

SEASONAL CONSEQUENCES FOR TEMPERATURE-SIZE RELATIONSHIPS IN THE
CORN EARWORM, *Helicoverpa zea* (LEPIDOPTERA: NOCTUIDAE)

By

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To my family

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LIST OF ABBREVIATIONS

CW	Critical weight: threshold weight for the hormonal cascade that results in pupation. Minimum weight a larvae must reach to have consistent timing for pupation.
DEW	Diapause entrance weight: minimum weight a larvae must reach to successfully enter pupal diapause.
DEX	Diapase exit weight: minimum weight a diapause-destined larvae must reach in the last larval instar to exit pupal diapause and survive to adult eclosion.
MVW	Minimum viable weight: minimum weight a larvae must reach in the last larval instar to survive to pupation.
PLM	Peak larval mass.
TSR	Temperature-size rule: general pattern observed in many ectotherms where body size decreases with increasing temperature.

Abstract of Thesis Presented to the Graduate School
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The majority of ectotherms exhibit a pattern of decreasing size with increasing temperature, known as the temperature-size rule (TSR). Despite the extent of this pattern, the effect of seasonality on the TSR is poorly understood. We use *Helicoverpa zea* (corn earworm) to determine the effects of seasonal dormancy programming (pupal diapause) on the TSR, lipid storage, and larval development. *H. zea* did not conform to the TSR over the seasonally-relevant temperature range where pupae entered diapause. *H. zea* increased in both lipid and lean mass with increasing temperature for 16°C to 20°C in both diapause-destined and non-diapause treatments. Diapausing *H. zea* were fatter across temperatures without having more lean mass, and the slope of the relationship between lean mass and temperature was not different between diapausing and non-diapausing pupae. Across temperatures, diapause-destined larvae increased their consumption but not their digestive efficiency, supporting increased pupal lipid mass. We found threshold weights for entrance to and exit from diapause, and diapause-destined larvae altered the timing for pupation, further reinforcing the importance of size to seasonal diapause. In conclusion, predictions of seasonal effects

on body size must incorporate cues other than temperature, to avoid inaccurate or incomplete models of development and body size.

CHAPTER 1 INTRODUCTION

What affects an animal's body size? How do animals determine when they have grown 'enough', and how do environmental factors alter optimal body size? These questions are critical to understanding development, but the physiological mechanisms underlying responses of body size to seasonal environmental changes remains a complex and understudied subject. It is well known that temperature is a major cause of body size variation in ectotherms (Angilletta 2009), with the majority of ectotherms conforming to the temperature-size rule of smaller body size at warmer temperatures (Atkinson 1994). Hypotheses explaining this relationship between temperature and size lack consensus, and often fail to explain exceptions to the temperature-size rule (Blanckenhorn and Demont 2004). Although seasonal shifts in environmental conditions are nearly ubiquitous across terrestrial habitats, the effects of environmental shifts other than temperature on temperature-size relationships are poorly understood.

Many organisms use phenotypic plasticity, in the form of cued dormancy, to escape from predictably poor environmental conditions. Diapause is a programmed state of arrested development that increases stress resistance and lowers metabolism. Diapause is often facultative, i.e. only occurs when the proper environmental "token stimuli" such as changes in photoperiod signal the onset of poor conditions (Danks 1987). Many insects escape from poor conditions by using diapause, but it is an energetically expensive strategy, and often results in reduced adult fitness after exiting diapause (Saunders 2000; Karlsson et al. 2008). Diapause is also a risky strategy because smaller individuals may exit diapause early while conditions are still poor or may fail to survive diapause (Danforth 1999; Matsuo 2006). Some individuals that are

small may side-step diapause entirely and directly develop even when presented with diapause-inducing conditions (Saunders 1997).

Given the costs of diapause, many insects that diapause in non-feeding stages, when presented with diapause-inducing conditions, will acquire more energy (lipid) storage. Insects preparing for diapause will likely possess a threshold weight for entering and exiting diapause – i.e. a weight below which insects will avert diapause even if they are exposed to diapause-inducing conditions (Saunders 1997; Hahn and Denlinger 2007). Diapause-bound larvae can increase their lipid stores by increasing their resource acquisition: by increasing the amount of food consumed, increasing their digestive efficiency, or a combination of both. Insects may be altering their resource acquisition by changing their larval development - many insects express developmental thresholds for pupation which may be manipulated to produce larger body sizes. For example, the minimum viable weight for pupation (MVW) is the threshold weight below which a larva will not survive to pupation and the critical weight (CW) is the threshold weight below which the timing for pupation will be disrupted. Diapause-bound larvae may alter these threshold weights to increase their resource acquisition.

The goal of this research was to determine the effects of seasonally-relevant changes in temperature and photoperiod on body size and lipid storage. I also tested how seasonal shifts affect larval development, specifically how resource acquisition and larval threshold weights were altered by temperature and photoperiod. I tested these effects by rearing *Helicoverpa zea* (corn earworm) larvae under different temperature, photoperiod, and feeding regimes. Over the range of temperatures in which individuals will diapause (Figure 1.1), *H. zea* demonstrated a response to temperature that was

opposite the general temperature-size rule, i.e. *H. zea* increased in size with increasing temperature. It is possible that *H. zea* actually demonstrated a dome-shaped interaction between temperature and size, with maximum size at an intermediate temperature. However, our data were too sparse at higher temperatures to assess this pattern (Figure 1.2). Nevertheless, it is informative to use this system to study temperature-size relationships because analysis of exceptions to general patterns can give valuable insight into the mechanisms that drive those patterns. Also, *H. zea* possesses an easy-to-manipulate and easily-studied diapause state, making this an excellent system for studying diapause effects on temperature-size relationships.

I found that, over the seasonally relevant range of temperatures in which pupae entered diapause, mass increased linearly with temperature in *H. zea*. In addition, diapausing individuals were fatter than non-diapausing individuals were. Temperature and diapause induction (photoperiod) altered both food consumption and digestive efficiency. Diapause induction also altered pupal threshold weights and introduced diapause threshold weights. Taken together, these data show that temperature alone did not adequately describe seasonal effects on body size.

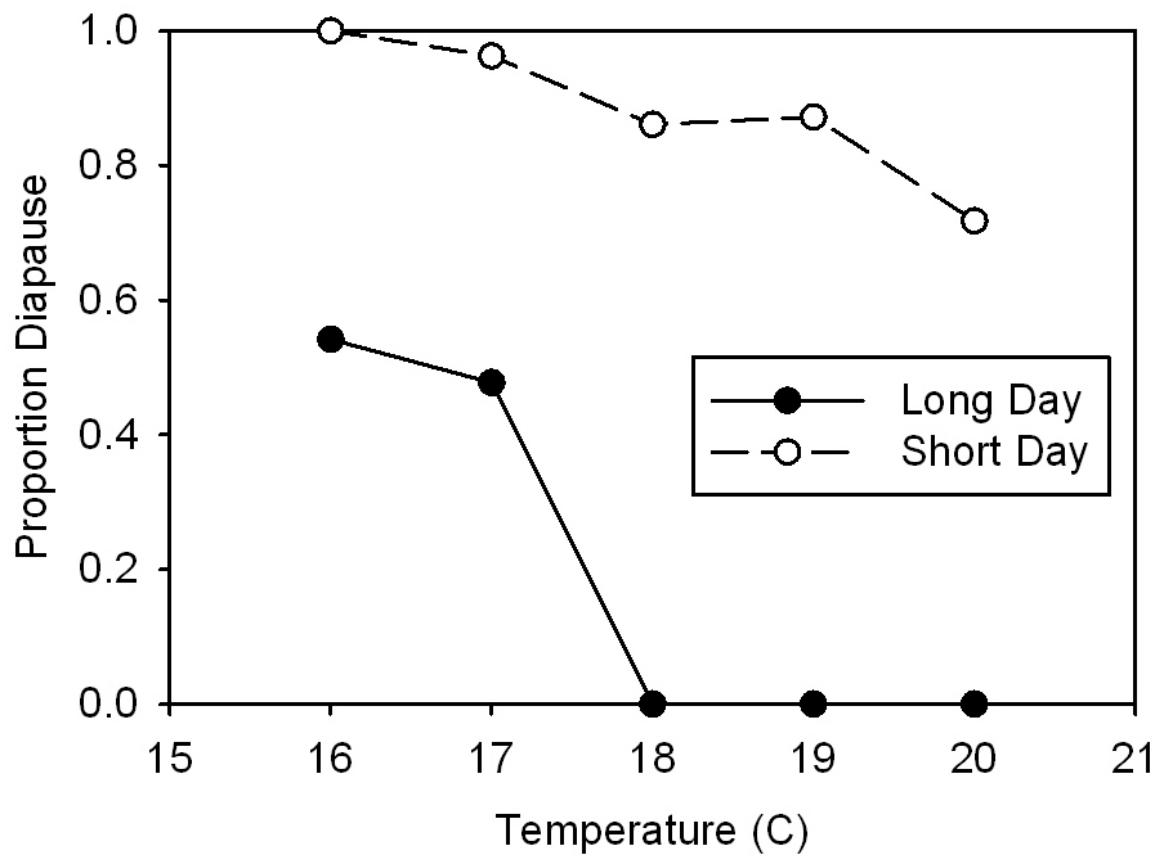


Figure 1-1. Diapause incidence in long day (14L:10D) and short day (8L:16D) conditions at different temperatures.

