# EFFECT OF LARVAL DIET ON ENDOGENOUS CARBON REPRODUCTIVE RESOURCES OF FIFTH INSTARS AND ADULT FEMALES OF THE MOTH, *HELIOTHIS VIRESCENS*

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**Title**

Effect of larval diet on endogenous carbon reproductive resources of fifth instars and adult females of the moth Heliothis virescens.

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

**MASTER OF SCIENCE**

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## ABSTRACT

Mostly adult Lepidoptera feed on plant nectar. That is, adults can only contribute to carbon, and not nitrogen, acquisition. The moth *Heliothis virescens* were used to explore the hypothesis that larval nutrition influences various adult carbon pools and that these, in turn, may affect pheromone production quantitatively. *H. virescens* larvae were reared on diets differing in carbohydrate, fat or protein content and resulting $5^{th}$ instars and adults were analyzed for carbon pools, hemolymph trehalose concentration (HTC) and lipid content. Across all the diet treatments, changes in carbohydrate content affected carbon pool the most. In particular, for insects reared on a high carbohydrate diet, adults had a greater lipid content, while for insects reared on a low carbohydrate diet, adults had a lower HTC, compared to insects reared on the control or other diets. By contrast, changes in protein and fat had little effect on HTC and lipid content of adults.

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## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

# CHAPTER 1. INTRODUCTION

## Insect Nutrition and Reproduction

An appropriate amount and balance of nutrients is crucial for the growth and development of all living organisms (Thompson, 1999). The basic nutritional requirements, protein, lipid, carbohydrates, minerals and vitamins, are the same for virtually all animals (Dadd, 1985; Nation, 2008). Failure to obtain specific nutrients in each stage of development can have profound effects on development, survival and reproduction. Therefore, when studying an animal's reproductive fitness, it is important to understand its nutritional requirements at different stages of the life cycle. In insects, larval feeding affects its growth, movement, and survival and adult feeding influences fecundity, movement, and survival in adults (Fig. 1; Slansky, 1982).

The role of nutrition in the growth and development of insects has been the subject of considerable study (Slansky, 1982; Simpson and Raubenheimer, 1995). Although insects have highly specific and diverse nutritional requirements throughout the different stages of development (e.g., sterols are required for producing molting hormone during juvenile stages; Nation, 2008). Like most animals, the bulk of their nutritional requirements are proteins, carbohydrates, and lipids, used for general cell and body growth, reproduction and maintenance (Joern and Behmer, 1997; Naya et al., 2007; van Huis et al., 2008). The ability of insects to maximize growth and reproductive output, while using limited nutritional sources across a range of different environments, is one of the major reasons that insects are such a successful taxon and frequent pests of human health and agricultural systems (McGavin, 2001; Nation, 2008).

