002; Ikeya *et al.*, 2002). To determine if this is the case in *M. sexta*, I investigated the effect of food withdrawal on the transcript levels of *Bbx* and *InR*. Larvae were deprived of food at two different time points of the feeding phase and *Bbx* and *InR* mRNA levels were measured 24h later. The effect of food deprivation on *Bbx* and *InR* differed depending on the time of larval development (Fig. 2.4A). Early in the instar (day 2 of the feeding phase) *Bbx* and *InR* mRNA levels fell dramatically when larvae were removed from food and are significantly different when compared to the expression of fed larvae (two-tailed t-test, *Bbx*:  $P=0.0022$ , *InR*:  $P=0.0083$ ). However, when larvae were starved later in the instar (day 4) starvation did not have a significant effect on either *Bbx* or *InR* mRNA levels (two-tailed t-test,  $P>0.05$ ,) (Fig. 2.4B). The simultaneous effect of food deprivation on *Bbx* and *InR* suggest that ligand and receptor vary in a coordinated way in response to nutrition.

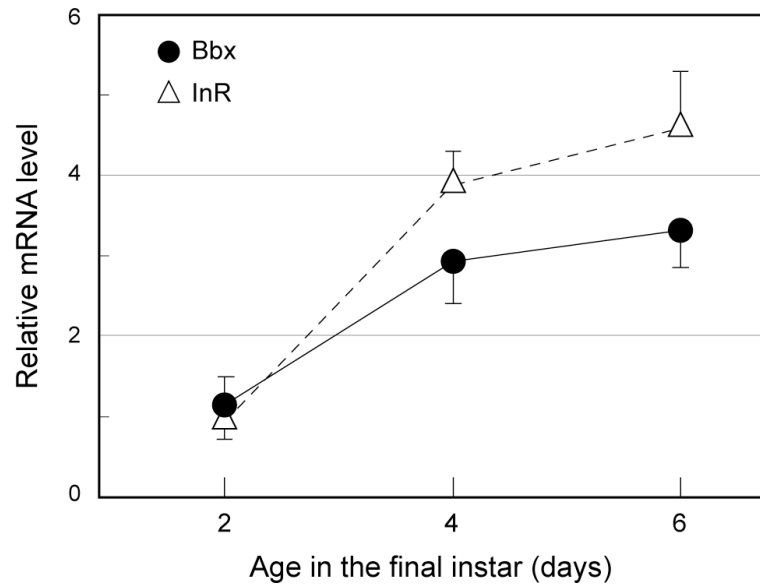


Figure 2.3. *Bbx* and *InR* mRNA levels during the last instar of *M. sexta* larvae. Each point represents the mean of 3-5 biological replicates measured in triplicate. Error bars represent SD.

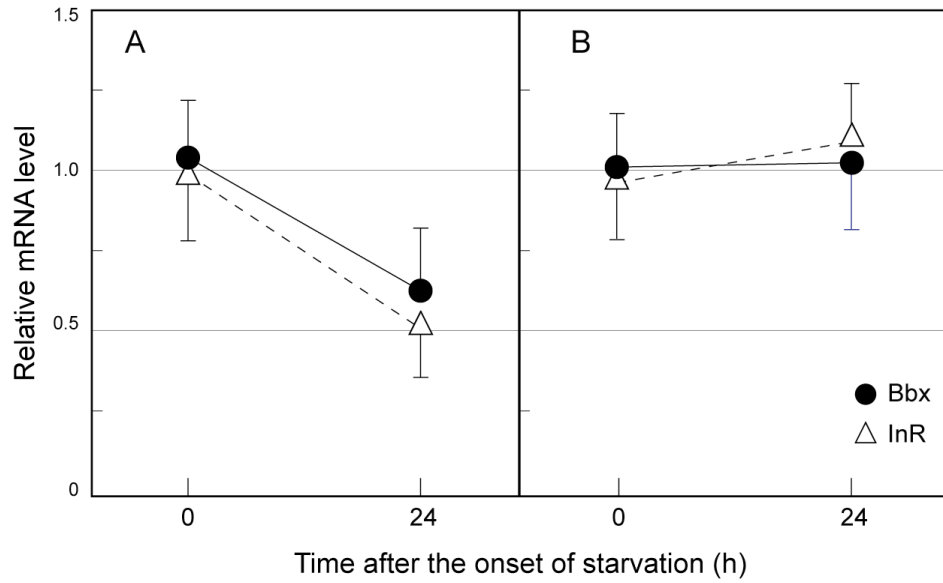


Figure 2.4. The effect of food deprivation on *Bbx* and *InR* mRNA levels. Larvae were deprived from food at (A) day 2 and (B) day 4 of the feeding phase. Expression levels were measured 24h after starvation. Each point represents the mean of 3-5 biological replicates measured in triplicate. Error bars represent SD.

### *Effect of 20E and Bbx on in vitro wing disk growth*

To determine the individual contributions and interaction of 20E and Bbx on wing disk growth I used an *in vitro* tissue culture system that allowed to add specific hormone cocktails and measure their effect on wing disk growth. Wing disks were removed from larvae on the first day of the wandering phase (day 6) and cultured for 48h. The results indicate that 20E alone can significantly stimulate wing disk growth, whereas, Bbx has a minor effect on wing disk growth (Fig. 2.5 and 2.6). The combined effect of 20E and Bbx had no significant increase in wing disk growth compared to the effect of 20E alone (one-tailed t-test,  $P > 0.05$ ).

20E stimulated wing disc growth during both the feeding and the wandering phase. However, the wing disk response to 20E differs markedly between the two phases (Fig. 2.7). In disks from feeding larvae, concentration of 20E between 0.01 - 1  $\mu\text{g}/\text{ml}$  resulted in a concentration dependent growth, and growth plateau at concentration above 1  $\mu\text{g}/\text{ml}$ . In contrast, disks from wandering larvae respond to 20E in a threshold fashion. Concentrations below  $\sim 0.1 \mu\text{g}/\text{ml}$  have very little effect and concentrations above this threshold show a dramatic stimulation in size compared to untreated disks. In contrast to previous studies (Dinan *et al.*, 1990; Koyama *et al.*, 2004; Mottier *et al.*, 2004), there was no inhibitory effect of high concentrations of 20E on disk growth neither from feeding nor from wandering larvae. The differences in the shape of the curve between feeding and wandering wing discs suggest that the mechanisms that control growth are different at these two stages.

To determine the interplay between ecdysone and the insulin-like pathway, we assayed the expression of *InR* on 20E stimulated cultured wing disks. Wing disks stimulated with 20E showed a significant increase in *InR* expression when compared to untreated disks ( $P = 0.021$ ) (Fig. 2.8). It is not clear whether the increase in *InR* levels is

due to the direct action of 20E. It is possible that observed increase in *InR* expression is an indirect effect of the increase in wing disk growth due to the cell proliferation activity of 20E.

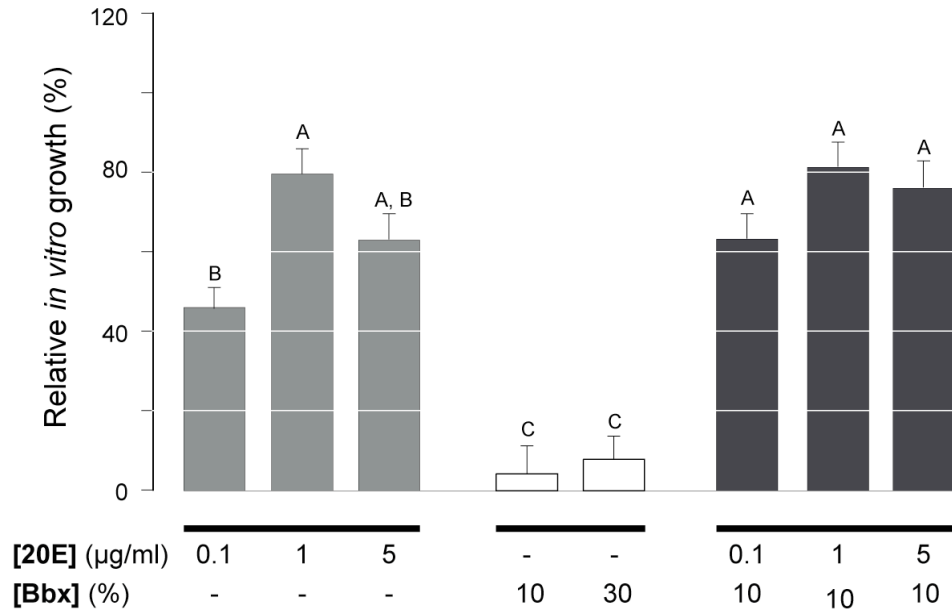


Figure 2.5. Effect of Bbx and 20E on *in vitro* wing disk growth. Wing disks from wandering larvae were stimulated with 20E, Bbx (heat treated hemolymph) or 20E and Bbx together. Relative growth was measured as the difference in growth between the treated and un-treated disks after 48h of culture. Each bar represents the average of 8-20 disks. Means connected by the same letter were not found to be statistically significant at  $p < 0.05$  by ANOVA and a post-hoc comparison of the means, with Tukey HSD ( $\alpha = 0.05$ ). Error bars represent SE.

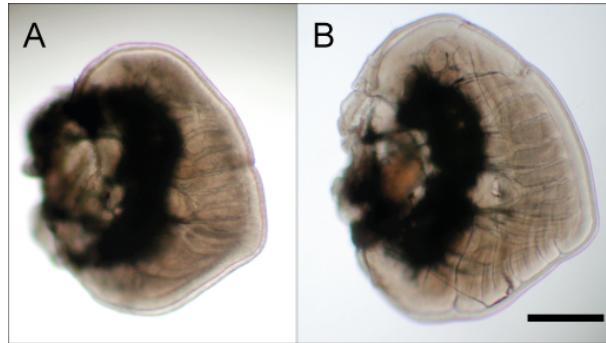


Figure 2.6. Effect of 20E on wing disk growth from wandering larvae. *In vitro* wing disk growth after 24 h of (A) untreated (B) supplemented with 20E 0.1 $\mu$ g/ml. Bar= 0.5mm.

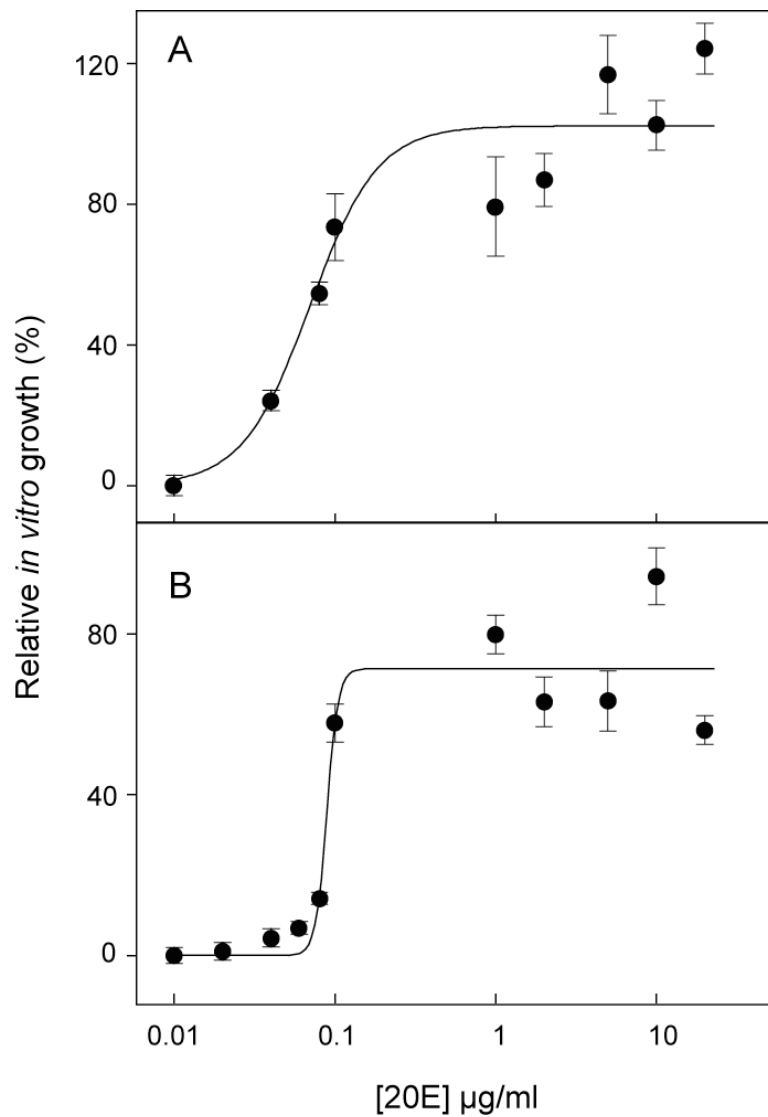


Figure 2.7. Dose response to 20E of wing imaginal disks. Disks take from (A) day 4 feeding and (B) day 1 wandering phase. Disks from the feeding phase were incubated for 72h, and disks from the wandering phase were incubated for 48h. The curves are fit by nonlinear regression to the best fitting sigmoid of the form  $y = a * x^b / (c^b + x^b)$ . For the feeding phase response:  $Growth(\%) = 102.3 * [20E]^{2.1} / (0.07^{2.1} + [20E]^{2.1})$ ,  $r^2 = 0.89$ ,  $P = 0.012$ . For the wandering phase response:  $Growth(\%) = 71.4 * [20E]^{12.3} / (0.09^{12.3} + [20E]^{12.3})$ ,  $r^2 = 0.92$  ( $P < 0.0001$ ). Each point represents the average of 8-20 samples. Error bars represent SE.