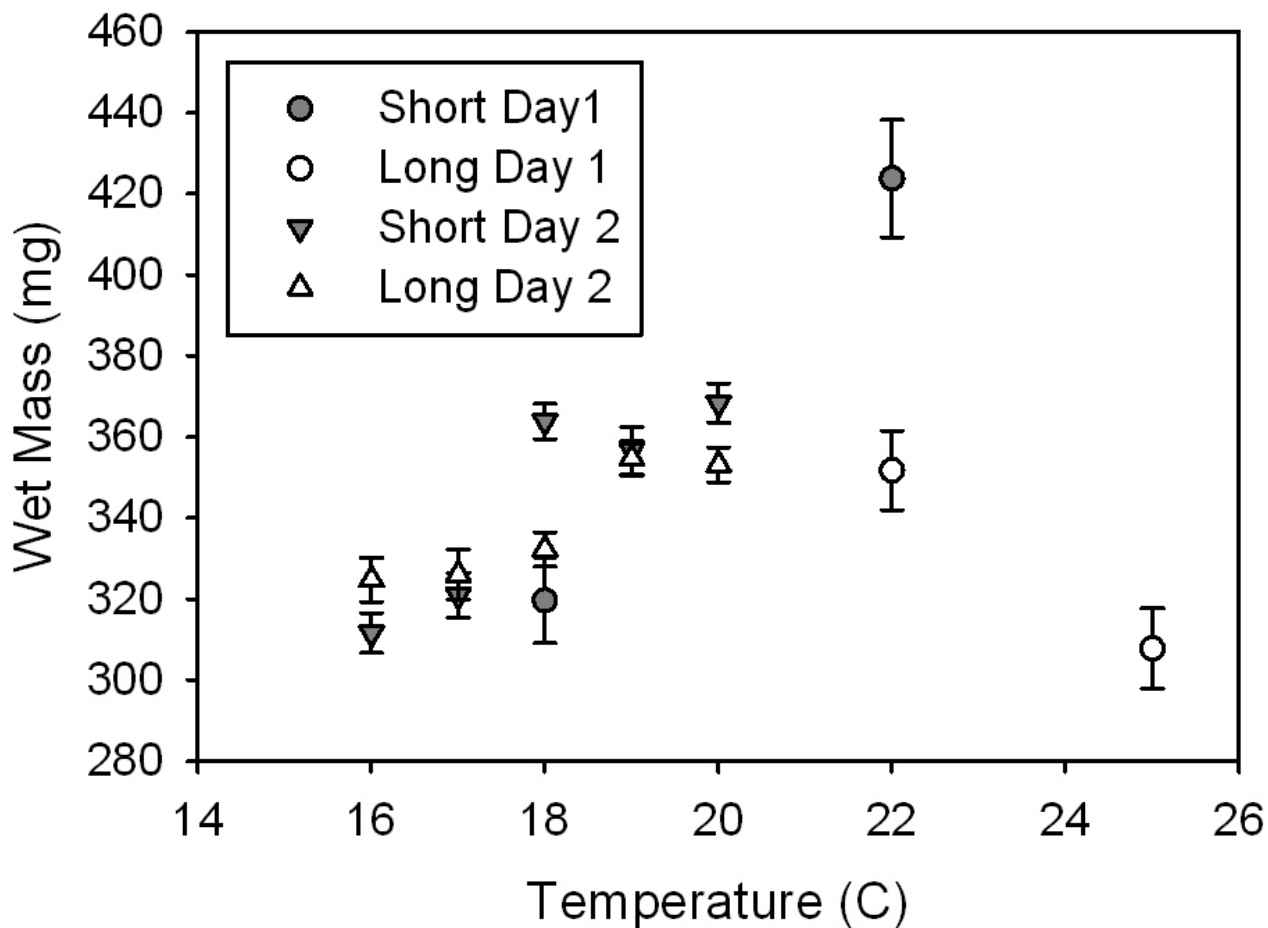


Figure 1-2. Temperature-size relationship for *Helicoverpa zea* (corn earworm) with pupal wet mass. Circles indicate a preliminary rearing experiment using 18, 22, and 25°C; triangles indicate data from experiment 1 (temperature-size experiment) at 16, 17, 18, 19, and 20°C.

CHAPTER 2

THE CORN EARWORM, *Helicoverpa zea* (LEPIDOPTERA NOCTUIDAE)

Helicoverpa zea (Boddie), the corn earworm, is a Noctuid moth species in the Heliothinae subfamily. *H. zea* has many common names, including tomato fruitworm, sorghum headworm, vetchworm, and cotton bollworm. The Heliothine group is an economically important Noctuidae subfamily because it contains species that cause severe damage to crops world-wide (Fitt 1989). In the U.S. an estimated \$1 billion in damage annually was caused by *Helicoverpa zea* and *Heliothis armigera* in the early 1980s (Reed and Kumble 1982). Most species within Heliothinae are host-specific; however, the pest species within this group are all highly polyphagous, which among other characteristics (high mobility, high fecundity, and a facultative diapause) is the probable reason for their incredible success as a pest species (Fitt 1989). *H. zea* in particular is a major pest of corn and cotton, and also survives on a variety of wild hosts (Neunzig 1963; Capinera 2001). Biological control has been ineffective in controlling *H. zea* activity, and therefore is a species of considerable economic and ecological interest (Capinera 2001).

Life History of *Helicoverpa zea*

The life cycle of *H. zea* is highly dependent on latitude and the length of the growing season; individuals generally complete their life cycle in 30 days, and can have between 1 and 7 generations per year (Capinera 2001). The high dispersal capabilities of adults lead to a yearly re-colonization of regions that are too cold for *H. zea* to overwinter successfully, higher than 40°N and 40°S latitude (Fitt 1989); resulting in incidence of *H. zea*, and its status as a pest species, throughout the continental United States and western Canada during the summer months (Capinera 2001). In areas

where *H. zea* successfully overwinters, adults emerge between March and April and begin mating.

Female moths lay eggs on leaf hairs or corn silk, and hatch 3 to 4 days later. The larvae are polyphagous and feed most often on plant reproductive structures but as they grow larger, larvae will become aggressive and will cannibalize other larvae. Individuals typically have 6 larval instars, but 5 or 7 instars can occur (Capinera 2001). After the growing period larvae drop off the host plant, burrow into the soil, and prepare a pupal chamber. In warm temperatures the pupal stage will last 13 days, with increased time in cooler temperatures and over 250 days in pupae that overwinter in the pupal stage (Capinera 2001).

H. zea can use facultative dormancy to avoid poor environmental conditions. Before the onset of winter, changes in temperature and photoperiod during the larval stage signal the end of the growing season and cue the individual to enter pupal diapause, a state of developmental arrest characterized by increased stress tolerance and depressed metabolism (Tauber et al. 1986). *H. zea* generally overwinters between 40°N and 40°S in North and South America, and emerges when temperatures signal the return of conditions suitable for growth (Fitt 1989). Adults emerge from pupal chambers and begin nocturnal mating and oviposition, usually correlated with lunar cycles (Parajulee et al. 1998). Females lay eggs in singletons, and can lay 500 – 3000 eggs over the reproductive period (Capinera 2001). Male and female adults are highly dispersive over local and geographic regions (Jackson et al. 2008; Westbrook 2008).

Phylogeny of Heliothinae

The subfamily Heliothinae (Lepidoptera: Noctuidae) contains several important pest species, including *Helicoverpa zea*. Constructing an accurate phylogeny can

assist in understanding what characters define these species and therefore what may be predictive traits for other potential pests (Fitt 1989). In addition, knowing how distantly lineages diverged enables more accurate predictions of organismal responses to various stimuli. Classifying the Families of the Order Lepidoptera is especially challenging because the fossil record for this group is so sparse (Grimaldi and Engel 2005; Kristensen et al. 2007). Constructing phylogenies of Lepidopterans depends on current morphological traits and more recently on molecular data.

It is difficult to be certain about origin of the first Lepidoptera species – the wing venation is particularly unlikely to survive intact under the conditions required for fossilization (Kristensen et al. 2007). It is hypothesized that the earliest Lepidopterans arose 190 mya in the early Lower Jurassic (Grimaldi and Engel 2005), leading to a large diversification in lineages. One of the major innovations within Lepidoptera is the use of hearing via tympanal organs to avoid predation by bats (Spangler 1988), organs with at least 8 independent origins in Lepidoptera (Grimaldi and Engel 2005). The first true Noctuid is estimated to have arisen between 50-60 mya and the clade has a synapomorphy in its unique tympanal organs situated on the metathorax between the postnotum and the epimeron (Grimaldi and Engel 2005).

Twenty years ago, the subfamilies of the Noctuidae Family was described by Poole as “in utter chaos”, and “too poorly known to attempt any type of a phylogenetic arrangement” (1989a). This is due to the relative simplicity of these derived groups – generally it is difficult to characterize the subfamilies of Noctuidae because they lack unambiguous synapomorphies (Mitchell et al. 2000). The first complete recording of all known Noctuidae genera was compiled by Poole, but no attempt at a phylogenetic

reconstruction was made at that time (1989b). Several attempts have been made to construct phylogenies for this Family, but the most current proposed phylogeny has yet to achieve a robust status.

Phylogenies were first based on the venation pattern of M2 on the hindwing (Mitter, Poole, and Matthews 1993). “Trifine” subfamilies are thought to be monophyletic, and “quadrafine” subfamilies were originally thought to be monophyletic but are now unresolved (Weller et al. 1994). Notodontidae is thought to be a proper outgroup, but the the monophyly of the Noctuidae is unclear both within the other derived ditrysians and in the Family, as it is currently thought to encompass other Families (Arctiidae and Lymantriidae) within the “Noctuid” clade (Grimaldi and Engel 2005; Mitchell et al. 2006).

Several attempts have been made to place Heliiothinae within Noctuidae, relying first on allozyme and then later on genetic divergence. The earliest phylogeny supported grouping Heliiothinae with Stiriinae as its sister group (Hardwick 1965), but in recent years Heliiothinae had been grouped with multiple other groups, including Hadeninae (Weller et al. 1994), Agaristinae (Poole 1995), Stiriinae (Kitching and Rawlins 1998), and most recently Condicinae (Mitchell et al. 2006). All proposed phylogenies group Heliiothinae within the trifine clade. Despite the lack of fossils characteristic of most Lepidoptera, Heliiothinae is thought to be a monophyletic group because of characteristic morphological features such as the setae arrangement and the spines on the larval cuticle (Lafontaine 1987).

The Heliiothinae phylogeny in use for many years was proposed by Hardwick (1965), and remained unchanged until the use of genetic data became feasible in the

field of systematics. The most recent phylogenies have drawn heavily on other research – when a new gene is used to make a phylogeny the investigators have strived for “convergence” parsimony, or phylogenies that use older data as a template for the next phylogeny hypothesis (Cho et al. 1995; Cho et al. 2008; Fang et al. 1997; Mitchell et al. 2000).

Notable genus changes have occurred within Heliothinae. The first example is the splitting of the *Heliothis* to include *Helicoverpa* – used by Poole (1989b) but not achieving widespread useage in the literature until some years later (Fitt 1989). There are currently no known fossils for the major pest genus *Helicoverpa*, making is difficult to estimate their origin; it is thought that *Helicoverpa* originated no earlier than the late Tertiary based on the limited allozyme divergence with other Heliothine groups (Mitter et al.1993). Another example of genus changes within Heliothinae is the addition of *Australothis* by Matthews (1991), suggested as a sister group to *Helicoverpa* and remaining stable since its introduction into the literature.