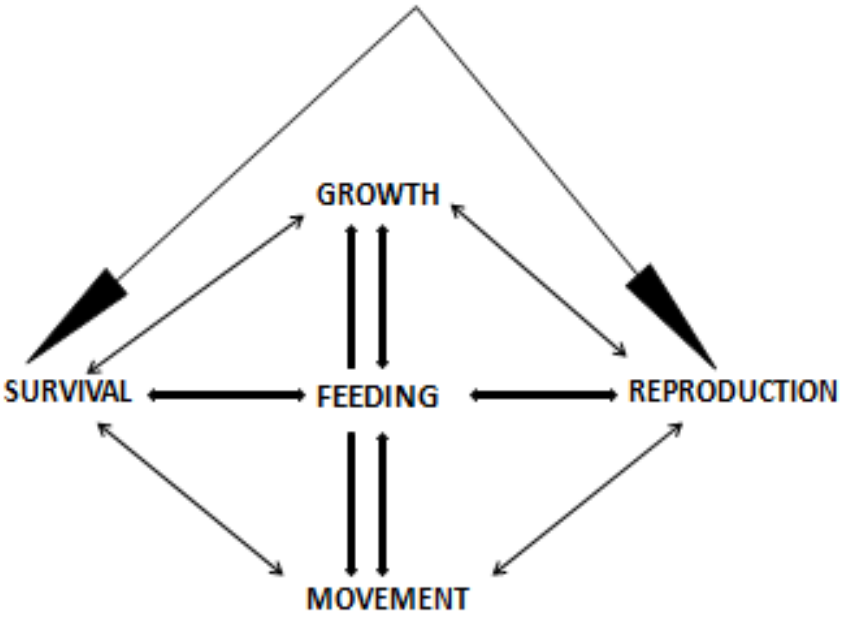



**Figure 1**. Effects of insect feeding behavior on its different important behaviors (after Slansky, 1982).

Insects may acquire nutrients throughout their life or during only specific stages. These nutrients must be sufficient for development until the larva emerges and can acquire (feed on) external nutrients. In most insects, the majority of the total nutrients acquired throughout the lifetime are obtained so during the larval stage (Boggs, 2009). Nutrients acquired during this stage are primarily used for somatic growth (growth of non-reproductive tissues) and survival (including defense) and may be carried through (to varying degrees) to the adult stage, depending on the longevity and feeding habits of the adult (Wheeler, 1996; Ramaswamy et al., 1997; Boggs, 2009). Depending on the species, an adult may not feed (i.e., all nutrients it uses

for reproductive activities must be acquired by the larva), feed on a limited range of nutrients (e.g., as in adults that feed only on carbohydrates in plant nectar), or feed on highly nutritious substances similar to, or different from, the juvenile form (Boggs, 2009).

Because adults of most insects (excluding eusocial insects) are primarily concerned with their own reproduction, a large portion of nutrients acquired throughout the lifetime is used for reproduction. If adults of a species do not feed, then all nutrients for reproduction must be acquired during the larval stage. However, if adults do feed, then these nutrients are generally used to supplement (to varying degrees) larval-acquired nutrients for reproductive behaviors/physiologies (O'Brien et al., 2004). Insects that acquire nutrients for reproduction during the larval stage are known as capital breeders, whereas insects that acquire the nutrients for reproduction through adult feeding are known as income breeders. A mixture of both is known as a mixed capital/income breeder (Johnson, 2006). Examples of capital breeders are species of Lepidoptera in which adults do not feed, such as the forest tent caterpillars, *Malacosoma disstri* (Colasurdo et al., 2009). The majority of insects are probably mixed income/capital breeders (Wheeler, 1996; Johnson, 2006; Boggs, 2009).

The most obvious distinction among insects with regard to nutritional acquisition and life stage is between hemimetabolous and holometabolous taxa. In hemimetabolous taxa, the immature (nymph) stage is similar to the adult stage, lacking development of adult structures such as wings and fully functional genitalia. Often, nymphs share the same habitat as adults, have similar mouthparts and feed on the same food (Wheeler, 1996). For example, both nymphal and adult stages of the bug *Rhodnius prolixus* (Reduviidae) have sucking-type mouth parts and consume vertebrate blood (Ampleford and Davey, 1989; Roberts and Janovy, Jr., 2000). By

contrast, holometabolous insects typically exhibit great differences in structure and function between larval and adult stages. Consequently, the feeding habits and food sources the two stages may be very different (Wheeler, 1996; Boggs 2009). In many species of holometabolous insects, larvae may have chewing mouthparts for consumption of solid plant material, whereas the adults may have no functional mouthparts or be capable of feeding only on liquids (e.g., Lepidoptera; Wheeler, 1996). The contrast between hemimetabolous and holometabolous taxa is best illustrated by hematophagous (blood-feeding) species. For example, both juveniles and adults of hemotophagous bugs, such as *R. proxilus* (see above) feed on blood. In contrast, in holometabolous insects, only one stage (often the adult) feeds on blood. For example, only adult female mosquitoes feed on the blood of vertebrate hosts, whereas larvae feed on algae and microbes in water (Merritt et al., 1992). However, in some holometabolous insects, such as carnivorous ladybug beetles (Coleoptera: Coccinellidae), both larvae and adults feed on the same food, even though larva and adult are structurally quite different (Michaud, 2001).