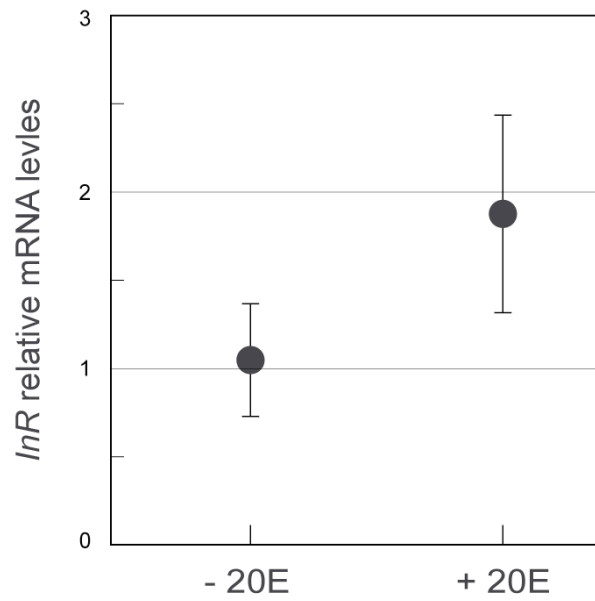


Figure 2.8. Effect of 20E on *InR* mRNA transcript levels in wing imaginal disks. Each point represents the mean of 3 biological replicates measured in triplicate. Error bars represent SD.

## Discussion

In *Drosophila*, the expression of insulin-like peptides are regulated by nutrient availability, and the down-regulation of this peptides causes a reduction in the animals growth rate (Britton *et al.*, 2002; Ikeya *et al.*, 2002), indicating that insulin-like peptides are involved in the coordination of nutrition with somatic growth. The results from the present study suggest that *Bbx* has retained the same function in *M. sexta* larvae; however, this function seems to be restricted, at least, to the beginning of the final larval instar. Later in the instar, in contrast, *Bbx* does not correlate with nutritional status and, therefore, does not seem to coordinate nutrition with growth. At some point during the final instar there is a switch that eliminates *Bbx* as the primary regulator of growth. This point seems to be associated with larva achieving critical weight – the irreversible commitment to pupation that occurs mid through the feeding phase of the final larval instar.

During the first part of the feeding phase of the final instar we found a strong correlation between larval food intake, hemolymph glucose concentration and *Bbx* transcript levels. Food withdrawal caused a sharp decline in hemolymph glucose concentration that was associated with a decrease in *Bbx* transcript levels. Starved larvae showed a significantly reduced *Bbx* transcript levels when compared with fed larvae (Fig. 2.4A). These results suggest that food intake causes a rise in hemolymph glucose levels and this in turn stimulates the synthesis of *Bbx*. Thus, *Bbx* in *Manduca*, at the beginning of the final instar, can serve as the signal for “fed” state, in agreement with a conserved function of the insulin-like peptides in other insects (Britton *et al.*, 2002; Masumura *et al.*, 2000).

During the second part of the feeding phase, however, glucose does not seem to regulate *Bbx* transcript levels. Food withdrawal causes a drop in glucose levels but has no effect on *Bbx* mRNA levels. The differential response to food withdrawal is correlated with the attainment of critical weight. Critical weight is the size at which larvae become irreversibly committed to pupation, and from that point on food is not necessary for a normal course of development (Nijhout *et al.*, 2006; Nijhout & Williams, 1974b). After the attainment of critical weight *Bbx* transcript levels are maintained high independent of glucose levels or food intake. Furthermore, when the larva enters the wandering phase *Bbx* levels are still high although glucose concentration is undetectable and the larva has ceased feeding. These findings suggest that *Bbx* transcript levels become independent of glucose and is no longer the signal for “fed” state after a larva passes the critical weight. Alternatively, *Bbx* may be regulated by other molecular signals more closely tied to the long-term nutritional status of the larva. One possibility for such a molecule is the disaccharide, trehalose.

Trehalose, a disaccharide of glucose, is the main carbohydrate circulating in the hemolymph of insects and serves as an energy storage molecule. In contrast to glucose, trehalose levels did not respond to food intake but seem to respond to the general storage availability. In *Manduca* food deprivation stimulates the trehalosemic hormone that is responsible for mobilizing carbohydrate storage from the fat body into the hemolymph (Meyer-Fernandes *et al.*, 2001; Satake *et al.*, 2000; Siegert & Ziegler, 1983). This explains why during food withdrawal hemolymph trehalose concentration is maintained high, because glycogen stored in the fat body is converted into trehalose and secreted into the hemolymph. Before the attainment of the critical weight trehalose levels drop in response to food withdrawal suggesting that starvation either fails to trigger the signal for carbohydrate mobilization or that the fat body is not competent to respond to the signal. It is possible that the attainment of the critical weight also

represents the point at which the larvae has accumulated sufficient energy to fulfill the energy demands required for the normal development to metamorphosis.

After the attainment of the critical weight *Bbx* transcript levels are correlated with hemolymph trehalose levels. Therefore I propose that a mechanism similar to the one that regulates trehalose levels also regulates the expression of *Bbx*. This signal must be generated in the fat body and travel to the brain to stimulate the medial neurosecretory cells to synthesize *Bbx*. In *Drosophila* suppression of metabolism in the fat body leads to the suppression of insulin-signaling activity (Colombani *et al.*, 2003). Taken together this supports my view that the fat body must produce a factor that stimulates *Bbx* activity.

To further explore the role of *Bbx* in stimulating wing disk growth I used an *in vitro* tissues culture. *In vivo*, *Bbx* and *InR* mRNA levels are correlated with wing disk growth. *Bbx* signaling decreased during growth arrest, and levels are maintained high when disks are growing. Surprisingly, the *in vitro* culture results showed that *Bbx* has a very small effect in promoting wing disk growth. This was unexpected since there is compelling evidence for insulin-like peptides as a growth factor (Bohni *et al.*, 1999; Britton *et al.*, 2002; Ikeya *et al.*, 2002; Rulifson *et al.*, 2002). *Manduca* *Bbx* stimulated *in vitro* wing disk growth of the butterfly *Precis coenia* (Nijhout & Grunert, 2002), however, the growth increase was not as dramatic unless 20E was supplemented to the culture media. In our cultures 20E was sufficient to stimulate wing disk growth and the presence of both hormones had no significant effect when compared to disks cultured with 20E alone (Fig. 2.5). Thus, the results indicate that in *Manduca* *Bbx* is not the primary factor that controls wing disk growth as has been shown in *Drosophila*.

The action of 20E on growth has been controversial. 20E has been shown as a growth promoting factor in several tissues such as eye primordia (Champlin & Truman, 1998b), muscle tissue (Champlin *et al.*, 1999) (Luedeman & Levine, 1996), and also wing

imaginal disks (Meyer *et al.*, 1980) (Nijhout & Grunert, 2002) and in this study); however, the optimal concentration at which 20E promotes growth is highly variable among these tissues. Here, I further show how the growth response to 20E in the same tissue can vary depending of the stage of development. We used wing disks from two developmental stages, before and after entering the wandering phase, and the growth response was different. In both groups low concentrations of 20E failed to stimulate wing disk growth and concentrations above 0.1 $\mu$ g/ml stimulated maximum growth. However, the response to varying levels of 20E of wing disks from the feeding phase was concentration dependent, while disks from the wandering phase responded in a threshold fashion. This shows that the same hormone can have differential effects depending on the time of development of the target tissue, and suggest that two different mechanisms are operating in the response to 20E before and after the larvae enters into the wandering phase.

I investigated the effect of 20E in the expression of *InR* in cultured wing disks. Wings stimulated with 20E showed an increased transcript levels of *InR*, suggesting an interaction between these two hormones. Similarly, Saegusa *et al.* (1992) showed that during the wandering phase an increase of Bbx secretion coincided with a big surge of 20E. However, Saegusa *et al.* (1992) suggested that Bbx might be stimulating 20E secretion. Recently Colombani *et al.* (2005) and Mirth *et al.* (2005) identify a functional interaction between insulin-like peptides and 20E. Both groups demonstrate that 20E opposes the action of insulin and inhibits growth. However, it is possible that this interaction is restricted to the tissue they were investigating, the prothoracic gland, and does not apply to all tissues in the larva. I propose, that in wing imaginal disks the growth promoting effect elicited by 20E is responsible for the up-regulation of *InR* and not by 20E per se.

In this study I have shown how two hormones Bbx and 20E can contribute to the regulation of wing disk growth. Furthermore, we have shown how the action of both hormones is context dependent. In the early days of the final instar Bbx acts as a primary factor controlling growth and also carbohydrate levels in the hemolymph, while later in the instar it acts as a secondary stimulator of growth and its involvement in carbohydrate levels is unclear. Also in *Drosophila* the requirements for insulin signaling seem to vary depending of the stage of the larval development (Shingleton *et al.*, 2005); Shingleton *et al.* (2005) associated these changes with the attainment of the critical weight. It is possible that attainment of critical weight is an indicator that the resources stored in the fat body are sufficient to meet the energy demands required for normal metamorphosis. Taken together, these results indicate that to understand the regulation of growth it is important to understand the contribution of each hormone in its varying temporal and developmental context.

## Chapter III

### Correlated wing–body allometric response to body size selection in *Manduca sexta*

## Introduction

Changes in the relative size of morphological traits account for most of the diversity observed in the animal morphology at taxonomic levels below the phylum. In the Lepidoptera, major characteristics that differentiate species are the differences in the relative sizes of the forewings and hindwings, and the size of the wings relative to the body. In general, organs and appendages are sized proportionally to the size of the animal. Within a population the relative sizes of body parts are relatively constant, large individuals bear larger wings than small individuals, which typically bear smaller wings.

Static allometry refers to the size relationship between two traits within a species, as individuals in a population differ in overall body size.. In most cases, this relationship is well described by a continuous function, most commonly a linear or a power function (Huxley, 1932). The contemporary thinking of allometry was developed by Huxley (Huxley, 1932; Huxley, 1924), but its origins date back to Galileo (Schlichting & Pigliucci, 1998). Huxley observed that the relationships between the sizes of body parts, in a broad diversity of animals, fit a simple power equation of the form:

$$y = a (x)^b,$$

here  $x$  and  $y$  are the dimensions of the two structures,  $a$  the constant, and  $b$  the constant differential growth-ratio, usually referred as the allometric coefficient.

This equation also applies to cases where  $y$  is the dimension of a body part and  $x$  is total body size (usually expressed as mass), so that the equation describes how a body part scales with variation in overall body size. The power equation can be expressed as a logarithmic equation:

$$\log y = \log a + b \log x$$

Using Huxley's equation on a logarithmic plot the relationships between two traits will appear as a straight line with  $b$  as the slope and  $\log a$  is the value of  $\log y$  when  $x = 1$ . The allometries of two populations can be compared by these parameters: differences in  $a$  reflect a difference in the relative sizes of the traits, and a difference in the slope ( $b$ ) reflects a difference in the scaling relationship between the traits as overall size varies. By this model, parameters  $a$  and  $b$  are sufficient to explain the differences in morphological variation among traits between populations of a species.

Huxley's allometric equation can be derived by considering how the growth of two structures is related. If we have two structures,  $x$  and  $y$ , that grow exponentially, their respective growth over time is expressed by:

$$\frac{dx}{dt} = \alpha x \quad \text{and} \quad \frac{dy}{dt} = \beta y ,$$

and their relative growth can be obtained as the ratio of these growth functions,

$$\frac{dy}{dx} = \frac{\beta y}{\alpha x} ,$$

which has the solution:

$$y = ax^{\beta/\alpha} ,$$

which is Huxley's power equation, where  $a$  is a constant and  $\beta/\alpha$  is the ratio of the two growth constants. There is no general agreement on the nomenclature of the parameters in this equation, so here I will call  $\beta/\alpha$ , the allometric coefficient, and  $a$  the scaling factor. If the relative growth rates of the two structures is constant over time, then  $\beta/\alpha = b$  and is a constant, and gives the slope of the linear regression of the logarithms of the two structures, as noted above.