There are several major monophyletic groups within Heliothinae. The Pyrrhia group is probably the earliest-diverging group in the subfamily, proposed by Matthews (1991) and supported by more recent data (Cho et al. 2008). The major genera currently placed in the Pyrrhia group include *Rhodoecia*, *Pyrrhia*, *Eutricopis*, and *Heliothodes* (Cho et al. 2008). The second monophyletic group is the Schinia group, containing the major genera *Schinia* and *Heliolonche* (Cho et al. 2008). The third major monophyletic group is the Heliothis group, which contains the smaller group of “major pest lineage” (*Helicoverpa*, *Australothis*, some *Heliothis*) as well as “typical” *Heliothis* (Cho et al. 2008).

In the “major pest lineage”, the *Heliothis virescens* group is ancestrally polyphagous (Poole 1995), yet within the group is *Heliothis subflexa* (Mitter et al. 1993; Fang et al. 1997) – a specialist species. The “loss” of polyphagy in this species is of considerable interest because it presents a relatively recent divergence (Poole 1995). Recent divergence allows close genetic and behavioral studies of specialist/generalist host preference (Sheck and Gould 1996; Oppenheim and Gould 2002), research that is critically important to understanding and predicting pest species (Fitt 1989).

New species of Heliethinae continue to be discovered in the field (Matthews and Patrick 1998). This is likely to continue for years to come because the total number of Lepidoptera species is probably double to quadruple the number of species currently described (Kristensen et al. 2007). Thus, the likelihood of discovering (or introducing) new pest species is high. At the same time, the distinction between species is difficult because the morphological traits are very similar, resulting in conflicting reports of “new” species that in actuality belong to already-established groups; as well as mis-identification of specimens in the field (Pogue 2004). Phylogenetic frameworks could play an important role in predicting or managing new pest problems, so it is critical that current phylogenies are as accurate as possible (Fitt 1989).

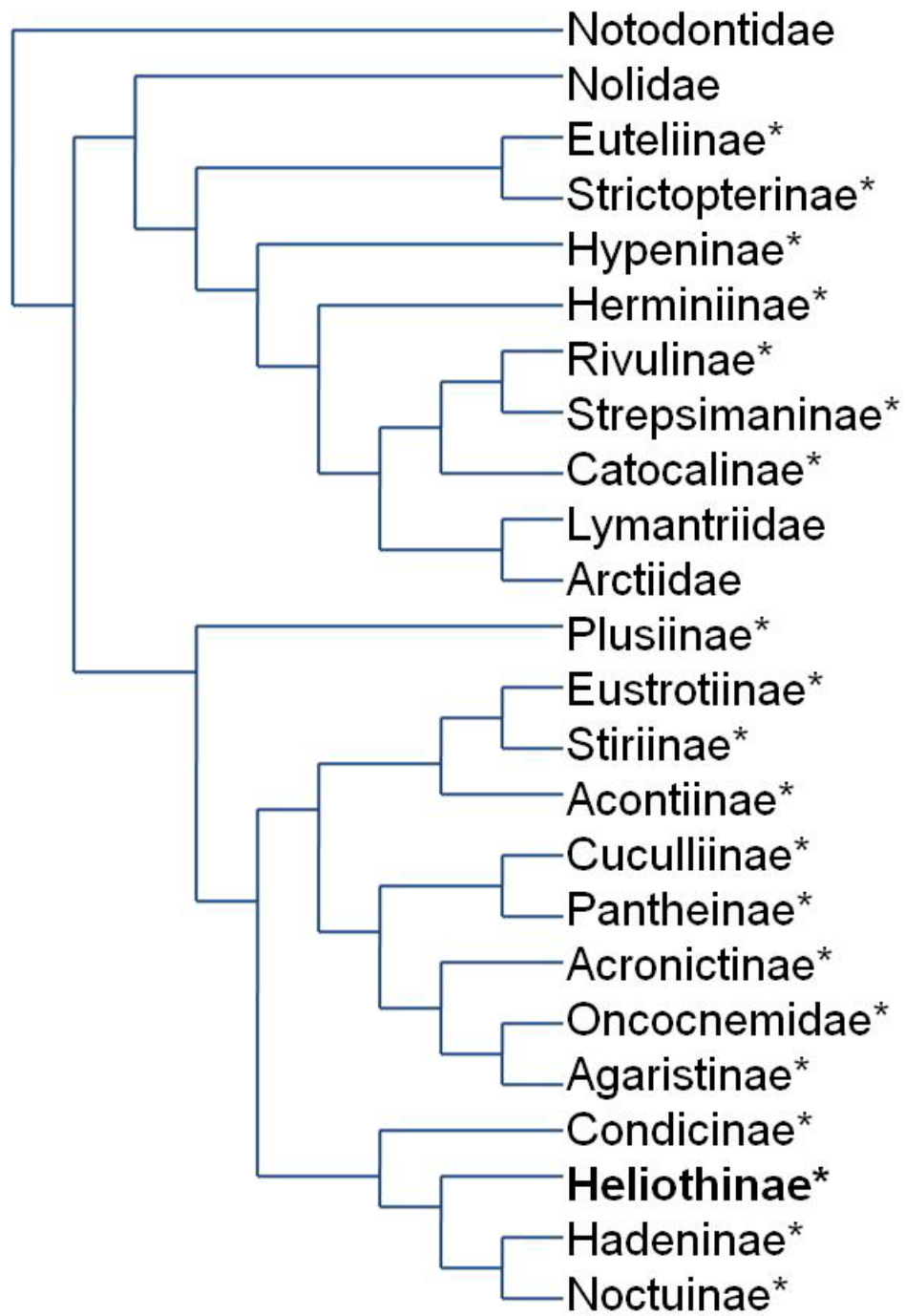


Figure 2-1. Phylogeny of Noctuidae, condensed from Mitchell et al. 2006. * indicates Noctuidae Families

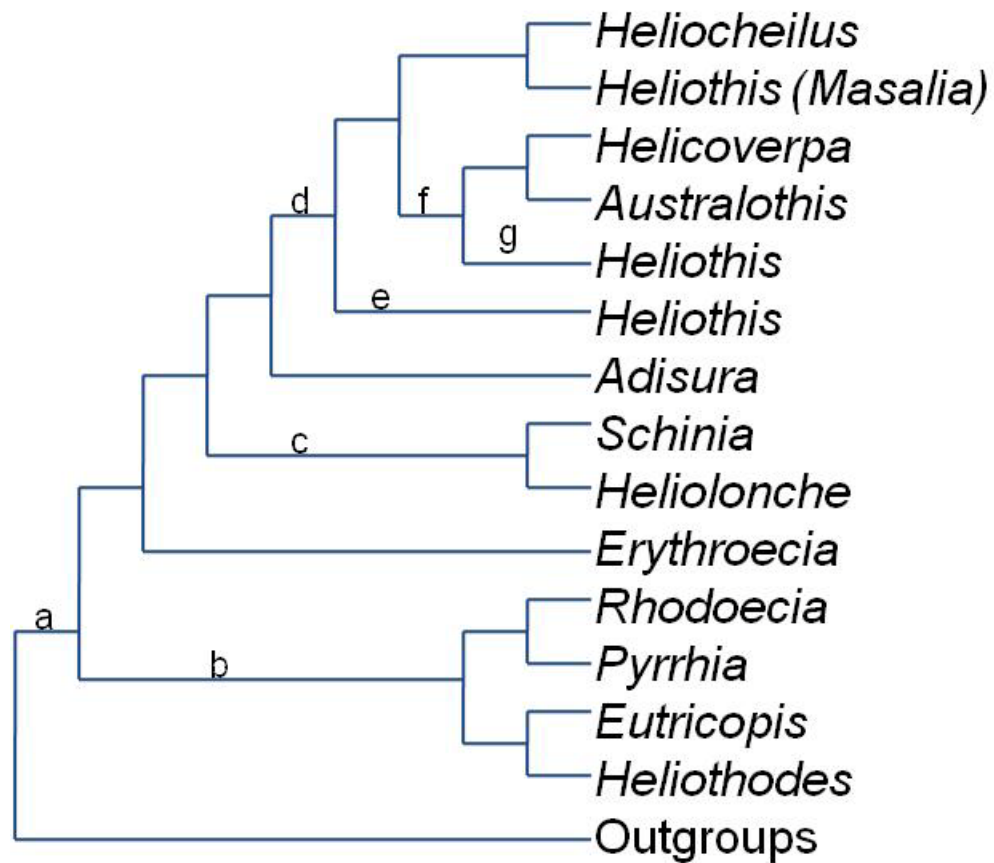


Figure 2.2. Phylogeny of Heliethinae subfamily, modified from Cho et al. 2008. a: Heliethinae, b: *Pyrrhia* group, c: *Schinia* group, d: Heliethis group, e: “typical” *Heliethis*, f: “major pest lineage”, g: *Heliethis virescens* group

CHAPTER 3

SEASONAL CUES ALTER THE TEMPERATURE-SIZE RELATIONSHIP IN THE MOTH *Helicoverpa zea*

Note: This chapter consists primarily of a manuscript prepared for submission to The American Naturalist, with the addition of figures (Fig 3.1 – 3.6) in the introduction for increased clarity (figures were also renumbered for consistency within the thesis, and the literature cited combined with the main references section).

Introduction

Body size is a critical aspect of an animal's life history because it has direct effects on numerous fitness correlates like survival and fecundity (Nylin and Gotthard 1998; Kingsolver and Huey 2008). Yet plasticity in body size is nearly ubiquitous, with body size responding to a myriad of factors, including developmental temperature (Kingsolver and Nagle 2007). The majority of ectotherms, some estimate 80%, show decreased body sizes as temperature increases, a response termed the temperature size rule (TSR) (Atkinson 1994). However, some ectotherms show no relationship or a reversed relationship with some species growing larger at higher temperatures (Atkinson 1994; Blanckenhorn and Demont 2004). The applicability of the temperature-size rule as a general rule and the proximate physiological and ultimate selective mechanisms that produce thermally-induced plasticity in size are sources for debate (Atkinson and Sibly 1997; de Jong 2010; Watt et al. 2010).