Given the often limited nature of adult nutrient acquisition in holometabolous insects, it follows that the nutrient quality of the larval diet is an important contributor to the reproductive potential of the adult (e.g., Chambers and Klowden, 1994; Karlsson and Wiklund, 1984; Karlsson, 1987; 1994, Braby and Jones 1995; Soliman et al., 1995; Tu and Tatar, 2003; Zhou et al., 2004). This is a tautology for insects in which the adult stage doesn't feed (e.g., Stern and Smith, 1960; Boggs and Ross, 1993). In holometabolous species in which adults feed, the quality of larval diet can also profoundly affect adult fitness, largely because important (for reproduction) nutrients are specifically acquired by the larva and not by the adult. In Lepidoptera (with the exception of *Heliconius* butterflies; see below), for instance, all protein is acquired by the larva, deficits of which cannot be compensated for by adults which only feed on nectar

(carbohydrate) (Wheeler, 1996; Boggs, 2009). Even in holometabolous species in which adults feed on protein, larval nutritional quality is important for adult reproductive fitness. For example, poor nutrition for larvae of anautogenous mosquitoes result in adults with reduced reproductive fitness, regardless of the size and number of adult female blood meals (Briegel, 1990; Renshaw et al., 1994; Brigel, 2003; Norieg, 2004; Telang and Wells 2004; Telang et al., 2006; Telang et al., 2007). However, females cannot mature and lay eggs without consuming proteins, from blood, during the adult stage (Engelmann, 1970). It is clear that in species in which both larvae and adults feed, reproduction must be considered within a context of both larval and adult nutrition.

**Nutrient Acquisition and Allocation to Reproduction in Lepidoptera**

The Lepidoptera, moths and butterflies, are a holometabolous taxon with four distinct stages: egg, larva (typically with 5–7 instars), pupa, and adult. Most species of moths have sexual reproduction with females exhibiting oviparity (i.e., laying eggs). The Lepidoptera are an excellent study system in holometabolous insect group for studying nutrient acquisition and allocation, particularly for reproduction (Miller, 1997; Boggs, 1997; 2003), primarily because the juvenile and adult stages have distinct feeding abilities and nutritional requirements. Juveniles have mouthparts and gut physiologies capable of feeding on and digesting solid foods (Nation, 2008). Most larval Lepidoptera are herbivorous (Baker and Baker, 1983; Boggs, 2009), with plant material providing them with the protein, carbohydrates, lipids and other nutrients necessary for growth and development. With the exception of some *Heliconius* butterflies, the adults of which can feed on and utilize amino acids in plant nectar and pollen (Gilbert, 1972), all

protein (excluding that provided by the female for embryonic development) acquired throughout the life of a typical Lepidopteran is done so through larval feeding.

In contrast to larvae, adult Lepidoptera typically do not feed, or feed only on plant nectar, which consists principally of sucrose and its component monosaccharide, glucose and fructose, at concentrations up to 35% (w/w) (Kingsolver and Daniel, 1993; Josens and Farina, 2001). These carbohydrates may be utilized by adult Lepidoptera for enhancing fecundity (females only) and longevity, and for fuelling highly energetic activities such as flight (Baker and Baker, 1973; Karlsson and Wickman, 1990; Caroll and Quiring, 1992; Leahy and Andow, 1994). Nectar is also a good source of water (Norris, 1936; Millar, 1988), which is important in egg production and prevention of mortality due to dehydration (Engelmann, 1970).

Fecundity in adult female moths and butterflies depends upon the availability and quality of both larval and adult food (Awmack and Leather, 2002). Food quality is described as the components e.g., levels of nitrogen, carbon that affects insect performances. Nitrogen is present in the form of amino acid that is protein and carbon comes from carbohydrate and lipid. So obtaining these components (protein-tissue building-blocks and carbohydrate & lipid-energy sources) in related ratios has significant effect on insect growth, development, survival, and fecundity (Naya et al., 2007; van Huis et al., 2008). For example, females of the pine beauty moth, *Panolis flammea*, fed as larvae on low quality food produce fewer, high quality eggs than those fed on high quality food (Leather, 1985). Since the availability of nitrogen (i.e., protein) during the larval stage is often a limiting factor in reproduction (Colasurdo et al., 2009), the potential fecundity (i.e., the possible, but not necessarily realized, number of eggs able to be developed and laid by a female) of a given moth or butterfly is likely to be determined by the acquisition of nutrients (primarily proteins) during the larval stage. Work by Colasurdo et al.

(2009) on forest tent caterpillars, *Malacosoma disstri*, demonstrated how a different balance of carbohydrates to protein in the larval diet influences nutrient allocation to reproduction. Larvae fed on a carbohydrate-rich diet allocated relatively more protein to somatic tissues than to reproductive tissues. By contrast, larvae fed a protein-rich or control **(**equal protein: carbohydrate) diet allocated relatively more protein mass to reproductive tissue over somatic tissue. In another study (Rossiter et al., 1993), gypsy moth, *Lymantria dispar*, fed as larvae on low quality food (oak and pine leaves) produced smaller amounts of egg protein (vitellogenins) compared to females that had been fed on high quality food (aspen leaves).