Note that time was eliminated in the derivation of this equation, and the equation is agnostic about how size variation comes about. The general assumption is

that individual variation in growth rate or development time do not affect the proportion of parts at a given absolute size (Reeve & Huxley, 1945), so presumably overall size variation comes about by variation in development time. The allometry equation, derived above, thus describes the relationship among parts during development as well as the relationship among the final sizes of the parts.

There are actually four different classes of allometric relationships that are differentiated by the type of morphological variation that they describe (Cock, 1966; Klingenberg, 1996; Schlichting & Pigliucci, 1998). *Ontogenetic allometry* describes the change in scaling of traits at different stages in development, and explains how the relationship between the traits will change during ontogeny. *Static allometry* refers to the scaling relationship between traits within a population and results from the inherent variability of body sizes among individual of the same population. *Evolutionary allometry* refers to the changes in relative size of different body parts among individuals from different species due to the evolutionary divergence of those traits. Finally, *plastic allometry* (also called the reaction norm) describes the variation among traits due to environmental effects. Thus allometric relationships can be used to understand the developmental and evolutionary basis of many kinds of morphological change, and the response of traits to different environments.

Given the assumptions used in deriving the allometric equation, developmental and evolutionary changes in allometry can come about only by changes in the scaling constant,  $a$ , and/or by changes in the allometric coefficient,  $b$ . Changes in the scaling constant will alter the ratio of the two parts at all body sizes, but will not change how those part scale with changes in body size. This would result in a parallel shift of the logarithmic allometry curve without a change in slope. Changes in the allometric coefficient due to a change in one or both growth exponents, will result in a change in the slope of the logarithmic allometry curve.

In the Lepidoptera, as in other flying insects, wing size and the relative size of wings and body are determinants of flight performance (Dudley, 2000; Frankino *et al.*, 2005; Srygley, 1993). Flight performance is relevant in many ecological aspects such as dispersal, predator avoidance, inter-sexual competition and mating success (for some examples Chai & Srygley, 1990; Marden & Chai, 1991; Thornhill & Alcock, 1983; Wickman, 1992). Therefore, wing shape, wing size, and the relation between wing size and overall body size are subject to strong selection pressures. One would therefore expect that the relationship between wing and body size should be robust to environmental, genetic and developmental perturbations, but at the same time it should be flexible so as to arrive to the optimal design when body size varies in response to changing environments.

In the Lepidoptera, wing size and body size have a strong genetic correlation (Frankino *et al.*, 2005), which is consistent with purifying selection on wing body allometries (Frankino *et al.*, 2005; Strauss, 1990). Therefore, if selection is applied only on body size, we would expect that wing size will change proportionally. Selection for smaller body size will generate small animals with smaller wings, but one would expect these small animals to have the same wing-body allometry as the group of larger animals. The same would be expected to be true for selection for large body size.

In this chapter I examine how selection on body size affected the relationship between wing and body size. I used three size strains of *Manduca sexta* that were generated for a previous study on the developmental basis of body size evolution. I use Huxley's model to explore the developmental processes that have led to the change in allometry. I show that selection on small body size led to a change in the intercept of the allometric equation, but that selection for large body size led to a change in the allometric coefficient. Furthermore, I illustrate that Huxley's model might not be the most appropriate to predict and understand insect allometries.

## Materials and Methods

### *Experimental animals*

All *Manduca sexta* lines were established from the same stock population designated as wild type (Wt) strain. All animals were reared on a standard laboratory diet in individual cups at 26°C under long-day conditions (16D: 8D) (Bell & Joachim, 1976). During the feeding phase, larvae were fed *ad libitum* except in the starvation experiments. After they initiated wandering, larvae were placed in holes bored in wooden blocks for pupation. Animal age during the fifth (final) instar was determined with reference to the transition from the feeding to the wandering phase, and was designated as day 0. Starved animals were deprived of food after passing the critical weight that usually occurs at about 55% of peak larval mass ( strain A: ~4g, strain Wt: ~5.5g, and strain B: ~7.2g) (Davidowitz *et al.*, 2003). Adults were collected 24h after eclosion and kept at -20°C until further use.

The big (B) and small (S) strains used in this study were derived from a previous experiment in which the Wt strain was submitted to divergent selection for body size. In this selection experiment larvae were selected for either large body size (strain B), or small body size (strain S), by truncating selection. Each generation the 25% most extreme individuals were selected for breeding and this selection process was continued for 10 generations. These strains were subsequently maintained by mild selection each generation for large and small body size, respectively.

### *Allometric measurements*

A randomly selected subset of individuals from each strain was collected and used to measure adult body and wing size. Dry mass of bodies and wings was used as a measure of body and wing size respectively. Fore- and hindwings were cut carefully from the moth and stored separately. Bodies and wings were dried at 60°C for seven days and weighed with a micro-analytical balance (AE50, Mettler Toledo) to the nearest 1mg for body, and 0.1mg for wings. Left and right fore- and hindwings were measured and averaged. For a subset of wings, the surface area was calculated by digitalizing the wing and using SigmaScan® Pro 5.0 (SPSS Inc., Chicago, IL).

#### *Larval body and wing disk growth rate*

Larval body and wing disk size were determined daily during the final larval instar. All measurements were performed 2-4h after lights-on. Wing disks were dissected out in insect saline after anesthetizing the larvae for 5min in CO<sub>2</sub>. Wing disk dry weight was determined by rinsing dissected wing disks in water, placing them on a small tared disc of aluminum foil, and drying them at 60°C for 48h. Wing disk dry weight was determined to the nearest 1μg on a Cahn-25 Electrobalance. Cell number estimates were obtained by dissociating wing disks in 0.35M citric acid and counting the cells in a hemocytometer (Martin, 1982). Cells were counted in duplicate in a total volume of 0.1μl.

#### *Wing disk surgery*

The right forewing imaginal disks from *Manduca* larvae were removed the first day of the final larval instar. At this stage, wing disks account for less than 0.1% of the larval mass. Larvae were submerged in sterile insect saline and the disk was gently

pulled with micro-scissors through a small incision in the mesothoracic region. After the surgery, larvae were placed in moistened Kimwipes for 1h and then transferred to individual rearing cups with fresh diet. Control larvae were sham operated by making a small incision without removing the disk. Larvae were anesthetized with CO<sub>2</sub> for 10min prior to any manipulation. To avoid confounding effects of sex-specific differences in body size, all measurements of the effect of wing removal were performed only on female individuals.

### *Statistical analysis*

To investigate the relationship between wing and body size I used Huxley's allometric equation:

$$\text{wing size} = a (\text{body size})^b,$$

a log transformation of this equation produces the linear equation:

$$\log(\text{wing size}) = \log a + b \log(\text{body size}),$$

where  $a$  is the scaling coefficient and  $b$  is the allometric coefficient (Huxley 1924). Under this model the allometric coefficient represents the difference between the relative growth rates of wings and body:

$$\text{Allometric coefficient } (b) = \frac{\text{growth rate (wing)}}{\text{growth rate (body)}}$$

Allometric coefficients were calculated by least squares regression using log-transformed values. Allometric equations for the different strains and treatments were compared using an analysis of covariance (ANCOVA) with body weight as the covariate. Growth rates for larval body and wing disks were compared using ANCOVA with age as the covariate. All statistical analyses were conducted using JMP®7.0.2 (SAS Institute Inc., Cary, NC).

## Results

### *Size distribution and allometries among the size strains.*

The distribution of body and wing sizes for the Wt, B and S strains is given in Figures 3.1 and 3.2 and summarized in Table 3.1. Mean body size was different for males and females (two-tailed t-test  $P < 0.0001$ ) in each of the three strains. Therefore, all analysis were performed separately for sex. Body sizes and wing sizes were significantly different for the three strains (two-tailed t-test  $P < 0.0001$ ). Mean body size (including wings) for the small (S) strain was about 40% smaller, and the big (B) strain was about 25% bigger than the wild type (Wt) strain. In all strains males were on average 17% smaller than females, a size difference that is common in insects.

The analysis of wing-body allometry indicates that the selection for a smaller size was accompanied by a proportional decrease in the wing size for both males and females (Fig. 3.3, Table 3.2). Interestingly, there was a change in the scaling coefficient,  $a$ , which shifted the allometry curve and shows that in the small strain the wings became smaller relative to the body at all body sizes. The allometric coefficient (see  $b$  in Table 3.2) did not change from the Wt strain, so the scaling of wings with variation in body size was unaltered, in spite of the fact that wings were smaller relative to the body at all body sizes. In contrast, selection for a larger size was not accompanied by a proportional change in wing size (Fig. 3.3, Table 3.2). In both males and females the allometric coefficient ( $b$ ) was significantly smaller for the B strain relative to the Wt strain (females:  $F_{(3,231)}=120.9$ ,  $P=0.0023$ ; Males:  $F_{(3,220)}=123$ ,  $P=0.0051$ ) (Table 3.2). This indicates that in animals from the B strain bear wing size becomes smaller relative to body size as body size increases. Males and females of a strain showed no differences in wing/body

allometries (Table 3.2). This indicates that, although sexes differed in body size, the wing/body allometry was the same for each gender.

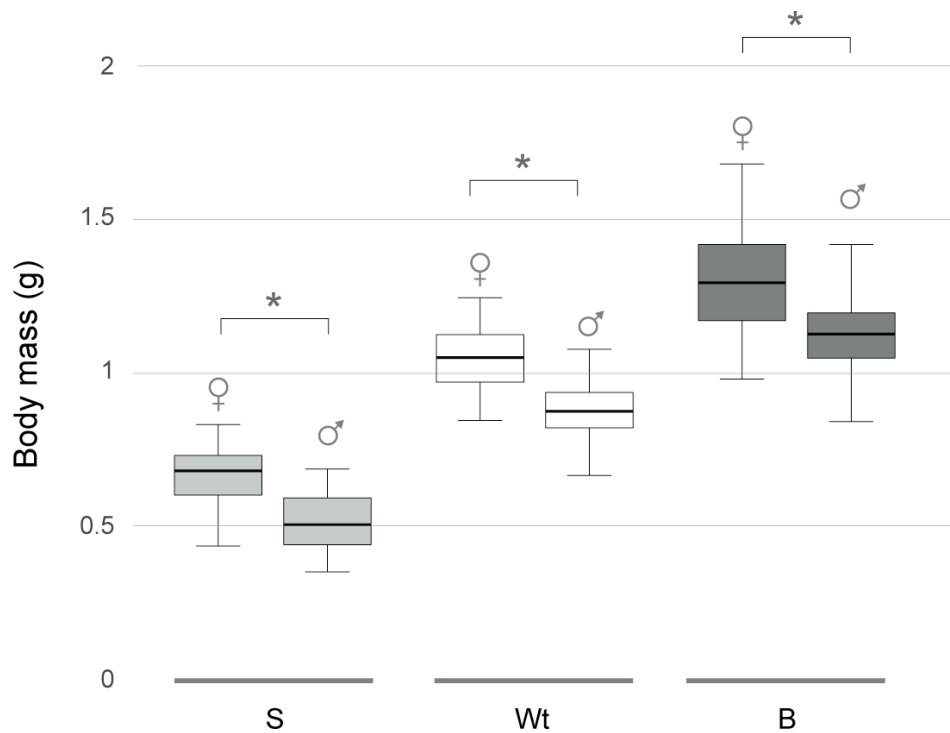


Figure 3.1. Body size distribution as a function of strain and sex. S small, Wt wild type, and B big strain. The box outline denotes the range of the middle 50% of the data, the horizontal line within each box indicates the median size, and the vertical lines show the dispersion of the data. Body size was significantly different between sexes (Two-tailed t-test,  $P < 0.001$ ) of the same strain and among the three size strains (Two-tailed t-test,  $P < 0.0001$ ).