Multiple factors can affect body size, and it is likely that interacting forces drive temperature-size patterns. It is also likely that the interaction of multiple forces explain species that show the opposite of the temperature-size rule (Atkinson 1995), and species that show reversals in the temperature-size rule across populations and environments (Kingsolver et al. 2007; Diamond and Kingsolver 2010). One model with

wide theoretical support posits that thermodynamic constraints drive organismal responses; specifically that increases in temperature act to increase growth rate and decrease development time (Clarke 2003; Walters and Hassall 2006). The interaction of these two factors can result in the temperature-size rule if growth rate increases are small relative to decreases in development time (Davidowitz and Nijhout 2004). While this model most often describes species that follow the temperature-size rule, the interaction between temperature, growth rate, and development time can also result in a reversal of the temperature-size rule – increased size with increasing temperature – if increases in growth rate are large relative to decreases in development time (Davidowitz and Nijhout 2004).

Some optimality models predict that the temperature-size rule is adaptive (Berrigan and Charnov 1994; Wilson 2009), resulting from an advantageous response to temperature (but see Stillwell et al. 2008). Selection can also act to alter physiological relationships between temperature and growth to compensate for temperature effects on body size. Thus adaptation can modify responses to thermodynamic constraints (Frazier et al. 2006; Angilletta et al. 2010; Chown and Gaston 2010). External forces can also modify the size response to temperature changes. The nutritional quality of a population's diet can act to reinforce the temperature-size rule if plant quality is greater at colder temperatures (Ho et al. 2010), or reverse the temperature-size rule when animals feed on low-quality plants (Diamond and Kingsolver 2010). Changes in diet quality may particularly affect animals towards the end of the growing season when both temperature and plant quality are decreasing. Specifically, at the end of the growing season, decreasing plant quality has a negative

effect on body size, while cooler temperatures may have a negative or positive effect on body size. Despite the huge impact that seasonal fluctuations have on development, few studies have examined how seasonality may act to modify the temperature-size rule in a single population (but see Atkinson 1995; Davidowitz et al. 2004).

Seasonal changes are ubiquitous and nearly all organisms experience some time during the year when conditions are too stressful for growth and reproduction. Many organisms have evolved facultative seasonal dormancy to avoid stressful periods of the year and time their life cycles to exploit good periods (Stearns 1992; Denlinger 2001; Roff 2001). Prior to the onset of poor conditions, many invertebrate ectotherms use predictable seasonal cues like photoperiod to enter diapause, a state of arrested development accompanied by enhanced stress resistance and metabolic suppression (Danks 1987). While seasonal diapause is advantageous, it is not without costs. Diapause during non-feeding stages (i.e. during post-growth larval or pupal stages) are especially costly for insects because they must not only have enough energy reserves to survive many months in diapause, but must also have enough reserves to complete their development after diapause is terminated (Ellers and van Alphen 2002; Hahn and Denlinger 2007).

Comparisons between individuals that have and have not undergone diapause show that diapause can result in reduced adult size (Saunders 2000; Matsuo 2006) and reduced fecundity (Karlsson and Wickman 1989; Ishihara and Shimada 1995; Karlsson et al. 2008). The costs of diapause are especially apparent in smaller individuals raised in diapause-inducing conditions, which are less likely to survive diapause (Pullin 1987; Ellers and van Alphen 2002; Matsuo 2006), may fail to diapause (Saunders 1997), or

may exit diapause sooner (Danforth 1999). Many insects are able to detect their nutritional status (Nijhout 2003; Stern 2003), and it has been suggested that individual condition will affect developmental decisions, with poor condition individuals avoiding diapause (Hahn and Denlinger 2007). The costs of diapause lead to the conclusion that an insect preparing to diapause that has low nutrient stores can either continue on its diapause-destined development or, if it is too small it can avert diapause and risk trying to complete development before the expected onset of poor environmental conditions (Saunders 1997; Hahn and Denlinger 2007). Thus there may be a minimum threshold size for entering diapause. After entering diapause, individuals may also have a threshold weight for exiting diapause, the minimum weight necessary for a diapausing pupa to survive to adult eclosion. Energetic costs should pressure insects to increase their body size as they prepare to diapause in poor conditions, which could alter the response to thermodynamic constraints. Diapause-induction could cause increased size across temperatures or could potentially reverse the temperature-size rule, because the diapause preparatory program would override thermodynamic constraints to meet the increased energetic cost of diapausing at warmer temperatures (Irwin and Lee 2003).

Some facultatively-diapausing insects compensate for the energetic costs of diapause by accumulating more nutrient stores than non-diapausing individuals, and fat is the most commonly stored resource (Hahn and Denlinger 2007). Temperature may alter fat storage and somatic size (lean mass) independently from each other, so it is important to consider both fat and lean mass relationships, instead of the overall body mass relationship, when placing temperature-size relationships in a seasonal context.

Individuals may accumulate greater fat storage prior to diapause in several ways. First, they could have an isometric increase in both lipid and their non-lipid “lean” mass (bigger and fatter, an isometric increase – Figure 3.1). Second, they could increase both their lipid and lean mass, but have a disproportionate increase in lipid mass (bigger and much fatter, an allometric increase – Figure 3.2). Third, they could increase lipid mass without increasing lean mass (just fatter – Figure 3.3). Finally, they could increase lipid mass at the expense of lean mass (a trade-off – Figure 3.4). The nutrient accumulation strategy employed will likely have distinct effects on developmental trajectories and resultant temperature-size relationships.

Temperature-induced increases in body size will necessarily increase nutrient acquisition. Nutrient acquisition needed to support greater growth and body size may come from eating more or eating better food (Bernays and Minkenberg 1997). Diapause-bound individuals can also increase their processing efficiency, so that instead of consuming more raw resources they produce less waste (Simpson et al. 1995). We expect that temperature and seasonal modes of temperature-size relationships (i.e. diapause induction) will affect resource acquisition by altering feeding and digestive patterns.

If diapausing pupae have more nutrient reserves than non-diapausing pupae, then they must be changing their larval development to acquire greater fat stores in order to survive diapause. One mechanism that holometabolous insects may use to alter their development is the alteration of their pupal threshold weights. Threshold weights for pupation, originally described for the tobacco hornworm moth *Manduca sexta*, have two components. The minimum viable weight indicates the weight below which a last-instar

larva will not survive to pupation (Figure 3.5; Nijhout 1975). The critical weight indicates the weight at which the animal reaches the physiological trigger for pupation, below the critical weight larvae will delay pupation (Figure 3.6; Nijhout and Williams 1974a; Nijhout and Williams 1974b; Nijhout 1981). Diapause-destined larvae can potentially extend their feeding period and acquire more resources prior to entering larval or pupal diapause by increasing their threshold weights. Shifts in threshold weights, potentially resulting in shifts in adult size, may therefore act to modify temperature-size relationships.

Here, we addressed several important questions regarding seasonal effects on body size and fat storage; and how nutrient acquisition and development changed to support size alterations. We used the Noctuid moth *Helicoverpa zea* (corn earworm) to test these relationships because its diapause stage was readily inducible, and its relative large size and short life cycle enabled large sample sizes. Over the temperature range where *H. zea* entered diapause, pupae showed a positive relationship between temperature and size (in opposition to the temperature-size rule, which predicts a negative relationship between temperature and size). We tested whether seasonal cues affect temperature-size relationships by raising larvae in different temperatures and photoperiods. We also determined relationships between lipid and lean mass of diapausing and non-diapausing individuals (isometric increase, allometric increase, only-lipid increase, or trade-off), and how this relationship changed over a range of physiologically-relevant temperatures. We evaluated nutrient acquisition by quantifying food intake and waste production to determine how seasonality (i.e. photoperiod) altered the relationship between temperature and food

consumption and processing. We then identified specific weight points that signaled the entrance to diapause and exit from diapause at a single temperature using a larval starvation experiment. Finally, we addressed whether photoperiod changed developmental thresholds in diapause-destined individuals by using the larval starvation experiment to compare minimum viable weights and critical weights between larvae raised in diapause-inducing or direct development conditions at a single temperature.

Methods

General Rearing Methods and Parameterizing the Photoperiodic-Diapause Response

Helicoverpa zea eggs were obtained from the North Carolina State University Insectary and maintained in colony at the USDA CMAVE facility at the University of Florida. Experimental eggs were kept at room temperature (21-22°C) until hatching. After hatching approximately 65 larvae per 5 oz cup were reared in a 25°C chamber at 14L:10D (long-day) conditions. Larvae were fed tobacco budworm diet from BioServ (Frenchtown, New Jersey; #F9781B, wheat germ base, Aureomycin antibiotic). Upon reaching the third of six instars, discernible by a change in head capsule size and color, larvae were individually reared in 1 oz cups in chambers at the treatment temperature in either summer-like long-day (14L:10D) or fall-like short-day (8L:16D) photoperiodic conditions. Temperatures within a treatment were kept constant with temperature fluctuation of +/- 0.5°C or less. Pupae were sexed using the location of the gonopore and anus and the size of the last abdominal segment; pupae were then scored for diapause by the presence/absence of pupal eyespots (Phillips and Newsom 1966). The disappearance of the pupal eyespots is concurrent with adult development in *H. zea*, so individuals that retain pupal eyespots have suspended their development and are in

diapause. Because lower temperatures slow development rates, non-diapausing pupae in lower temperatures took longer to lose their pupal eyespots, so individuals were scored for diapause at an interval that was physiologically relevant to the temperature they were experiencing (between 8 and 12 days after pupation).

To parameterize the relationship between temperature and diapause for *H. zea*, larvae were initially reared in different temperatures and photoperiods: 18, 19, 20, 22, and 25°C in long-day (14L:10D) and short-day (8L:16D) conditions. Larvae were provided unlimited food, and were weighed 2 days after pupation. All larvae were scored for diapause 8-10 days after pupation. In this system, we found an interaction between photoperiod and temperature on diapause incidence. High temperatures will override photoperiodic programming and individuals will not diapause if raised in short-day (8L:16D) conditions at or above 22°C. Therefore further experiments were done below 22°C, to obtain a seasonally-appropriate diapause response.