Depending upon the species, nectar feeding by adult Lepidoptera can also have major effects on female fecundity (Ramaswamy et al., 1997; Wackers et al., 2007). The prevalence and effect of adult feeding on fecundity in Lepidoptera appears related to the egg maturation and mating strategies of a given species. Ramaswamy et al. (1997) divided egg maturation strategies in Lepidoptera into four groups. In the first group, including species such as *Bombyx mori* and *Lymantria dispar*, adult females eclose with their full complement of mature eggs. Species in this group mate shortly after eclosion, do not feed as adults, typically mate only once (i.e., are usually monandrous), and are relatively short-lived. Females of the second group, including many species of Pyralidae and Crambidae, also eclose with a full complement of mature eggs. Females in this group mate shortly after eclosion, but may, depending on species, feed and exhibit limited polyandry (i.e., mate more than once). The third group includes species such as *Manduca sexta*. Females of this group emerge with some mature eggs but also mature a portion of their eggs as adults. Females mate soon after eclosion, but feed and exhibit polyandry. The fourth group, including papillionid butterflies and noctuid moths, eclose with no mature eggs. Egg maturation is completed sometime after eclosion and may be accelerated by mating and feeding. These

females are polyandrous, typically interspersing mating events with bouts of egg laying. In these species, multiple mating generally results in increased fecundity, in part through transfer of increased amounts of male resources via a spermatophore (Ramaswamy et al., 1997), although the mechanisms behind this remain largely unexplored.

Feeding on plant nectar by adult female Lepidoptera typically results in increased fecundity. However, relatively little is known about the underlying mechanisms. Using isotope ratio mass spectrometry (IR-MS) and different $^{12}C/^{13}C$ isotopic ratio diets for larvae and adults, O'Brien and coworkers (2000, 2003, 2004, 2005) examined the relative contributions of larval- and adult-acquired carbon to eggs in a range of Lepidoptera, including the moth *Amphion floridensis* (Sphingidae) and the butterflies *Speyeria mormonia* (Nymphalidae: Heliconiinae: Heliconiini: Argynniti), *Euphydryas chalcedona* (Nymphalidae: Nymphalinae; Melitaeini), *Heliconius charitonia* (Nymphalidae: Heliconiinae: Heliconini: Heliconiiti), and *Colias eurytheme* (Pieridae). Essentially, there was an increasing contribution of adult-acquired carbon (i.e., in the form of nectar feeding) as the number of eggs laid increased. The maximum percentage of adult-acquired carbon contributing to egg carbon varied according to species, ranging from 80% in *S. mormonia* to 39% in *E. chalcedona*. Using compound-specific IR-MS, O'Brien et al. (2005) analyzed for incorporation of adult-acquired carbon into egg amino acids. In general, 20 amino acids are required to make proteins in insects (Nation, 2002), with 12 out of the 20 required from dietary sources (i.e., are essential amino acids). O'Brien et al. (2005) found that carbon in the essential amino acids of eggs was derived solely from larval carbon, whereas non-essential amino acids were derived from a mixture of larval and adult carbon. As for their studies on total carbon, O'Brien et al. (2005) found that an increasing amount of adult carbon

was incorporated into non-essential amino acids as the numbers of eggs laid increased, before eventually plateauing.

Adult reproductive physiologies such as mating (through producing pheromone) and egg development are fueled by several nutrients, including protein (nitrogen), carbohydrate (carbon), and fat (carbon). Within the Lepidoptera, moths are especially useful for studying the allocation of these nutrients to different physiologies, as allocations both to mating and egg production can be quantitatively determined.