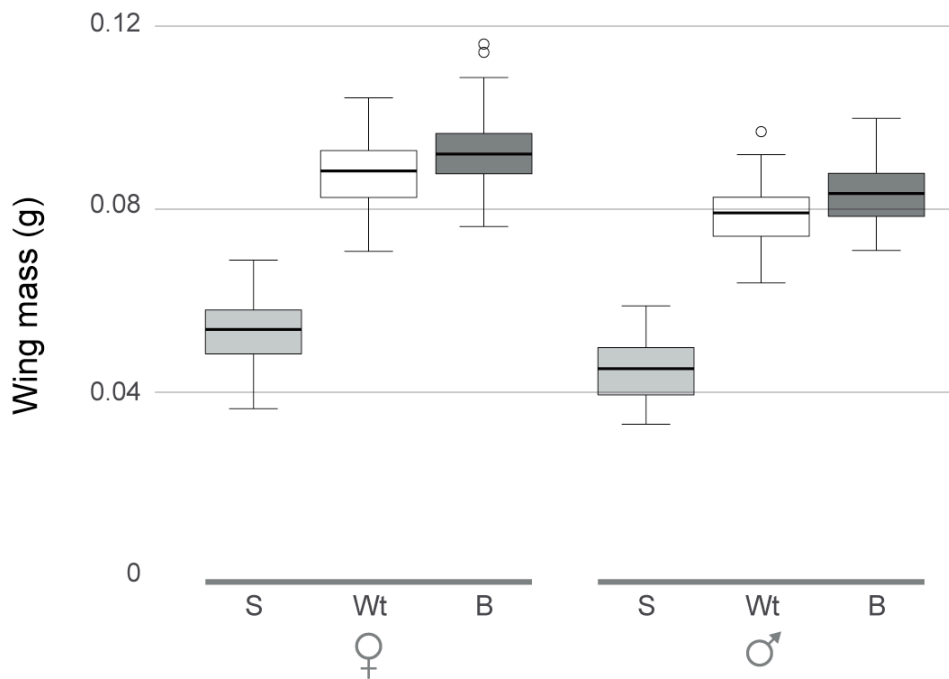


Figure 3.2. Wing size distribution as a function of strain and sex. S small, Wt wild type, and B big strain. The box outline denotes the range of the middle 50% of the data, the horizontal line within each box indicates the median size, and the vertical lines show the dispersion of the data. Points beyond this range are shown as open circles. Wing size is significantly different among the strains (Two-tailed t-test,  $P < 0.0001$ ).

Table 3.1. Body size and wing size for the three *M. sexta* size strains (mean  $\pm$  SD).

Strain	Sex	Body size (g)	Wing size (g)	<i>N</i>
S	F	0.662 $\pm$ 0.094	0.0536 $\pm$ 0.0067	84
	M	0.516 $\pm$ 0.096	0.0445 $\pm$ 0.0061	86
Wt	F	1.050 $\pm$ 0.096	0.0879 $\pm$ 0.0074	120
	M	0.883 $\pm$ 0.085	0.0783 $\pm$ 0.0063	118
B	F	1.307 $\pm$ 0.179	0.0925 $\pm$ 0.0077	115
	M	1.134 $\pm$ 0.129	0.0836 $\pm$ 0.0062	106

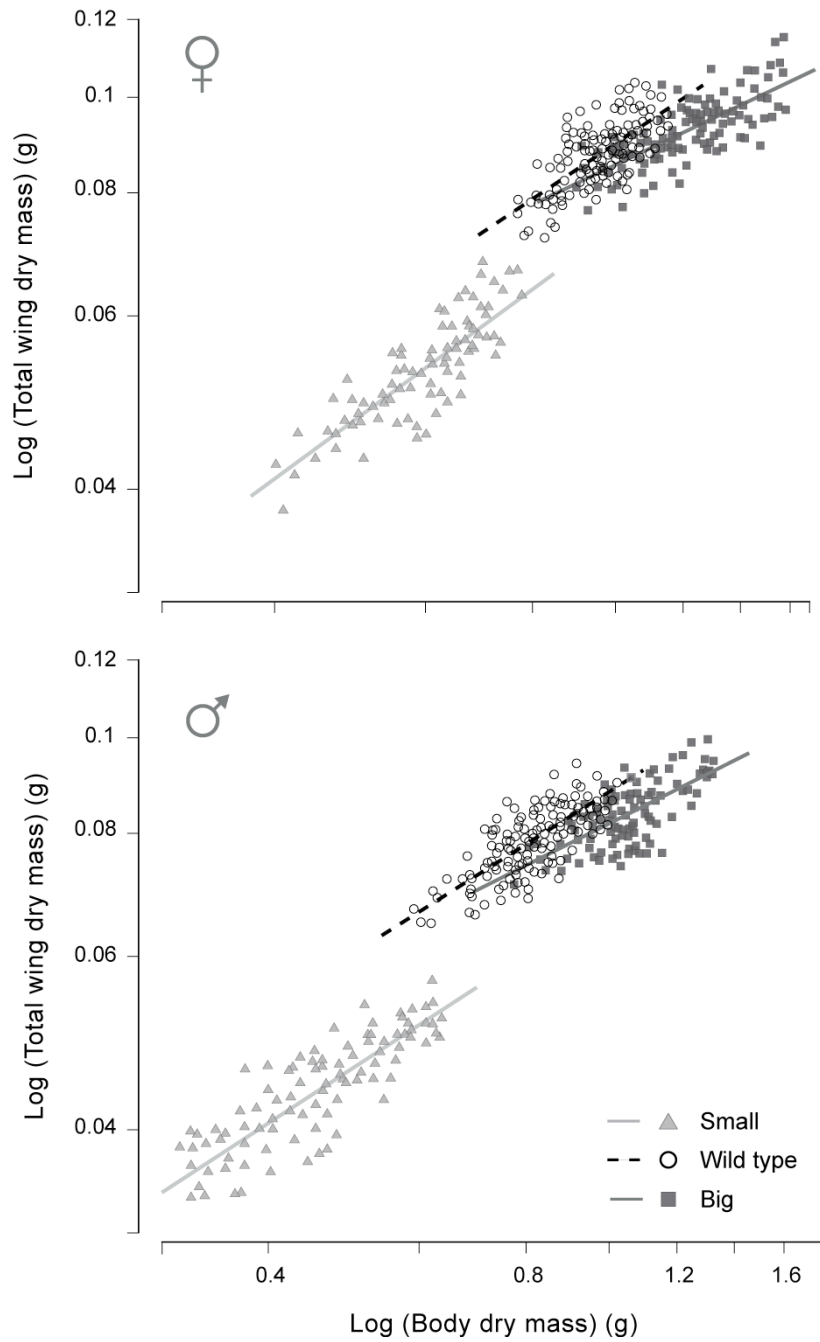


Figure 3.3. Allometric relationships between wing and body size for the three size strains of *M. sexta* adults. Allometric equations were determined by the least square method on log-transformed body and wing dry weights. Parameters for the allometric equations are given in Table 3.2.

Table 3.2. Allometric parameters for the wing-body relationship for individuals from the three size strains of *M. sexta*. Parameters are calculated for the allometric equation:  $\text{Log}(\text{wings}) = \log(a) + b(\log \text{body})$ ; where  $b$  represents the slope and specifies the allometric coefficient, and  $\log(a)$  represents the intercept of the equation. Allometric coefficients marked with different letters in the exponent indicate significant differences between samples ( $p > 0.05$ , ANCOVA).

Strain	Sex	Allometric parameters		$r^2$	N
		$b$	$a$		
S	F	$0.66^a \pm 0.054$	$-0.069 \pm 0.029$	0.71	84
	M	$0.65^a \pm 0.047$	$-0.068 \pm 0.037$	0.73	86
Wt	F	$0.69^a \pm 0.051$	$0.084 \pm 0.005$	0.66	120
	M	$0.63^a \pm 0.048$	$0.082 \pm 0.012$	0.65	118
B	F	$0.41^b \pm 0.033$	$0.080 \pm 0.008$	0.67	115
	M	$0.41^b \pm 0.036$	$0.076 \pm 0.005$	0.69	106

### *Body and larval growth rates*

In holometabolous insects, adults do not grow, so adult size is determined by the growth that occurs during the larval life. In *M. sexta* most of the body and wing growth occurs during the final instar. The last larval instar is divided into two distinct phases: the feeding phase and the wandering phase. Body growth is restricted to the feeding phase, but the wing imaginal disks (the structures that will give rise to the adult wings), grow continuously during the feeding and wandering phases, and during the early days of the pupal stage. In the Wt and B strains, the fifth instar usually lasts 10 days, five days in the feeding phase followed by five days in the wandering phase. In contrast, the S strain lasts eight days in the fifth instar with four days in each phase.

Because overall larval growth is exponential, in *Manduca* 90% of the growth in mass occurs during the final larval instar. Growth rate exponents for wing disk and larval body in the final larval instar are given in Table 3.3. Larval body and wing disk growth in the three strains was nearly exponential (Fig. 3.4 and Fig. 3.5). Body growth rate for the S strain was the highest followed by the B and the H strains, respectively. Body growth rates between the S-Wt, and B-Wt are significantly different (S-Wt:  $F_{(3,748)}=2786, P<0.0001$ ; B-Wt:  $F_{(3,901)}=3764, P=0.013$  respectively) (Table 3.3).

Wing disk growth rates show a similar pattern. Strain S has the highest wing disk growth rate followed by the B and the Wt strains. Wing disk growth rates between the S-Wt, and B-Wt are significantly different (S-Wt:  $F_{(3,357)}=5813, P<0.0001$ ; B-Wt:  $F_{(3,388)}=8650, P=0.0028$  respectively) (Table 3.3). In all three strains wing disk cell proliferation occurred at a slightly faster rate than the increase in mass (Table 3.4) indicating that cells must be becoming smaller with time. The B strain showed the highest cell proliferation rate followed by the S and Wt strains, respectively, and all rates were significantly different (S-Wt:  $F_{(3,142)}=1340, P=0.0189$ ; B-Wt:  $F_{(3,166)}=1608, P=0.025$

respectively). The ratio of cell number to wing mass was different for the three strains, indicating that at the end of the growing period the B strain had more cells per unit wing area than the Wt and S strain, and the S strain had the fewest cells per area (Table 3.4). Although the S strain has the fastest wing and body growth rates, it arrives to a smaller size because it takes a shorter time to develop.

Based on Huxley's allometric model, the relative growth rates of wings and body size should account for the differences in the allometric coefficients observed between the three size strains. The allometric coefficients predicted from the wing-body growth rate ratio differ dramatically from the coefficients obtained from the regression lines (Table 3.3). The predicted allometric coefficients are  $\sim 1$ , while the coefficients obtained from the regression lines range between 0.41-0.69. An allometric coefficient close to 1 would indicate that the size of the wings in relation to the size of the body is in isometry. This means, that the relative size of the wings and the body is constant irrespective of the absolute size. Our data, in contrast, indicate that wing and body size in all three size strains are in negative allometry, or hypometry; that means that as the size of the body becomes larger, the relative size of the wings become smaller.

However, it is worth noticing that the coefficients predicted from the growth rates are the same for the S and the Wt strain, but different in the B strain. These values predict that indeed the S and the Wt strain have the same wing body allometries, while the B strain has changed. Furthermore, the values also predict, that the relative size of wings and body is smaller in the B strain than in the other two strains.

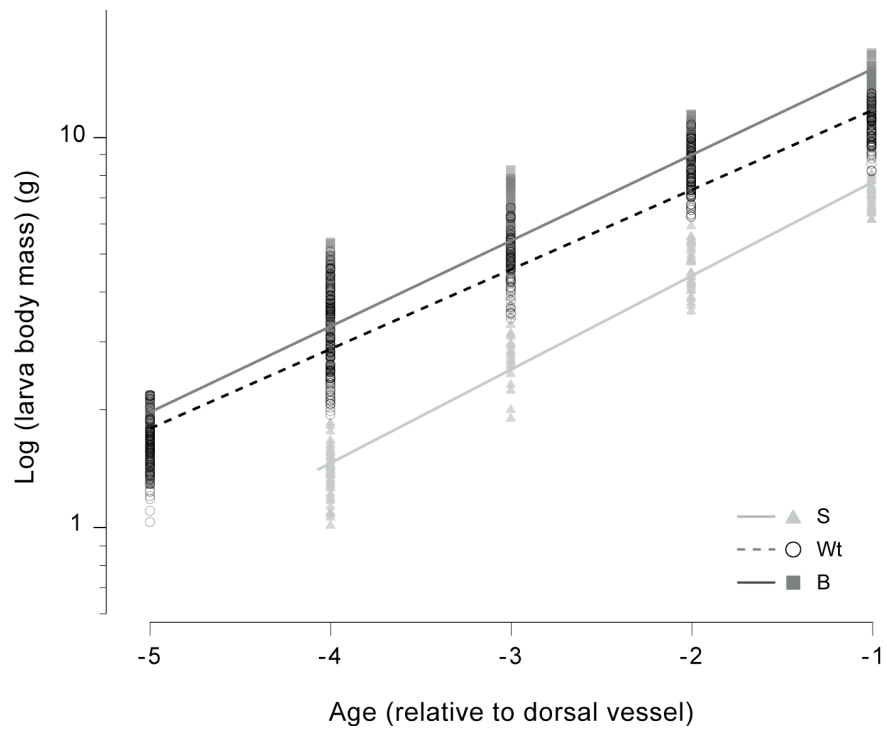


Figure 3.4. Larval body growth rate for the final instar in the three size strains. Linear regressions were determined by the least square method. Coefficients for the regression lines are given in Table 3.3.