Experiment 1

To determine effects of diapause on the temperature-size relationship, larvae were raised at 5 different temperatures (16 – 20°C) and 2 photoperiods (14L:10D long-day and 8L:16D short-day) with 90 larvae per treatment. Larvae were fed *ad libitum* and after reaching the last instar were checked daily for pupation. Individuals in the long-day 18, 19, and 20°C conditions were weighed and moved to a -20°C freezer 2 days after pupation because trial rearings at these temperatures under long-day conditions showed zero diapause incidence (n = 60 individuals/treatment). All other treatments were scored for diapause at the biologically relevant time after pupation for a given temperature (12 days at 16°C, 10 d at 17 and 18°C, 9 d at 19°C, and 8 d at 20°C), determined by daily observations of subsamples of individuals until the disappearance

of pupal eyespots; pupae were then frozen at -20°C. Subsamples of all treatment groups were freeze-dried and weighed for total mass; then placed in perforated gelatin capsules in a Soxhlet extractor and extracted with diethyl ether for 48 hrs to remove neutral-lipids (Newman et al. 1972; Tschinkel 1993). After extraction, pupae were freeze-dried again and weighed for lean mass. Lipid mass was calculated by subtracting the lean mass from the total mass.

We also measured food consumption and waste production for each larva. Wet mass for all food provided to the larvae was measured, and estimates of the dry mass of diet provided were obtained by using a standard curve of fresh diet to dry diet mass. Uneaten food and waste were each collected and placed separately in a drying oven for 5 days at 40°C. Food consumption was calculated by subtracting the dry mass of uneaten diet from the estimated dry mass of diet provided to each larva.

Experiment 2

This experiment limited food consumption in groups of larvae to determine threshold (minimum) weights for pupation and diapause. Forty-five larvae were raised in each treatment group; all were raised in 19°C in either long-day (14L:10D) or short-day (8L:16D) conditions because this temperature minimized the possibility of individuals entering diapause in long-day conditions while maximizing the number of individuals that entered diapause in short-day conditions. After molting to the 5th instar (determined by checking the head capsule size) larvae were observed twice a day for the molt to the 6th (last) larval instar. Larvae were then moved from the diet to a 5% agar block to prevent desiccation. The first group was moved a half day after molting to the last instar, the second group 1 day after, the third group 1.5 days after molting, and so on up to 4.5 days after molting. A final group was never removed from the *ad libitum*

diet, for a total of 10 food-removal groups in each photoperiod treatment. Peak larval mass (PLM) was obtained by weighing each larva immediately after removal from the diet for all starvation groups. Larvae were observed twice a day until they either molted to pupae or until death. Pupae were removed from the agar block and weighed 2 days post-pupation. Pupae in the short-day (8L:16D) treatment were scored for diapause 9 days after pupation. Pupae in all groups were observed until exit from diapause (disappearance of the pupal eyespots) and adult eclosion, or death.

Statistical Analyses

With the exception of the critical weight calculation in experiment 2, all statistical analyses were performed using JMP v7 (SAS Institute, Cary, North Carolina). In experiment 1, lean and lipid mass were analyzed in 352 individuals. Lean mass was evaluated using analysis of variance (ANOVA), with temperature, sex, and photoperiodic response (long-day non-diapause or short-day diapause) as fixed effects and extraction group as a random effect (REML). Interactions among all fixed effects were initially included in the model, and the analysis was repeated after excluding non-significant interactions. Lipid mass was evaluated with an analysis of covariance (ANCOVA) with lean mass as a covariate; temperature, sex, and photoperiodic response as fixed effects; and extraction group as a random effect. Food consumption was analyzed with an ANOVA model with temperature, diapause status, sex, and temperature by diapause status interaction effects (after excluding non-significant interactions). Waste production was initially analyzed with an ANCOVA with food consumption as a covariate and temperature, sex, diapause status, and all interactions as fixed effects. A significant interaction between food consumption and sex rendered the analysis invalid for evaluating other effects. Therefore, to evaluate digestive

efficiency an ANOVA for the ratio for waste production to food consumption was used with temperature, diapause status, sex, and interaction effects.

In experiment 2, diapause entrance weight (DEW), diapause exit weight (DXW), and minimum viable weight (MVW) were calculated as the 50% inflection points of logistic regressions. The DEW was calculated by comparing the peak larval mass (PLM) to the number of diapausing and non-diapausing pupae in the short-day groups. The DEX was calculated by comparing the PLM to the number of individuals that did and did not successfully eclose in the short-day group. The MVW was calculated by comparing the PLM to the number of successful and unsuccessful pupal molts. The MVWs for long-day and short-day conditions were compared by evaluating overlap of 95% confidence intervals.

Critical weights for long-day non-diapausing and short-day diapausing pupae in experiment 2 were first calculated by segmented linear regressions in R v2.10.1 (R Foundation for Statistical Computing, Vienna, Austria). Segmented regressions are sensitive to initial conditions, so we repeated the regressions with different start points and performed a second analysis using cubic splines to estimate critical weight; with the expectation that we will have higher confidence in critical weight estimates where the 2 procedures match. Analyses of the relationship between the PLM and time from starvation to pupation were also performed using an ANCOVA with PLM, PLM^2 , class (diapause or non-diapause), and PLM by class interaction effects.

Results

Although most individuals reared in short-day conditions entered diapause, and most individuals raised in long-day conditions did not diapause, some pupae did not perform as predicted. Pupae that did not enter diapause in short-day conditions and

entered diapause in long-day conditions occurred in small percentages; therefore, we excluded these pupae and only used long-day non-diapause and short-day diapause individuals in subsequent analyses.

Experiment 1

As expected, body size (both lipid and lean mass) increased linearly with temperature, showing the opposite of the general temperature-size rule (Table 3.1, Table 3.2, Figure 3.7). Photoperiodic induction of diapause did not change the scaling relationship between temperature and size, i.e. there was no interaction between temperature and photoperiod to alter size. However, diapausing pupae had substantially greater fat masses, but not lean mass, over the entire temperature range. Therefore, photoperiodic cues altered the temperature-size relationship by changing the elevation, but not the slope, of fat mass. Diapausing individuals acquired more lipid mass without changing their lean mass compared to non-diapausing individuals, so they fit the “just fatter” model of increased seasonal energy storage. Across temperatures, diapausing pupae of both sexes had greater lipid mass and males had greater lipid mass than females in both diapause and non-diapause groups. Across temperatures, females had greater lean mass than males in both diapause and non-diapause groups.

Food consumption increased linearly with temperature across all groups, and the photoperiodic induction of diapause did affect the relationship between temperature and food consumption (Table 3.3). As temperature increased, diapause-destined individuals' consumption increased disproportionally more than individuals that did not enter diapause (Figure 3.8A). Across temperatures, diapause-destined individuals consumed more food than individuals that did not enter diapause. Photoperiodic induction of diapause changed the relationship between temperature and the ratio of

waste consumption to food consumption, the digestive efficiency (Table 3.4). As temperature increased, diapause-destined individuals had higher digestive efficiency than individuals that did not enter diapause (Figure 3.8B, 3.8C). Sex also altered the temperature by diapause status interaction, but additional analyses could not detect how the slope of the relationship between temperature and the waste/food ratio was changed.

Experiment 2

We estimated the diapause entrance weight (DEW) at 382.37 mg [95% CI = 337.93 – 421.20] and the diapause exit weight (DEX) at 490.29 mg [95% CI = 425.46 – 1781.31]. The minimum viable weight (MVW) was 260.64 mg [95% CI = 251.72 – 269.79] for larvae raised in long-day (direct development) conditions, and 269.71 mg [95% CI = 262.10 – 277.39] for larvae raised in short-day (diapause-inducing) conditions; the MVWs for larvae in long-day and short-day conditions were not different from each other. The critical weight for diapause-bound individuals was 404.81 mg [95% CI = 379.80 – 429.82]. Unfortunately we could not identify a discrete critical weight (CW) for larvae in the long-day (14L:10D) treatment, so we could not directly compare critical weights between diapause-bound larvae and larvae that did not enter diapause. Photoperiodic induction of diapause changed the relationship between peak larval mass (PLM) and the timing of pupation ($r^2_{\text{adj}} = 0.58$). Across PLM, diapause-destined individuals took longer to pupate than individuals that did not enter diapause (Figure 3.9). For short-day diapause individuals, the minimum viable weight (MVW) was less than all other thresholds. The diapause entrance weight (DEW) and the critical weight (CW) overlapped, as did the CW and the diapause exit weight (DEX), but the diapause entrance and exit weights did not overlap (Figure 3.10).

Discussion

Diapause Effects on the Temperature-Size Relationship

Contrary to most models of temperature-size relationships in ectotherms, *Helicoverpa zea* (corn earworm) does not show a negative relationship between temperature and body size over a temperature range relevant to the seasonal diapause response, and instead shows a positive relationship between temperature and body size. We have shown that seasonal cues (i.e. photoperiod) affected the elevation of the relationship between temperature and body size by causing diapause-destined individuals to acquire more lipid stores than their non-diapausing counterparts. Seasonal cues did not affect the slope of the scaling relationship between temperature and body size. Photoperiod was a critical variable when evaluating the temperature-size relationship in *H. zea*, and photoperiod will likely be important in other ectotherms that enter seasonal dormancy to avoid poor conditions. Future research on seasonality should therefore incorporate non-thermal variables into temperature-size models.

We also found that females were larger than males, but males had more lipid mass than females. It is not clear why males had more lipid storage than females – both sexes engage in seasonal migrations to reach higher latitude areas where they cannot successfully overwinter (Westbrook 2008). While males are more active during mating, females are also active in oviposition behavior (Fitt 1989). Adult *H. zea* feed on nectar, so it is possible that males and females exhibit different investment in time spent feeding during their larval and adult stages. Females may invest more larval resources into somatic growth than lipid storage, and recoup “lost” fat by nectar feeding.

Both diapause-bound larvae and larvae not bound for diapause increased their food consumption at higher temperatures. As part of the diapause development

program, short-day larvae consumed more overall and relatively more at higher temperatures than larvae that did not diapause. Increased consumption in diapause-bound larvae reinforces the observed increases in lipid mass when compared to larvae that did not enter diapause. Increased consumption at higher temperatures in *H. zea* could represent an adaptive mechanism for overcoming thermodynamic constraints on body size, or could represent part of the physiological mechanisms that reverse the temperature-size rule in a small proportion of ectotherms. In either case, diapause modified the temperature effects on food consumption, and further reinforces the importance of non-thermal seasonal cues on temperature-size relationships.