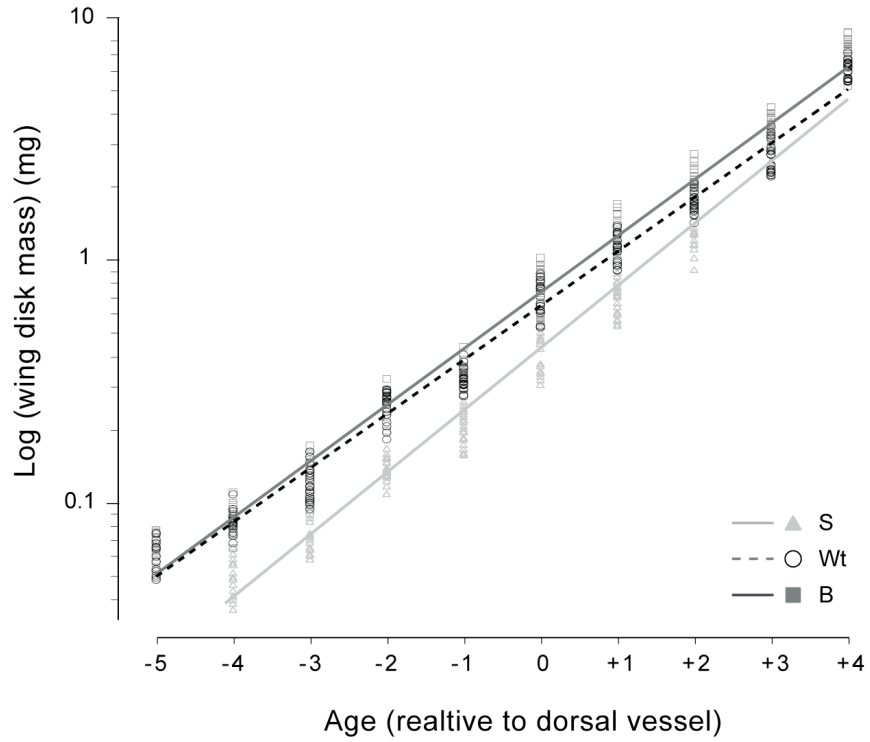


Figure 3.5. Wing disk growth rate for the three size strains. Linear regressions were determined by the least square method. Coefficients for the regression lines are given in Table 3.3.

Table 3.3. Wing disk and larval body growth rates for the three size strains. Growth rates marked with (\*) in the exponent indicate significant differences from the wild type (mean  $\pm$  SE) ( $p > 0.05$ , ANCOVA).

Strain	Body growth rate		Wing disk growth rate		Wing / Body (range)
		$r^2$		$r^2$	
S	0.54* $\pm$ 0.011	0.95	0.592* $\pm$ 0.0080	0.97	1.096 (1.062-1.132)
WT	0.47 $\pm$ 0.006	0.92	0.514 $\pm$ 0.0045	0.98	1.094 (1.070-1.117)
B	0.51* $\pm$ 0.009	0.93	0.531* $\pm$ 0.0049	0.98	1.041 (1.014-1.070)

Table 3.4. Wing disk mass and cell doubling time for the three size strains (mean  $\pm$  SE).

Strain	Doubling time (h)		Cell number / mass
	Mass	Cell number	
S	28 $\pm$ 2	27 $\pm$ 4	0.96
Wt	32 $\pm$ 3	30 $\pm$ 4	0.93
B	31 $\pm$ 3	27 $\pm$ 2	0.87

*Effect of food deprivation on allometries*

Nutritional status is a major regulator of organ and body size. Therefore, the mechanisms that coordinate nutrition with growth might be involved in the regulation of allometries. In the previous chapter I showed that food deprivation in larvae that have attained critical weight had a differential effect on larval body and wing disk growth. Food withdrawal causes an arrest in larval body growth, while wing disks continued to grow, although, at a reduced growth rate. I expected that the differential response of wing disks and larval body to food deprivation would result in a change in the adult wing-body allometry. I predicted that food deprived animals would in general be smaller, but the size of their wings would be relatively larger to the size of the body. Food restriction would have a larger effect on body size than on wing size, thus the wing-body allometric coefficient would be larger when compared to the coefficient of fed animals.

Our prediction was realized in both the S and the B strains, although in the S strain the change was restricted to the females (Fig. 3.6 and Table 3.5). In these three cases the wing-body allometric coefficient was statistically higher in food-deprived animals than in fed animals. It is not clear why food deprivation had an effect on the S and the B strain but had no effect on the allometry of the Wt strain. The S and the B strain have a higher wing growth rate than the Wt strain, and therefore, it is possible that having a higher growth rate increases the sensitivity to nutrition.

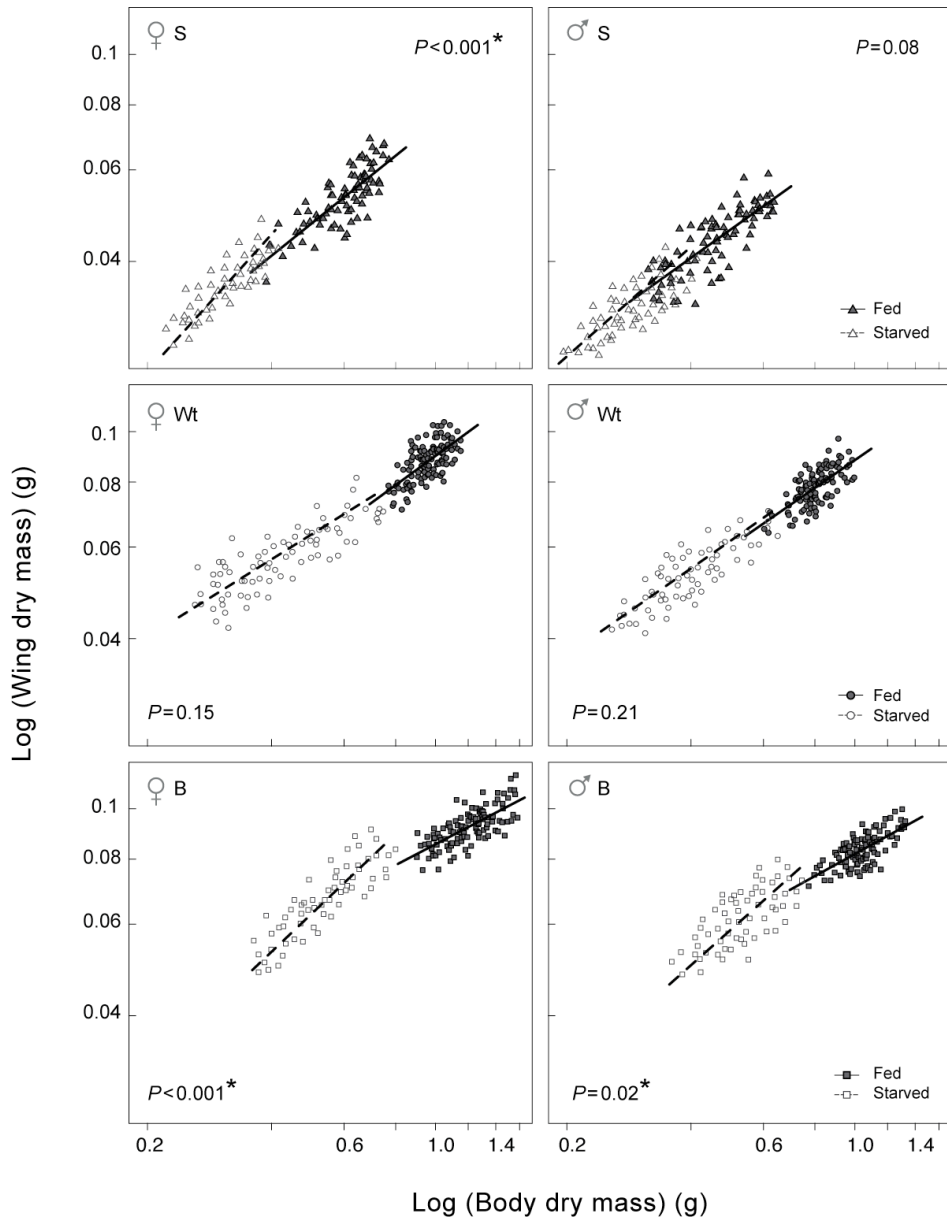


Figure 3.6. Effect of food deprivation on wing-body relationship in the three size strains of *M. sexta*. Regression lines were fitted by the least square method. Coefficients for the respective lines are given in Table 3.5. Statistically significant differences between fed and starved animals are marked with (\*) (ANCOVA,  $p < 0.05$ ).

Table 3.5. Effect of food deprivation on the wing-body size allometry for the three size strains. Comparison between slopes for the food deprived and fed animals. Slopes from food deprived animals that are significantly different from the slopes of the fed animals are marked with (\*) (ANCOVA).

Strain	Sex	<i>b</i> (slope)	<i>F</i> ( <i>df</i> )	<i>P</i>
S	F	0.76* ± 0.031	481.6 (3, 132)	<0.0001
	M	0.69 ± 0.034	308.8 (3,139)	0.079
Wt	F	0.71 ± 0.041	732.5 (3,195)	0.151
	M	0.66 ± 0.036	681.8 (1,183)	0.208
B	F	0.47* ± 0.027	478.6 (3, 173)	0.0212
	M	0.50* ± 0.026	694.1 (3,164)	<0.0001

*Effect of removing a wing on wing-body allometries*

Most of *Manduca* wing disk growth occurs when the larvae has ceased feeding. Therefore, the resources for imaginal disc growth come from fixed metabolic stores or body tissues. It is likely then that the growth of one trait occurs at the expense of growth in the other trait. Thus the absence of one disk should free up resources that can become available for the other disks, and result in an increase in growth in the remaining disks. The available resources would be allocated to the growing organs and not to the larval body and thus the remaining disks should have an increased growth that is not accompanied by an increase in the body growth. This disproportional change in wing and body growth should then result in a change in the allometric relationship between wings and body.

I expected to see an increase in the allometric coefficient in moths whose right forewing had been removed compared to sham-operated moths. Our results, in contrast to our expectations, showed that removing a wing disk had no effect on the wing-body allometry (Fig. 3.7, Table 3.6). It is possible that disks in *Manduca* larvae are not resource limited as observed in disks from other insects (Klingenberg & Nijhout, 1998; Nijhout & Emlen, 1998). Wing disks in *Manduca* may have an upper limit for wing disk size that is genetically encoded and cannot be exceeded by increasing nutrient storage. Another possibility is that given the small size of the wing disk relative to the large size of the larvae, the resources that are “freed up” from the absence of the wing are negligible compared to the resources available for growth. Large bodies can pose different limits on growth than do small bodies since large bodies have a much larger amount of reserves. Previous studies that demonstrated competition for resources between organs used insects that were substantially smaller (Klingenberg & Nijhout, 1998; Nijhout & Emlen, 1998).

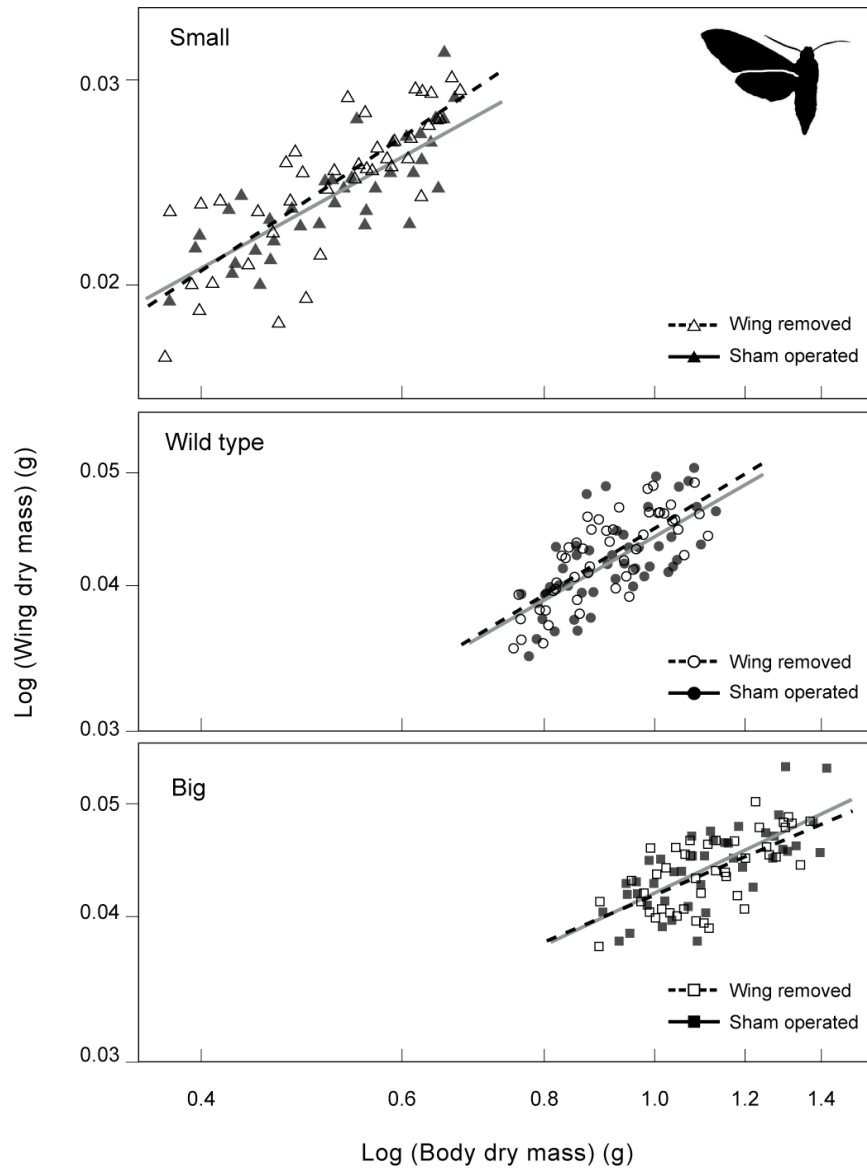


Figure 3.7. Effect of wing disk removal on the allometric relationship of the three size strains. During eclosion the right hindwing had minor damages due to the absence of the forewing and therefore, analysis was performed only on the fore- and hindwings of the left side. Regression lines were calculated by the least square method. Coefficients for the relationships are given in Table 3.6. Regression lines between removed and sham operated show no significant differences (ANCOVA,  $p=0.05$ ).