As temperature increased, diapause-destined individuals had better digestive efficiency than individuals that did not enter diapause. This interaction reveals an effect that would have been overlooked if we had only considered the effect of temperature on digestive efficiency. It is likely that diapause-destined individuals are obtaining more lipids from their diet than non-diapausing individuals are. Future research could compare the relative amounts of macronutrients (lipid, carbohydrate, and protein) utilized by diapause-destined individuals and individuals that do not enter diapause.

Diapause effects on Threshold Weights

The minimum viable weight was not different between larvae raised in diapause-inducing or direct development conditions; however, the relationship between peak larval mass (PLM) and time from PLM to pupation was different between these two groups (Figure 3.9). Even though we could not directly compare CW for diapause-destined individuals and individuals that did not enter diapause, it is likely that the CW of diapause-destined individuals was larger than the CW of individuals that did not enter diapause. The diapause entrance weight (DEW) was not different from the critical

weight (CW) in larvae raised in diapause-inducing conditions. Because the commitment to pupate and commitment to diapause occurred at the same weight, and the timing for pupation was altered after this point, it is likely that larvae commit to diapause using the same cues that trigger the physiological processes that result in pupation.

Unexpectedly, diapause exit weight (DEX) was distinct from DEW. The overlap between DEX and the peak larval mass (PLM) likely indicates that diapause survival depends on the mass a larvae can acquire after they have already committed to diapause, and is not tightly linked to the diapause entrance weight. The identification of weight thresholds divides the last larval instar into discrete development periods or modules, each of which can potentially be used to identify when individuals raised in diapause-inducing conditions alter their development in relation to individuals raised in direct development conditions. These development modules can also be used to determine when temperature will have the greatest impact on larval development, and how growth translates into temperature-size relationships.

Conclusion

To our knowledge, empirical studies of seasonally programmed dormancy effects on temperature-size relationships have not been done before. The addition of diapause-induction (photoperiod) to analysis of temperature and body size demonstrated that temperature-size relationships can be modified by seasonal cues other than temperature. For biologists interested in seasonal effects on temperature-size relationships, the key to fully understanding these relationships may lie in integrating multiple seasonal cues.

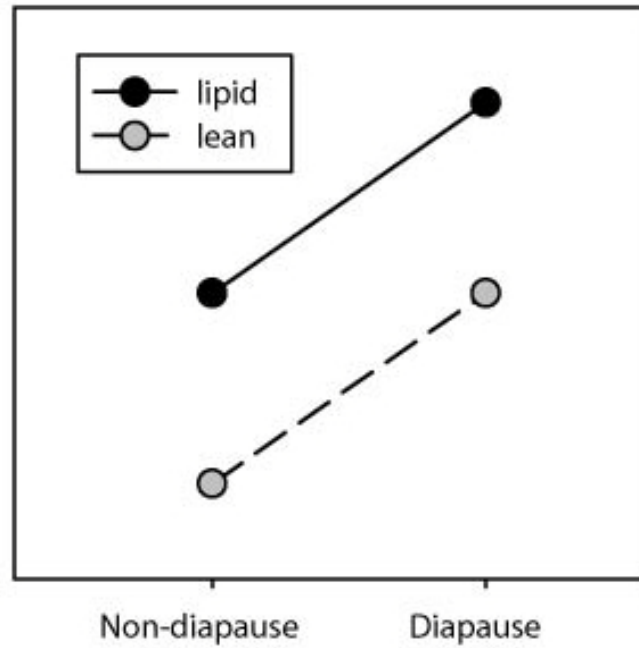


Figure 3-1. Hypothetical isometric increase in somatic and lipid mass (no change in lipid/lean proportion)

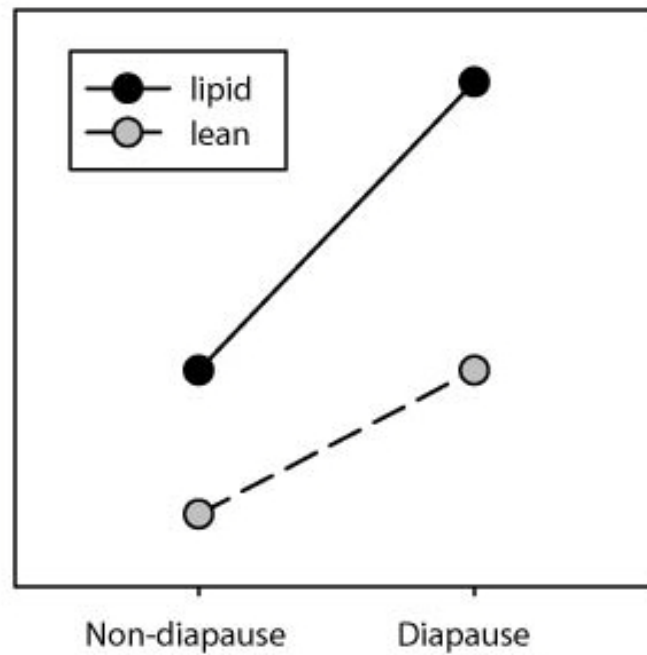


Figure 3-2. Hypothetical allometric increase in somatic and lipid mass

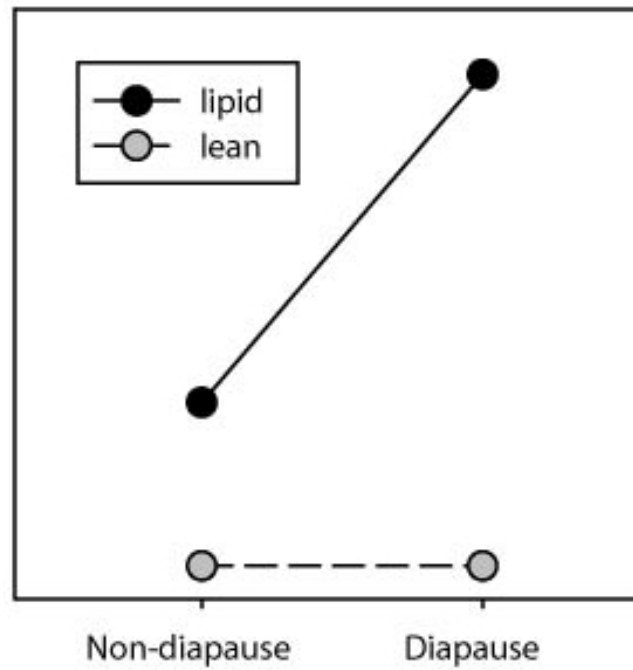


Figure 3-3. Hypothetical increase in lipid mass only

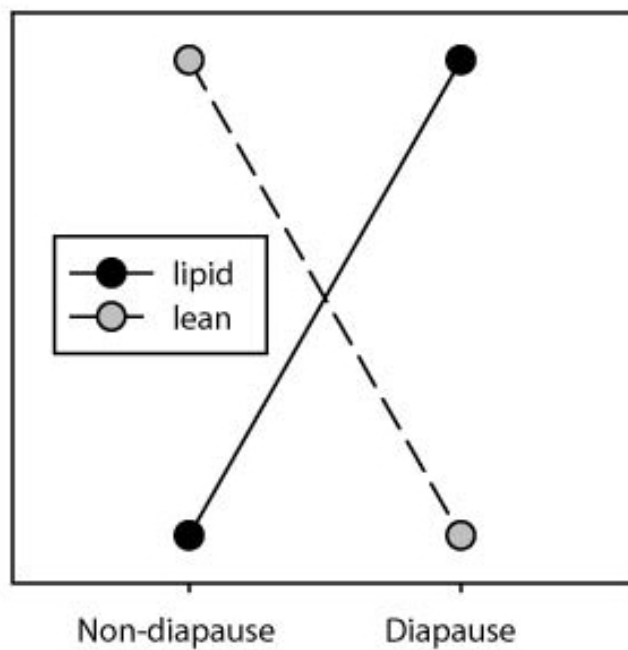


Figure 3-4. Hypothetical trade-off – increase in lipid mass, decrease in somatic mass

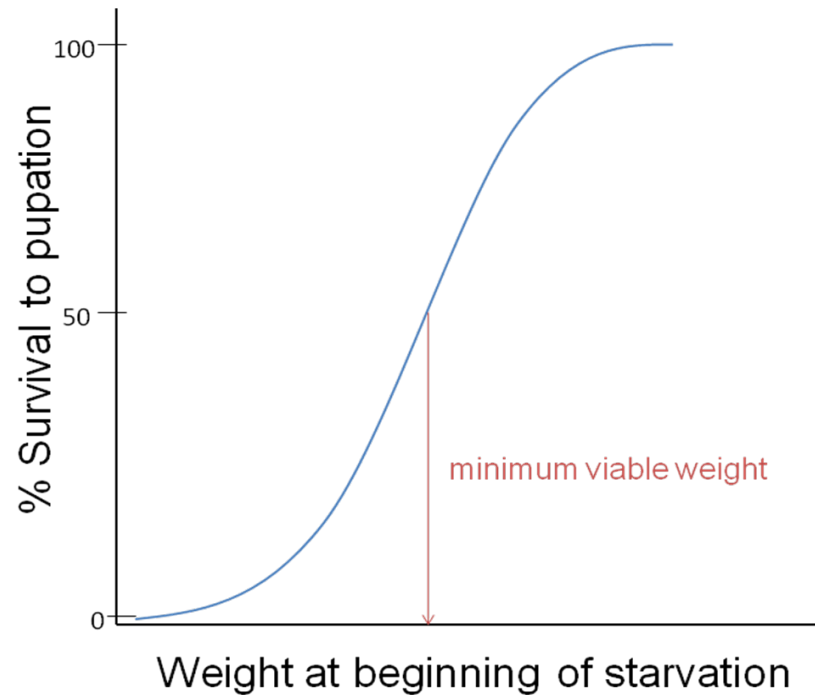


Figure 3-5. The minimum viable weight

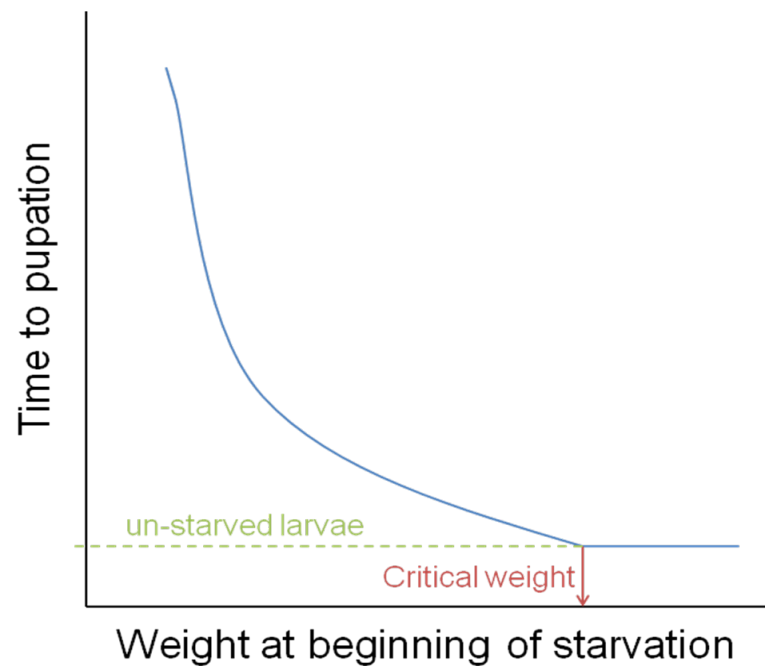


Figure 3-6. The critical weight

Table 3-1. ANCOVA table for lipid mass ($r^2_{adj} = 0.70$). Sex and temperature effects were significant, and lipid mass showed a significant effect of diapause status, but there were no interaction effects

Source	DF	F-ratio	Prob>F
Temperature (C)	4	3.21	0.013*
Lean Mass (mg)	1	451.17	< 0.001*
Sex	1	16.85	< 0.001*
Diapause/Non-diapause	1	6.38	0.012*
Temperature x Diapause/Non-diapause	4	1.62	0.169

Table 3-2. ANCOVA table for lean mass ($r^2_{adj} = 0.28$). Sex and temperature effects were significant, but there were no interaction effects

Source	DF	F-ratio	Prob>F
Temperature (C)	4	16.32	< 0.001*
Sex	1	9.34	0.002*
Diapause/Non-diapause	1	0.005	0.943
Temp x Diapause/Non-diapause	4	1.81	0.127

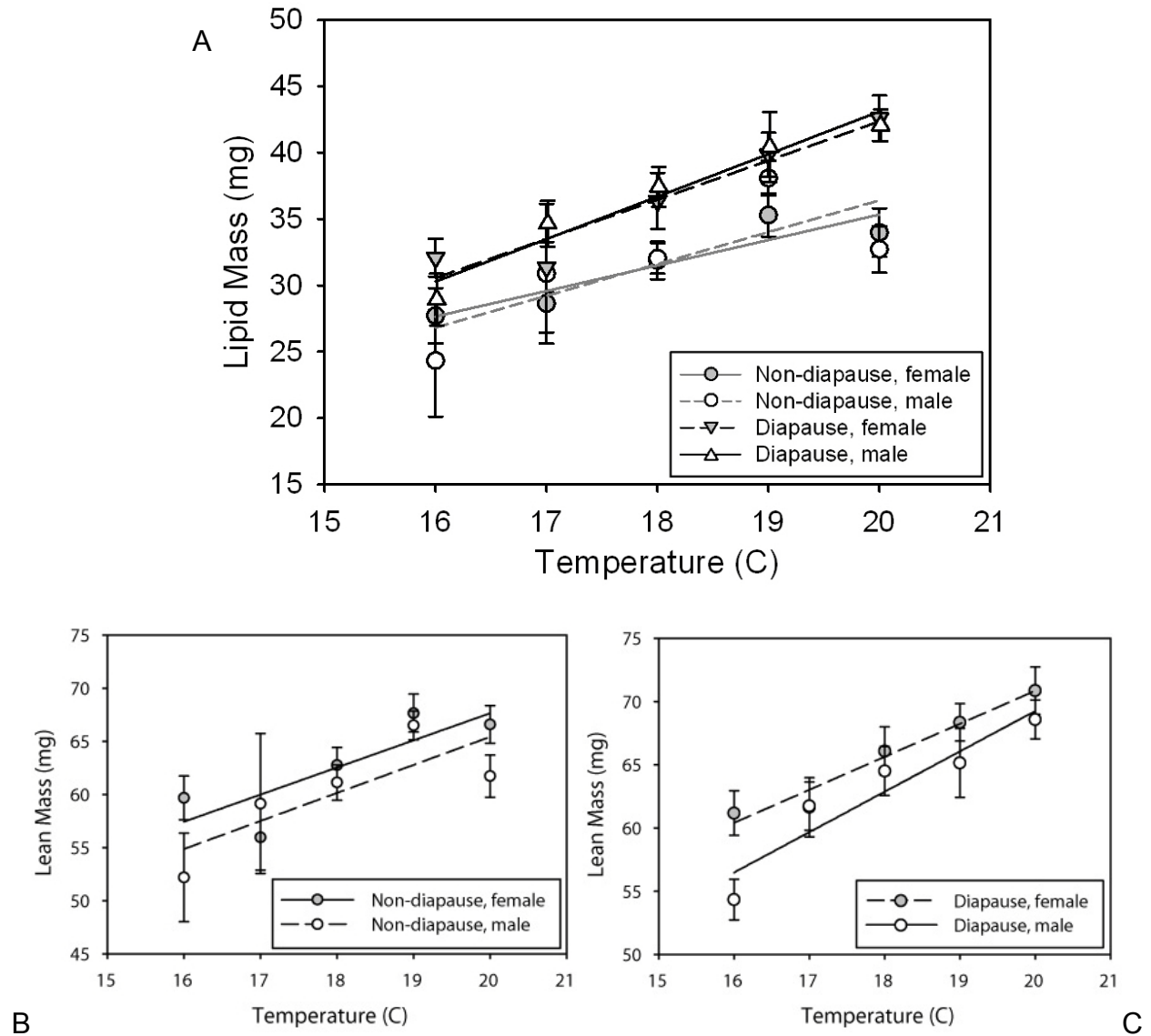


Figure 3-7. Temperature effects on lipid and lean mass: lean and lipid mass increased with temperature, but had opposite sex effects (females had more lean mass; males were fatter). Diapause individuals were fatter than non-diapause individuals, but lean mass was not altered by diapause. (A) Mean lipid mass and (B, C) mean lean mass with standard error bars plotted against temperature for diapause and non-diapause males and females

Table 3-3. ANOVA table for food consumption ($r^2_{adj} = 0.21$, $p < 0.001$). Temperature, diapause status, and the temperature by diapause status interaction were significant for food consumption, but there was no significant sex effect

Source	DF	F-ratio	Prob>F
Temperature	4	20.76	< 0.001*
Sex	1	0.88	0.350
Diapause/Non-diapause	1	4.79	0.029*
Temperature x Diapause/Non-diapause	4	3.31	0.011*

Table 3-4. ANOVA table for the ratio of waste production to food consumption ($r^2_{adj} = 0.09$, $p = 0.001$). There were significant interactions for temperature by diapause status and temperature by diapause status by sex, but no other significant effects

Source	DF	F-ratio	Prob>F
Temperature	4	1.75	0.137
Sex	1	0.63	0.426
Diapause/Non-diapause	1	2.46	0.118
Temperature x Diapause/Non-diapause	4	2.63	0.034*
Temperature x Sex	4	2.32	0.056
Sex x Diapause/Non-diapause	1	2.01	0.157
Temperature x Sex x Diapause/Non-diapause	4	3.96	0.004*

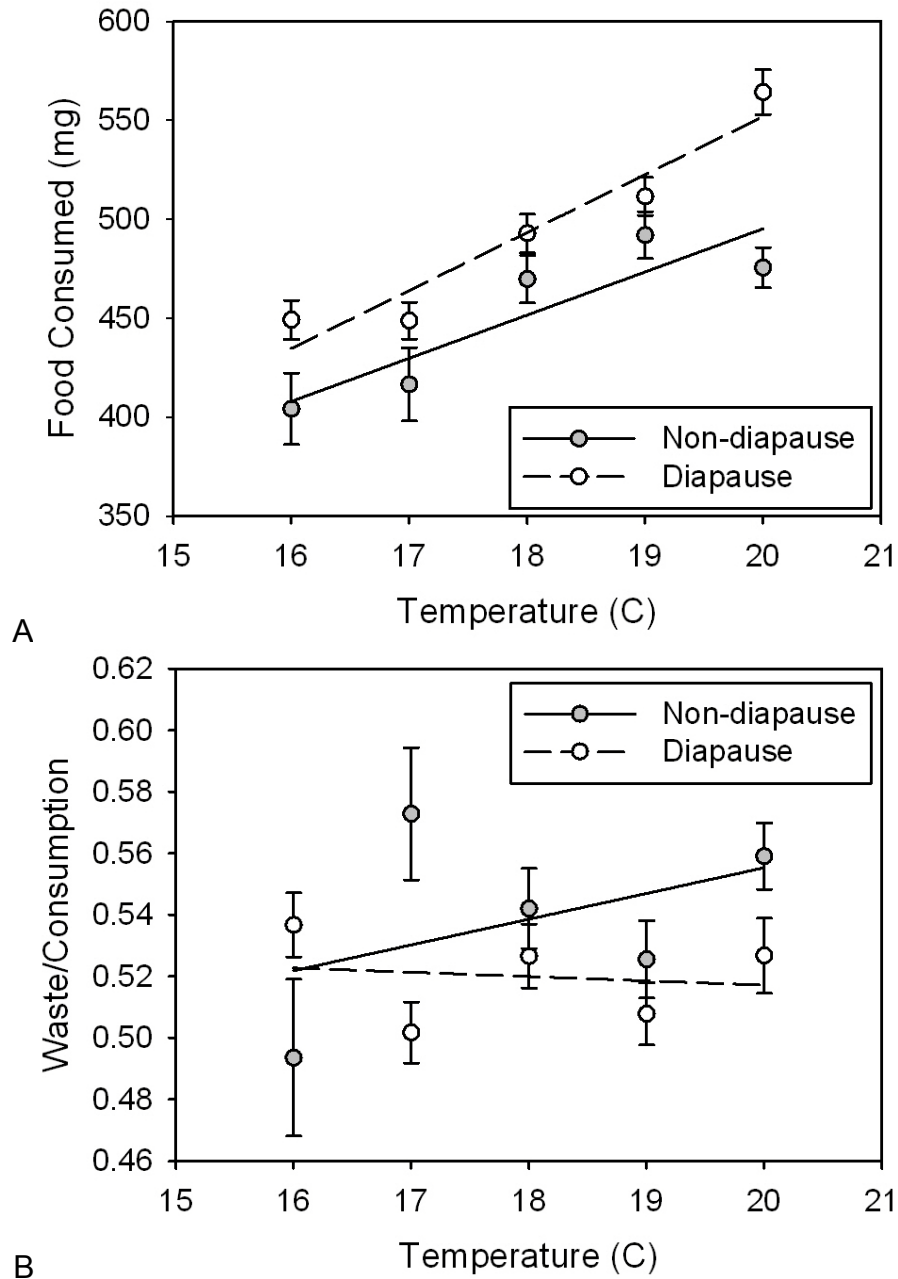


Figure 3-8. Temperature effects for food consumption (A) and the ratio of waste production to food consumption (B), plotted using least square means. Diapause-destined individuals consumed more than individuals that did not go into diapause, and also increased their consumption more than individuals that did not go into diapause as temperature increased. Diapause-destined individuals had higher digestive efficiency (smaller waste/food ratio) as temperature increased than individuals that did not go into diapause