Table. 3.6. Effect of wing disk removal on the allometric relationship for females of the three size strains. Removing the forewing had no significant effect on the wing body allometry for any of the three size strains (ANCOVA  $p > 0.05$ ).

Strain	$F$ ( $df$ )	$P$
S	44.5 (3, 73)	0.32
Wt	35.0 (3, 95)	0.86
B	24.2 (3, 81)	0.67

#### *Wing mass and wing area*

I selected a random subset of wings to determine if dry mass was correlated with wing area. In all three strains there was a strong correlation between wing mass and wing area (Fig. 3.8 and Table 3.7). However, the slope for the B strain was slightly significantly different from the S and the Wt strains. These results indicate that wings of the B strain are larger relative to their mass than those of the S and the Wt strains.

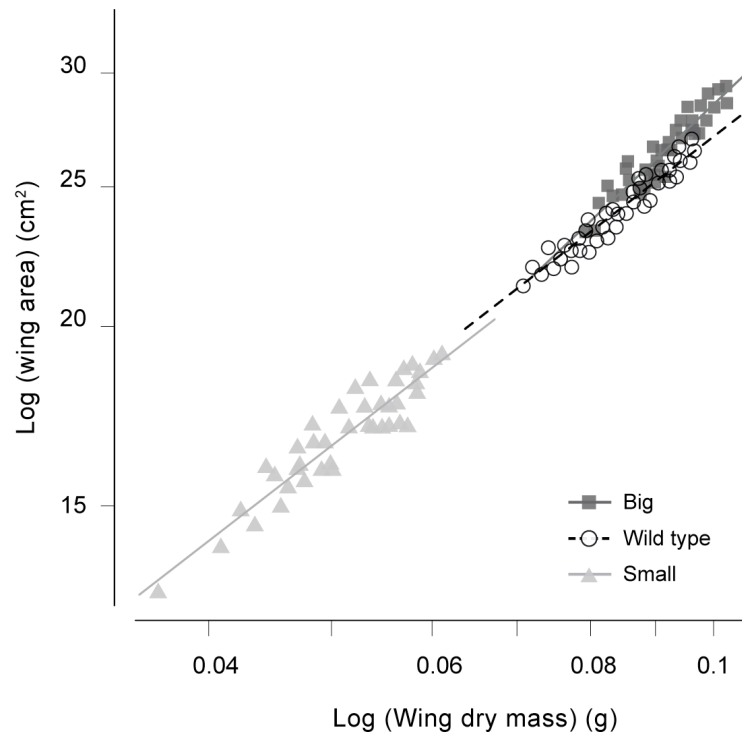


Figure 3.8. Correlation between wing area and wing mass for the three size strains. Linear regressions were determined by the least square method. Coefficients for the regression lines are given in Table 3.7.

Table 3.7. Correlation between wing area and wing mass for the three size strains. Slopes marked with different letters in the exponent indicate significant differences between samples ( $p > 0.05$ , ANCOVA).

Strain	$b$ (slope)	$r^2$	$F$ (df)	$P$
S	$0.67^a \pm 0.043$	0.91	249.2 (1, 38)	<.0001
Wt	$0.68^a \pm 0.044$	0.94	264.1 (1, 38)	<.0001
B	$0.84^b \pm 0.056$	0.92	215.5 (1, 38)	<.0001

## Discussion

In most animals, body parts and organs are sized proportionally to the size of the individual. In general, within a population of butterflies, larger individuals bear larger wings compared to smaller individuals. In a selection experiment that generated a small (S) and a big (B) strain, selection was only applied for an increase or decrease in body size. Based on evidence of strong genetic correlations between wing and body size in other Lepidopteran species (Frankino *et al.*, 2005), I expected a concomitant response in the size of the wings. I observed that selection for a smaller body size (S strain) led to a change in the scaling coefficient ( $a$ ) for the wing and body size allometry, while the allometric coefficient stayed the same. In contrast, selection for a larger size (B strain), led to a change in the allometric coefficient ( $b$ ).

A change only in the scaling coefficient, as seen for the S strain, indicates that although the animals are smaller and their wings are smaller with respect to body size, the allometric relationship between wings and body in the two strains did not change. In contrast, a change in the allometric coefficient of the allometric equation, as seen in the B strain, indicates that the scaling relationship between wings and body in this strain is different from the other strains. These two classes of allometric change imply different consequences for the flight ability of the strains, and imply changes in the developmental regulation of wing-body relationship in both strains.

To investigate the developmental basis for the observed changes in allometry, I used the general allometric model proposed by Huxley (1932). Huxley's model is based on the differential growth rate of the traits under consideration; the difference between the two growth rates should predict the scaling relationship between the traits. Using empirical data, Huxley's model accurately predicted the coordinated change in wing and body growth rates. In the case in which allometric coefficients did not change, as

between the S and the Wt strains, changes in growth rate should be in the same direction. This means that if body growth rate increased, wing growth rate must have proportionally increased as well. This is what I observed (Fig. 3.6 and Table 3.5). In the case in which allometric coefficients did change, as observed between the B and the Wt strain, an increase in the body growth rate should not be accompanied by a concomitant increase in wing growth rate. For the B strain I observed that although body and wings became larger, wings were smaller relative to the body size than in the Wt strain. This indicates that changes in growth rates are indeed relevant for determining the proportionality between structures.

The growth rates of wing disk and body, however, failed to predict the actual values of the wing-body allometric coefficients for the three strains. The growth rate coefficients for wings and body size were very close to each other, which would lead to an allometric coefficient close to 1, whereas the empirically measured allometric coefficients were invariably significantly smaller. This smaller allometric coefficient implies that the growth rate for the wings should be larger than that of the body. This discrepancy may be explained by the fact that Huxley's equation assumes that both structures grow for the same period of time, whereas in *Manduca* the wings grow for a much longer period of time than the body. Thus if the wings grew for the same amount of time as the body they would have to grow faster to achieve the same expected final size. Thus a longer growth period should have the same effect on the allometry as a greater growth rate.

I identified an additional reason why Huxley's model might not have been the most appropriate to explore the relationships between wings and body size in *M. sexta*. The allometric equation is a power function and is the solution of the differential equations relating the specific growth rates of two traits with respect to time. Therefore, it assumes that the traits under consideration follow pure exponential growth. It is

generally assumed that growth is exponentially (for some examples Berger *et al.*, 2006; D'Amico *et al.*, 2001; Margraf *et al.*, 2003). For *M. sexta*, the wings and body growth data do fit an exponential equation well with high coefficients of determination ( $r^2 > 0.9$ ). However, when plotting the instantaneous growth rate for body mass it becomes apparent that body growth rate at the last larval instar actually decreases with time (Fig. 3.9). Growth rate for wing disks, by contrast, also has a good fit to an exponential equation, however, as seen in chapter one, the wing disk growth coefficient is not constant throughout the final instar. The same growth pattern as observed for wing disks from the Wt strain (see chapter 1) is observed for wing disks from the S and the B strain (data not shown).

Finally, allometric relations between body parts and the whole body are complicated by the fact that organs grow at the expense of the body and not in parallel with it. Huxley's model assumes that body parts are autonomous and don't communicate with each other. Although I was not able to show that wings were competing with each other for resources (Fig. 3.7), it is evident that wing disks must grow using resources from the larvae. This is in part corroborated by our food deprivation experiments. In the case where wing and body growth rates are independent, food deprivation would affect both growth rates proportionally, thus would not change the wing-body allometries. In our experiments food deprivation resulted in a change in the wing body allometry in the S and the B strain, therefore, indicating that the growth of the wings is in competition for available resources with larval body growth.

Two different models have been proposed that take into account the above-mentioned issues. These models differ substantially in the primary factors that account for the allometric change (Nijhout & Wheeler, 1996; Shingleton *et al.*, 2007; Stieper *et al.*, 2008). Nijhout and Wheeler's (1996) model is restricted to the period when body growth

has ceased and shows that competition for nutrients among body parts can lead to allometric changes. In contrast, Shingleton *et al.* (2007) and Stieper *et al.* model (2008), predicts that the major factor affecting allometric changes is the interaction of growth hormones with the growing tissue, therefore, in this model the determining factor for allometric relationships is the duration of the growth period when structures are sensitive to the growth hormone. Although both models simplify the complexity of growth regulation, they contain parameters that are empirically difficult to measure. Here, I have shown that even Huxley's simplified model does offer important explanatory power for understanding the evolution of allometries in holometabolous insects.

In this study I investigated the evolution of wing-body allometries by investigating the relationship between mass of the wings and mass of the body. However, the wing mass might not be the phenotype most selectively or developmentally constrained relative to body size. Biomechanical models predict that an increase in body mass must be accompanied with an increase in wing surface area to generate enough lift to fly (Dudley, 2000). While a more massive wing tends to have a larger surface area, a close inspection of the relationship of mass and surface area indicates that for the B strain this relationship has changed (Fig. 3.8). In this strain, the wing surface area is larger than expected for wing mass, at least when compared to the other two strains. Wings from individuals of the B strain also showed a faster cell doubling-time than a mass doubling time than wings of the Wt and S strains (Table 3.3). This difference in cell/mass doubling-time ratio indicates that either the cells of the wing in the B strain become smaller than those of the other two strains, or that the wing becomes lighter relative to the number of cells it contains. The latter could be accomplished if the wing surface area increased relative to its mass, which is what happened here. The B strain appears to have compensated for the relative small wing

mass with a larger surface area, which implies that surface area and mass are regulated separately in development, and that there may exist a homeostatic mechanism that attempts to maintain a proportional wing surface area, which is functionally required for flight.

An interesting observation is that the B strain has a lower allometric coefficient (Fig. 3.3) and is also more sensitive to food deprivation (Fig. 3.6) than the other two strains. A possible explanation for both effects is that although body size of this strain has increased, the period for wing growth has remained about the same, so if the wings are growing at their maximal rate, they are not able to attain a fully proportional size to the body. The fact that the wings of the B strain are more sensitive to reduced nutrition supports this interpretation. If this interpretation is correct, it implies that the B strain is pushing up to the limit to which wings can scale with body size, and that the intrinsic growth rate of the wing disks poses a severe developmental constraint on the scaling of wings with body size

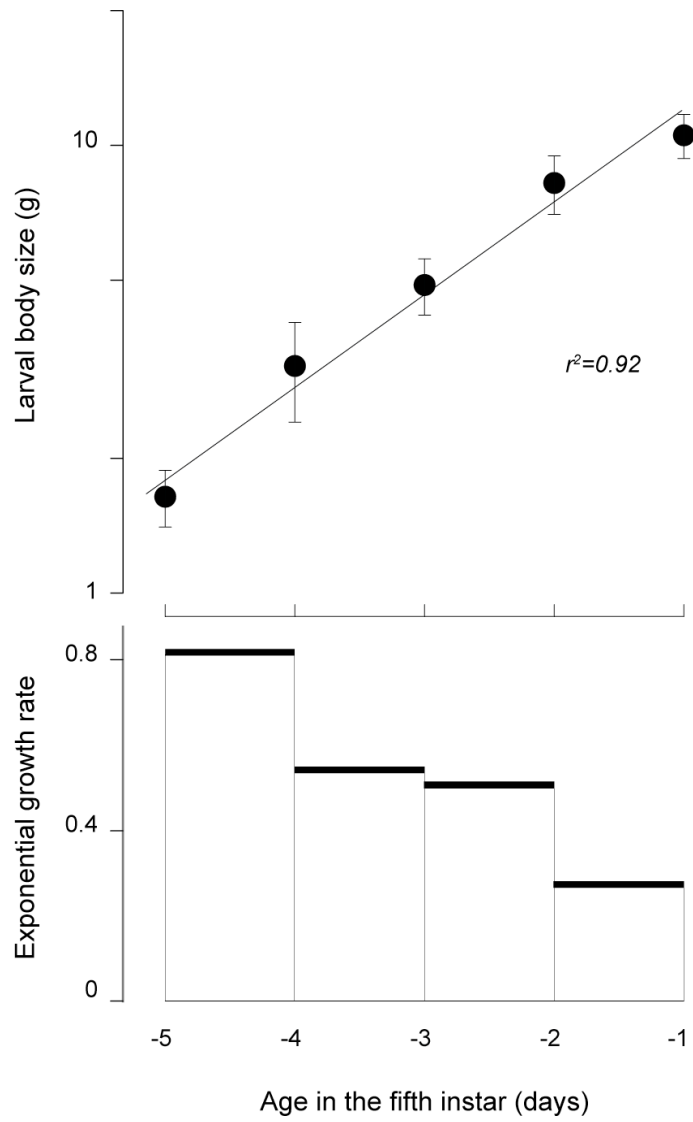


Figure 3.9. Larval body growth rate for the Wt strain during the fifth instar.

## References

- Affolter, M. & Basler, K.** 2007. The Decapentaplegic morphogen gradient: from pattern formation to growth regulation. *Nature Reviews Genetics* **8**(9): 663-674.
- Baker, F., Lw, T., Reuter, C. & Schooley, D.** 1987. *In vivo* fluctuation of JH, JH acid, and ecdysteroid titer, and JH esterase activity, during development of fifth stadium *Manduca sexta*. *Insect Biochemistry* **17**(7): 989-996.
- Bell, R. & Joachim, F.** 1976. Techniques for rearing laboratory colonies of tobacco hornworms and pink bollworms. *Annals of the Entomological Society of America* **69**: 365-373.
- Berger, D., Walters, R. & Gotthard, K.** 2006. What keeps insects small?—Size dependent predation on two species of butterfly larvae. *Evolutionary Ecology* **20**(6): 575-589.
- Bertalanffy, L.** 1960. Principles and theory of growth. In *Fundamental aspects of normal and malignant growth* (ed. W. Nowinski), pp. 137-259. Elsevier, New York.
- Bohni, R., Riesgo-Escovar, J., Oldham, S., Brogiolo, W., Stocker, H., Andruss, B. F., Beckingham, K. & Hafen, E.** 1999. Autonomous control of cell and organ size by CHICO, a *Drosophila* homolog of vertebrate IRS1-4. *Cell* **97**(7): 865-875.
- Bollenbacher, W. E., Smith, S. L., Goodman, W. & Gilbert, L. I.** 1981. Ecdysteroid titer during larval-pupal-adult development of the tobacco hornworm, *Manduca sexta*. *General and Comparative Endocrinology* **44**(3): 302-6.
- Britton, J. S. & Edgar, B. A.** 1998. Environmental control of the cell cycle in *Drosophila*: nutrition activates mitotic and endoreplicative cells by distinct mechanisms. *Development* **125**(11): 2149-58.
- Britton, J. S., Lockwood, W. K., Li, L., Cohen, S. M. & Edgar, B. A.** 2002. *Drosophila*'s insulin/P13-kinase pathway coordinates cellular metabolism with nutritional conditions. *Developmental Cell* **2**(2): 239-249.
- Brogiolo, W., Stocker, H., Ikeya, T., Rintelen, F., Fernandez, R. & Hafen, E.** 2001. An evolutionarily conserved function of the *Drosophila* insulin receptor and insulin-like peptides in growth control. *Current Biology* **11**(4): 213-221.
- Caldwell, P. E., Walkiewicz, M. & Stern, M.** 2005. Ras activity in the *Drosophila* prothoracic gland regulates body size and developmental rate via ecdysone release. *Current Biology* **15**(20): 1785-1795.
- Chai, P. & Srygley, R. B.** 1990. Predation and the flight, morphology, and temperature of neotropical rain-forest butterflies. *American Naturalist* **135**(6): 748-765.

- Champlin, D. T., Reiss, S. E. & Truman, J. W.** 1999. Hormonal control of ventral diaphragm myogenesis during metamorphosis of the moth, *Manduca sexta*. *Development Genes and Evolution* **209**(5): 265-274.
- Champlin, D. T. & Truman, J. W.** 1998a. Ecdysteroid control of cell proliferation during optic lobe neurogenesis in the moth *Manduca sexta*. *Development* **125**(2): 269-277.
- Champlin, D. T. & Truman, J. W.** 1998b. Ecdysteroids govern two phases of eye development during metamorphosis of the moth, *Manduca sexta*. *Development* **125**(11): 2009-2018.
- Cock, A. G.** 1966. Genetical aspects of metrical growth and form in animals. *The Quarterly Review of Biology* **41**(2): 131-190.
- Colombani, J., Bianchini, L., Layalle, S., Pondeville, E., Dauphin-Villemant, C., Antoniewski, C., Carre, C., Noselli, S. & Leopold, P.** 2005. Antagonistic actions of ecdysone and insulins determine final size in *Drosophila*. *Science* **310**(5748): 667-670.
- Colombani, J., Raisin, S., Pantalacci, S., Radimerski, T., Montagne, J. & Leopold, P.** 2003. A nutrient sensor mechanism controls *Drosophila* growth. *Cell* **114**(6): 739-49.
- Crickmore, M. A. & Mann, R. S.** 2008. The control of size in animals: insights from selector genes. *Bioessays* **30**(9): 843-53.
- Cymborowski, B., Bogus, M., Beckage, N. E., Williams, C. M. & Riddiford, L. M.** 1982. Juvenile-hormone titers and metabolism during starvation-induced supernumerary larval molting of the tobacco hornworm *Manduca sexta* L. *Journal of Insect Physiology* **28**(2): 129-135.
- D'amico, L., Davidowitz, G. & Nijhout, H.** 2001. The developmental and physiological basis of body size evolution in an insect. *Proceedings of the Royal Society of London, Series B: Biological Sciences* **268**(1476): 1589-1593.
- Dai, J., Mizoguchi, A. & Gilbert, L.** 1994. Immunoreactivity of neurosecretory granules in the brain-retrocerebral complex of *Manduca sexta* to heterologous antibodies against *Bombyx* prothoracicotropic hormone and bombyxin. *Invertebrate Reproduction and Development* **26**: 187-187.
- Davidowitz, G., D'amico, L. J. & Nijhout, H. F.** 2003. Critical weight in the development of insect body size. *Evolution & Development* **5**(2): 188-197.
- Davis, K. & Shearn, A.** 1977. In vitro growth of imaginal disks from *Drosophila melanogaster*. *Science* **196**(4288): 438-440.

- Day, S.** 2000. Measuring dimensions: the regulation of size and shape. *Development* **127**(14): 2977-2987.
- Dean, R., Locke, M. & Collins, J.** 1985. Structure of the fat body. In *Comprehensive Insect Physiology, Biochemistry, and Pharmacology*, vol. 3 (ed. G. Kerkut and L. Gilbert), pp. 155–210. Pergamon Press, New York.
- Dinan, L., Spindler-Barth, M. & Spindler, K.** 1990. Insect cell lines as tools for studying ecdysteroid action. *Invertebrate Reproduction and Development* **18**: 43-53.
- Dudley, R.** 2000. *The biomechanics of insect flight: form, function, evolution*. Princeton University Press, New Jersey.
- Dutkowski, A. & Oberlander, H.** 1974. Interactions between beta-ecdysone and fat body during wing disk development *in vitro*. *Journal of Insect Physiology* **20**(4): 743-9.
- Edgar, B. A.** 2006. How flies get their size: genetics meets physiology. *Nature Reviews Genetics* **7**(12): 907-916.
- Fain, M. & Riddiford, L. M.** 1975. Juvenile hormone titers in the hemolymph during late larval development of the tobacco hornworm, *Manduca sexta* (L.). *The Biological Bulletin* **149**(3): 506-521.
- Franco, M., Bohbot, J., Fernandez, K., Hanna, J., Poppy, J. & Vogt, R.** 2007. Sensory cell proliferation within the olfactory epithelium of developing adult *Manduca sexta* (Lepidoptera). *PLoS ONE* **2**(2): e215.
- Frankino, W., Zwaan, B., Stern, D. & Brakefield, P.** 2005. Natural selection and developmental constraints in the evolution of allometries. *Science* **307**(5710): 718-720.
- Garofalo, R.** 2002. Genetic analysis of insulin signaling in *Drosophila*. *Trends in Endocrinology & Metabolism* **13**(4): 156-162.
- Hauerland, N. & Shirk, P.** 1995. Regional and functional differentiation in the insect fat body. *Annual Review of Entomology* **40**(1): 121-145.
- Hufnagel, L., Teleman, A., Rouault, H., Cohen, S. & Shraiman, B.** 2007. On the mechanism of wing size determination in fly development. *Proceedings of the National Academy of Sciences* **104**(10): 3835.
- Huxley, J.** 1932. *Problems of relative growth*. Johns Hopkins University Press, Baltimore.
- Huxley, J. S.** 1924. Constant differential growth-ratios and their significance. *Nature* **114**(2877): 895-6.

- Ikeya, T., Galic, M., Belawat, P., Nairz, K. & Hafen, E.** 2002. Nutrient-dependent expression of insulin-like peptides from neuroendocrine cells in the CNS contributes to growth regulation in *Drosophila*. *Current Biology* **12**(15): 1293-1300.
- Iwami, M., Furuya, I. & Kataoka, H.** 1996. Bombyxin-related peptides, cDNA structure and expression in the brain of the hornworm *Agrius convolvuli*. *Insect Biochemistry and Molecular Biology* **26**(1): 25-32.
- Kimura-Kawakami, M., Iwami, M., Kawakami, A., Nagasawa, H., Suzuki, A. & Ishizaki, H.** 1992. Structure and expression of bombyxin-related peptide genes of the moth *Samia cynthia ricini*. *General and Comparative Endocrinology* **86**(2): 257-268.
- Kingsolver, J. & Pfennig, D.** 2004. Individual-level selection as a cause of Cope's rule of phyletic size increase. *Evolution* **58**(7): 1608-1612.
- Kirschenbaum, S. R., Higgins, M. R., Tveten, M. & Tolbert, L. P.** 1995. 20-Hydroxyecdysone Stimulates Proliferation Of Glial-Cells In The Developing Brain Of The Moth *Manduca-Sexta*. *Journal Of Neurobiology* **28**(2): 234-247.
- Klingenberg, C.** 1996. Multivariate allometry. In *Advances in morphometrics* (ed. L. Marcus), pp. 23-49. Springer, New York.
- Klingenberg, C. P. & Nijhout, H. F.** 1998. Competition among growing organs and developmental control of morphological asymmetry. *Proceedings of the Royal Society of London Series B* **265**(1401): 1135-1139.
- Knutze, H.** 1935. Die Flügelentwicklung bei *Philosamia cynthia drury*, mit besonderer berücksichtigung des Geäders, der Lakunen und der Tracheensysteme. *Zoomorphology (Berlin)* **30**(4): 544-72.
- Kopec, S.** 1922. Studies on the necessity of the brain for the inception of insect metamorphosis. *Biological Bulletin* **42**(6): 323-342.
- Koyama, T., Iwami, M. & Sakurai, S.** 2004. Ecdysteroid control of cell cycle and cellular commitment in insect wing imaginal discs. *Molecular And Cellular Endocrinology* **213**(2): 155-166.
- Kremen, C. & Nijhout, H.** 1998. Control of pupal commitment in the imaginal disks of *Precis coenia* (Lepidoptera: Nymphalidae). *Journal of Insect Physiology* **44**(3-4): 287-296.
- Labarbera, M.** 1989. Analyzing body size as a factor in ecology and evolution. *Annual Review of Ecology and Systematics* **20**(1): 97-117.