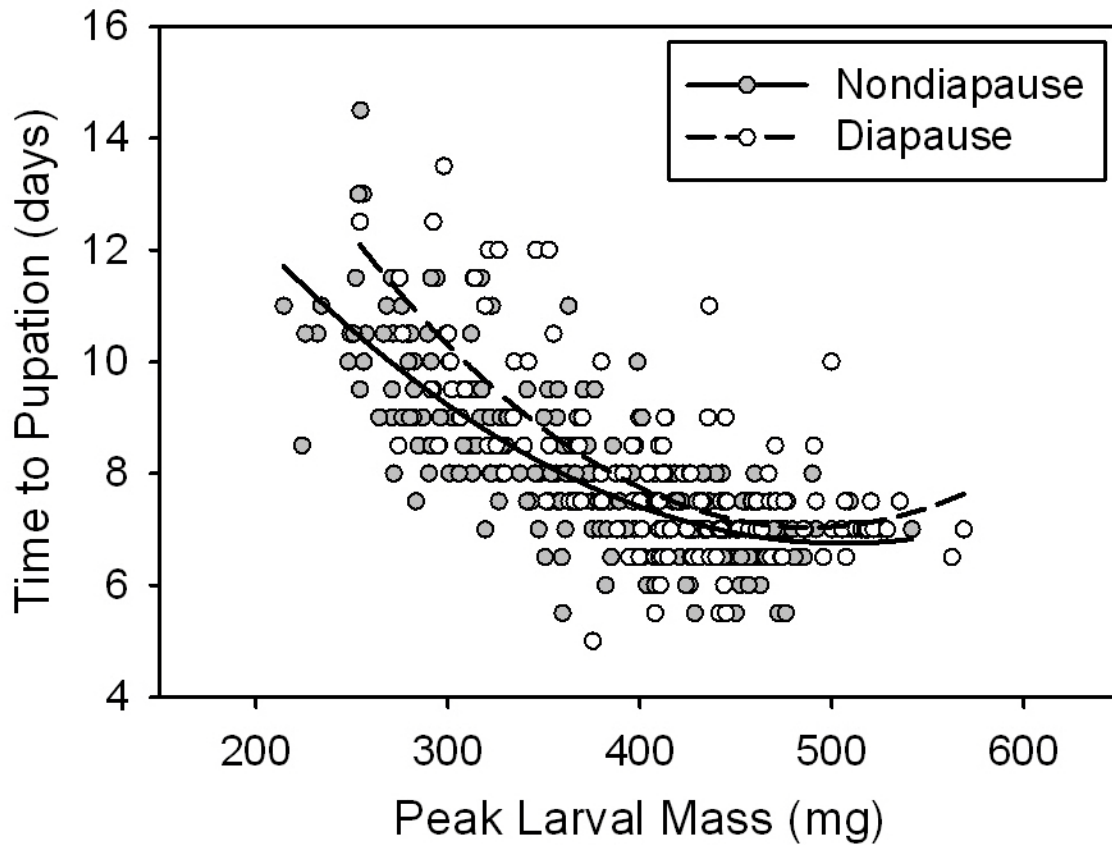


Figure 3-9. Critical weight analysis: diapause-destined larvae had a different relationship between mass and development time ($r^2_{\text{adj}} = 0.58$, $p < 0.001$). A quadratic relationship between peak larval mass (PLM), measured as the mass at the beginning of starvation, and the time from starvation to pupation for diapause-destined individuals and individuals that did not enter diapause

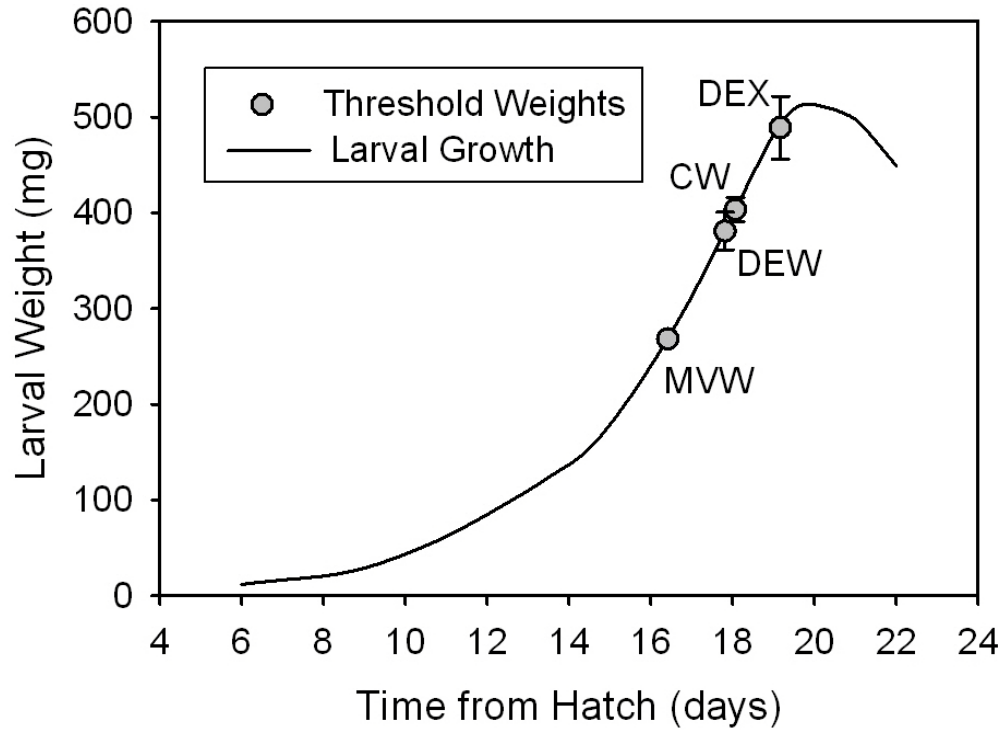


Figure 3-10. Threshold weights: plot of threshold weights for larvae raised in diapause-inducing conditions (19 °C, 8L:16D) had separation between the different thresholds. Larval growth trajectory drawn via a smoothing spline from a typical individual. MVW: minimum viable weight for pupation ($X^2 = 422.10$, $p < 0.001$, $n = 399$), DEW: diapause entrance weight ($X^2 = 15.11$, $p < 0.001$, $n = 125$), CW: critical weight ($r^2_{adj} = 0.53$, $p < 0.001$, $n = 125$), DEX: diapause exit weight ($X^2 = 4.69$, $p = 0.030$, $n = 98$). Standard error for MVW is too small to be visible on the graph

CHAPTER 4 CONCLUSION

This research demonstrated that photoperiodic induction of diapause did alter the temperature-size relationship in *Helicoverpa zea* (corn earworm). Diapausing individuals were consistently fatter than non-diapausing individuals across temperatures. Diapause-destined individuals also consumed more food and improved in digestive efficiency as temperature increased; supporting increased fat storage in diapausing individuals. This research can be expanded upon in the future by studying diapause effects in systems that do conform to the general temperature size rule (hotter is smaller) to determine if the effect of diapause induction in this system is a general pattern or one specific to *H. zea*. A more detailed study of nutrient utilization in diapausing systems would likely explain some of the mechanisms driving the alteration of temperature-size relationships by diapause.

Females were larger than males, but males had more fat than females. This phenomenon is potentially explained by adaptation for local dispersal or migration, if males are more active local fliers or migrate for longer distances they would need more energy reserves than females. The pattern of lean and lipid mass could also be explained by increased female investment in adult body size: if there is stronger selection for larger adult size in females than in males, females would invest more of their larval resources into somatic growth and use adult nectar-feeding to build fat reserves. However, the actual causal relationships are unclear in these experiments. Future research in this area is required before hypotheses are more than speculative. Potential experiments could test relationships between flight activity, metabolism,

feeding behavior, and adult size and fat content to determine how males and females allocate their energy.

There was a clear threshold weight for diapause induction. Thresholds for entrance to diapause and commitment to pupation overlapped, possibly indicating that these thresholds used the same cues “decide” to pupate and diapause. Future research in this area should focus on the known pupation triggers in Lepidoptera, such as photoperiodic gates and Juvenile Hormone (JH) titers, and determine if these mechanisms also drive diapause commitment (Nijhout 1975). The addition of diapause-induction (photoperiod) to analysis of temperature and body size demonstrated that temperature-size relationships can be modified by other factors. For biologists interested in seasonal effects on temperature-size relationships, the key to understanding these relationships may lie in integrating multiple cues that signal seasonal shifts.

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BIOGRAPHICAL SKETCH

Sharon Clemmensen was born in 1985 in Arlington, Virginia, and spent the rest of her formative years in Great Falls, Virginia. Sharon always loved the outdoors, but did not become interested in biology until she was in high school, when she was first introduced to the concepts of inheritance and evolution. She majored in biology at McGill University in Montreal, Canada. Sharon was interested in teaching, so she also minored in education. In May of 2007, Sharon received her Bachelor of Science degree and was sure she wanted to go on to graduate study. She completed a year-long internship with the Student Conservation Association in Warm Springs, Georgia with the United States Fish and Wildlife Service, and then applied to graduate school. Sharon received her master's degree in entomology at the University of Florida in August 2010, and is in a doctoral program in at the University of Tennessee in Knoxville, Tennessee.