- Livak, K. J. & Schmittgen, T. D.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ddCT method. *Methods* **25**(4): 402-408.
- Luedeman, R. & Levine, R. B.** 1996. Neurons and ecdysteroids promote the proliferation of myogenic cells cultured from the developing adult legs of *Manduca sexta*. *Developmental Biology* **173**(1): 51-68.
- Marden, J. & Chai, P.** 1991. Aerial predation and butterfly design: how palatability, mimicry, and the need for evasive flight constrain mass allocation. *American Naturalist* **138**(1): 15-36.
- Margraf, N., Gotthard, K. & Rahier, M.** 2003. The growth strategy of an alpine beetle: maximization or individual growth adjustment in relation to seasonal time horizons? *Functional Ecology*: 605-610.
- Martin, P. F.** 1982. Direct determination of the growth rate of *Drosophila* imaginal discs. *Journal of Experimental Zoology* **222**(1): 97-102.
- Masumura, M., Satake, S. I., Saegusa, H. & Mizoguchi, A.** 2000. Glucose stimulates the release of bombyxin, an insulin-related peptide of the silkworm *Bombyx mori*. *General and Comparative Endocrinology* **118**(3): 393-399.
- Mckinney, M. L.** 1997. Extinction vulnerability and selectivity: combining ecological and paleontological views. *Annual Review of Ecology and Systematics* **28**(1): 495-516.
- Meyer, D. R., Sachs, F. N. & Rohner, R. M.** 1980. Parameters for growth of the imaginal wing disk in last instar larvae of *Galleria mellonella* L. *Journal of Experimental Zoology* **213**(2): 185-197.
- Meyer-Fernandes, J., Clark, C., Gondim, K. & Wells, M.** 2001. Fat body fructose-2, 6-bisphosphate content and phosphorylase activity correlate with changes in hemolymph glucose concentration during fasting and re-feeding in larval *Manduca sexta*. *Insect Biochemistry and Molecular Biology* **31**(2): 165-170.
- Miner, A. L., Rosenberg, A. J. & Nijhout, H. F.** 2000. Control of growth and differentiation of the wing imaginal disk of *Precis coenia* (Lepidoptera : Nymphalidae). *Journal of Insect Physiology* **46**(3): 251-258.
- Mirth, C., Truman, J. & Riddiford, L.** 2005. The role of the prothoracic gland in determining critical weight for metamorphosis in *Drosophila melanogaster*. *Current Biology* **15**(20): 1796-1807.
- Mottier, V., Siaussat, D., Bozzolan, F., Auzoux-Bordenave, S., Porcheron, P. & Debernard, S.** 2004. The 20-hydroxyecdysone-induced cellular arrest in G2 phase is preceded by an inhibition of cyclin expression. *Insect Biochemistry and Molecular Biology* **34**(1): 51-60.

- Nagasawa, H., Kataoka, H., Isogai, A., Tamura, S., Suzuki, A., Mizoguchi, A., Fujiwara, Y., Suzuki, A., Takahashi, S. Y. & Ishizaki, H.** 1986. Amino acid sequence of a prothoracicotropic hormone of the silkworm *Bombyx mori*. Proceedings of the National Academy of Sciences of the United States of America **83**(16): 5840-5843.
- Nijhout, H. F.** 1994. Insect hormones. Princeton University Press, New Jersey.
- Nijhout, H. F.** 1979. Stretch-induced moulting in *Oncopeltus fasciatus*. Journal of Insect Physiology **25**: 277-282.
- Nijhout, H. F.** 1984. Abdominal stretch reception in *Dipetalogaster maximus* (Hemiptera: Reduviidae). Journal of Insect Physiology **30**(8): 629-633.
- Nijhout, H. F.** 2003a. The control of body size in insects. Developmental Biology **261**(1): 1-9.
- Nijhout, H. F., Davidowitz, G. & Roff, D.** 2006. A quantitative analysis of the mechanism that controls body size in *Manduca sexta*. Journal of Biology **5**(5): 16.
- Nijhout, H. F.** 1981. Physiological control of molting in insects. American Zoologist **21**(3): 631-640.
- Nijhout, H. F.** 2003b. The control of growth. Development **130**(24): 5863-5867.
- Nijhout, H. F. & Emlen, D. J.** 1998. Competition among body parts in the development and evolution of insect morphology. Proceedings Of The National Academy Of Sciences Of The United States Of America **95**(7): 3685-3689.
- Nijhout, H. F. & Grunert, L. W.** 2002. Bombyxin is a growth factor for wing imaginal disks in Lepidoptera. Proceedings of the National Academy of Sciences of the United States of America **99**(24): 15446-15450.
- Nijhout, H. F., Smith, W. A., Schachar, I., Subramanian, S., Tobler, A. & Grunert, L. W.** 2007. The control of growth and differentiation of the wing imaginal disks of *Manduca sexta*. Developmental Biology **302**(2): 569-576.
- Nijhout, H. F. & Wheeler, D. E.** 1996. Growth models of complex allometries in holometabolous insects. American Naturalist **148**(1): 40-56.
- Nijhout, H. F. & Williams, C. M.** 1974a. Control of moulting and metamorphosis in the tobacco hornworm, *Manduca sexta* (L.): cessation of juvenile hormone secretion as a trigger for pupation. The Journal of Experimental Biology **61**(2): 493-501.

- Nijhout, H. F. & Williams, C. M.** 1974b. Control of moulting and metamorphosis in the tobacco hornworm, *Manduca sexta* (L.): growth of the last-instar larva and the decision to pupate. *The Journal of Experimental Biology* **61**(2): 481-91.
- Oldham, S., Stocker, H., Laffargue, M., Wittwer, F., Wymann, M. & Hafen, E.** 2002. The *Drosophila* insulin/IGF receptor controls growth and size by modulating PtdInsP(3) levels. *Development* **129**(17): 4103-4109.
- Peters, R.** 1983. *The ecological implications of body size.* Cambridge University Press, Cambridge, U.K.
- Reeve, E. C. R. & Huxley, J. S.** 1945. Some problems on the study of allometric growth. In *Essays on Growth and Form Presented to D'Arcy Wentworth Thompson* (ed. W. E. LeGross Clark and P. B. Medawar). Clarendon Press, Oxford.
- Rountree, D. B. & Bollenbacher, W. E.** 1984. Juvenile hormone regulates ecdysone secretion through inhibition of PTTH release. *American Zoologist* **24**(3): A31-A31.
- Rulifson, E. J., Kim, S. K. & Nusse, R.** 2002. Ablation of insulin-producing neurons in flies: growth and diabetic phenotypes. *Science* **296**(5570): 1118-1120.
- Saegusa, H., Mizoguchi, A., Kitahora, H., Nagasawa, H., Suzuki, A. & Ishizaki, H.** 1992. Changes in the titer of bombyxin-immunoreactive material in hemolymph during the postembryonic development of the silkworm *Bombyx mori*. *Development, Growth & Differentiation* **34**(5): 595-605.
- Satake, S., Kawabe, Y. & Mizoguchi, A.** 2000. Carbohydrate metabolism during starvation in the silkworm *Bombyx mori*. *Archives of Insect Biochemistry and Physiology* **44**(2): 90-98.
- Satake, S., Masumura, M., Ishizaki, H., Nagata, K., Kataoka, H., Suzuki, A. & Mizoguchi, A.** 1997. Bombyxin, an insulin-related peptide of insects, reduces the major storage carbohydrates in the silkworm *Bombyx mori*. *Comparative Biochemistry and Physiology B* **118**(2): 349-357.
- Schlichting, C. D. & Pigliucci, M.** 1998. *Phenotypic evolution: a reaction norm perspective.* Sinauer Inc., Sunderland, MA.
- Shingleton, A. W., Das, J., Vinicius, L. & Stern, D. L.** 2005. The temporal requirements for insulin signaling during development in *Drosophila*. *PLoS Biol* **3**(9): e289.
- Shingleton, A. W., Frankino, W. A., Flatt, T., Nijhout, H. F. & Emlen, D. J.** 2007. Size and shape: the developmental regulation of static allometry in insects. *Bioessays* **29**(6): 536-48.

- Siegert, K. & Ziegler, R.** 1983. A hormone from the corpora cardiaca controls fat body glycogen phosphorylase during starvation in tobacco hornworm larvae. *Nature* **301**(5900): 526-527.
- Simmons, R. E. & Scheepers, L.** 1996. Winning by a neck: sexual selection in the evolution of giraffe. *American Naturalist* **148**(5): 771-786.
- Smaghe, G., Loeb, M. & Tirry, L.** 2001. *In vitro* and *in vivo* effects of a fat body extract on *Spodoptera littoralis*. *In Vitro Cellular & Developmental Biology-Animal* **37**(2): 90-92.
- Srygley, R. B.** 1993. Correlations of the position of center of body mass with butterfly escape tactics. *Journal of Experimental Biology* **174**(1): 155-166.
- Stieper, B. C., Kupershtok, M., Driscoll, M. V. & Shingleton, A. W.** 2008. Imaginal discs regulate developmental timing in *Drosophila melanogaster*. *Developmental Biology* **321**(1): 18-26.
- Strauss, R. E.** 1990. Patterns of quantitative variation in Lepidopteran wing morphology: the convergent groups Heliconiinae and Ithomiinae (Papilionoidea: Nymphalidae). *Evolution* **44**(1): 86-103.
- Thornhill, R. & Alcock, J.** 1983. The evolution of insect mating systems. Harvard University Press Cambridge, MA.
- Truman, J., Riddiford, L. & Safranek, L.** 1974. Temporal patterns of response to ecdysone and juvenile hormone in the epidermis of the tobacco hornworm, *Manduca sexta*. *Developmental Biology* **39**(2): 247-62.
- Truman, J. W., Hiruma, K., Allee, J. P., Macwhinnie, S. G., Champlin, D. T. & Riddiford, L. M.** 2006. Juvenile hormone is required to couple imaginal disc formation with nutrition in insects. *Science* **312**(5778): 1385-8.
- Weinkove, D. & Leivers, S. J.** 2000. The genetic control of organ growth: insights from *Drosophila*. *Current Opinion in Genetics & Development* **10**(1): 75-80.
- Weinkove, D., Neufeld, T. P., Twardzik, T., Waterfield, M. D. & Leivers, S. J.** 1999. Regulation of imaginal disc cell size, cell number and organ size by *Drosophila* class I-A phosphoinositide 3-kinase and its adaptor. *Current Biology* **9**(18): 1019-1029.
- Wickman, P.** 1992. Sexual selection and butterfly design- a comparative study. *Evolution* **46**(5): 1525-1536.

**Zhang, H., Stallock, J., Ng, J., Reinhard, C. & Neufeld, T.** 2000. Regulation of cellular growth by the *Drosophila* target of rapamycin dTOR. *Genes & Development* **14**(21): 2712-2724.

## Biography

Alexandra Tobler Rodriguez

### Born

Bogotá, Colombia, 14 June 1969

### Education

2009 PhD Duke University, Durham, NC  
2002 MS Biology University of Puerto Rico, San Juan, PR  
1995 BS Biology, University of the Andes, Bogotá, Colombia.

### Publications

- Nijhout, H.F., W.A. Smith, I. Schachar, S. Subramanian, A. Tobler and L.W. Grunert. 2007. The control of growth and differentiation of the wing imaginal disks of *Manduca sexta*. *Developmental Biology*, 302 (2): 569-576.
- Kapan, D. D., N. S. Flanagan, A. Tobler, R. Papa, R. D. Reed, J. Acevedo, M. Ramirez, L. Martinez, K. Maldonado, C. Ritschoff, D. Heckel and W. O. McMillan. 2006. Localization of Müllerian mimicry genes on a dense linkage map of *Heliconius erato*. *Genetics*, 173 (2): 735-757.
- Tobler, D. D. Kapan, N S Flanagan, C. Gonzalez, E. Peterson, C. D. Jiggins, J. S. Johnstson, D. G. Heckel & W. O. McMillan. 2005. First-generation linkage map of the warningly colored butterfly *Heliconius erato*. *Heredity* 94, 408-417.
- Salazar, C. A., C. D. Jiggins, C. F. Arias, A. Tobler, E. Bermingham, and M. Linares. 2005. Hybrid incompatibility is consistent with a hybrid origin of *Heliconius heurippa* Hewitson from its close relatives, *Heliconius cydno* Doubleday and *Heliconius melpomene* Linnaeus. *Journal of Evolutionary Biology* 18 (2): 247-256.
- Flanagan, N., A. Tobler, A. Davison, O. G. Pybus, D. D. Kapan, S. Planas, M. Linares, D. Heckel and W. O. McMillan. 2004. Historical demography of Müllerian mimicry in the neo-tropical *Heliconius* butterflies. *Proceedings of the National Academy of Sciences* 101(26): 9704-9709.
- Cavelier, J and A. Tobler. 1998. The effect of abandoned plantations of *Pinus patula* and *Cupressus lusitanica* on soils and regeneration of tropical montane rainforest. *Biodiversity and Conservation* 7(3): 335-347.

### Awards

- 2007 Duke University. Graduate School Conference Travel Award  
2006 Duke University. Biology Department Grant-in-Aid.  
2004 Duke University. Graduate School International Research Travel Award