**Mating Behavior and Pheromone Production in Moths**

In general, moths and butterflies exhibit significantly different mating behaviors. Most butterflies use visual cues to locate and recognize conspecific mates, using chemical cues only (if it all) when the male and female are in close proximity during courtship (Fordyce et al., 2002)**.** By contrast, most species of moth use olfaction as the primary mode for mate location and recognition (Hartlieb and Anderson, 1999). Typically, a female moth produces a long-range sex pheromone, released from a specialized gland between the $8^{th}$ and $9^{th}$ abdominal segments (Tamaki, 1985), which attracts conspecific males over a long distance (tens to hundreds of meters; Schneider, 1989, 1999). Upon locating a female, a male engages in some form of close-range 'courtship', which may or may not enhance recognition by the female and eventual mate recognition and/or selection (e.g., as in the ornate moth, *Utethesia ornatrix;* Dussourd et al., 1991; LaMunyon and Eisner, 1994). In some species, a male-produced pheromone maybe used during courtship, and can result in greater copulatory success for individual males (e.g., as in the European corn borer; Pelozuelo et al., 2007). During copulation, sperm are transferred to the female, usually encased in a spermatophore. A spermatophore is a gelatinous capsule, comprised

largely of proteins originating from male accessory glands, which is formed inside the female's bursa copulatrix prior to transfer of sperm (Van der Reijden et al., 1997; Rooney and Lewis, 1999; Demary 2005). Following the withdrawal of the male aedeagus from the female, contractions of the bursa copulatrix cause a rupture of the spermatophore, liberating the sperm, which move to the spermathecae, where sperm are stored until fertilization. Eggs are fertilized during ovulation, immediately prior to external deposition (oviposition).

Female-produced sex pheromones of moths usually comprise a single chemical, or blend of chemicals in a specific ratio, that is highly specific to a given species (e.g., Morse and Fabrias, 1987; Lofstedt, 1993; Ando et al., 2004). A wide range of compounds has been identified as sex pheromone components of female moths (Byers, 2005; Ando, 2007; El-Sayed, 2007, http://pheromone.net/). Moth pheromone compounds basically sort into two structural types. Type 1 compounds, the more prevalent structural type, are typically unsaturated, even-numbered straight-chain carbon skeletons, with an aldehyde, alcohol, or acetate ester as a terminal oxygenated functional group (Blomquist et al., 2011). These compounds are typically produced *de novo* in a specialized gland, located between the $8^{th}$ and $9^{th}$ abdominal segments, with biosynthesis involving synthesis of saturated fatty acids from cytosolic acetyl CoA (Jurenka, 2003). Following synthesis of a saturated fatty acid, usually hexadecanoicacid (Roelofs and Wolf, 1988), the acid is modified by several unique enzymatic steps, including desaturation (insertion of one or more double bonds) (Bjostad et al., 1987; Knipple and Roelofs, 2003), cytosolic β-oxidation (limited two-carbon chain shortening) (Wolf and Roelofs, 1987), reduction (to an alcohol), acetylation (to produce acetate esters) (Jurenka and Roelofs, 1989), and/or oxidation (to produce aldehydes) (Teal and Tumlinson, 1986). Figure 2a shows the biosynthetic

pathways for a typical Type 1 pheromone compound, bombykol, the sex pheromone of the silkworm moth, *Bombyx mori*.

Type 2 pheromone compounds (Ando et al., 2004), are composed of unsaturated hydrocarbons and their epoxy derivatives. These compounds are biosynthesized from linoleic or linolenic acids, which cannot be biosynthesized *de novo* by Lepidoptera, and therefore must be derived from the larval diet (Stanley-Samuelson et al., 1988). Biosynthesis of hydrocarbons with an odd-numbered carbon skeleton (C17, 19, or 21) occurs via reductive decarboxylation of even-numbered acyl precursors of linoleic or linolenic acids, while biosynthesis of even-numbered carbon skeletons (C18, 20, or 22) occurs via reduction of the carboxyl group after 0, 1, or 2 additions of acetate units to linoleic or linolenic acids (Millar, 2000). Unlike Type 1 compounds, Type 2 compounds are not produced entirely within the pheromone gland. Instead, the hydrocarbon structure is produced in oenocytes (cells located in the abdomen), and is then transported to the pheromone gland by the lipid carrier protein lipophorin (Schal et al., 1998). The epoxidation step takes place within the pheromone gland, prior to release of the pheromone blend (Miyamoto et al., 1999). Figure 2b shows the biosynthetic pathways for typical Type 2 pheromone compounds.

Females usually biosynthesize sex pheromone in a daily cycle, with peak production and release generally occurring during the sexually active period (i.e., just prior to and during when mating occurs) of the day (Raina, 1988; Foster and Anderson, 2011). In most of the species studied, biosynthesis of Type 1 compounds is controlled by the release of the pheromone biosynthesis-activated neuropeptide (PBAN) from the corpora cardiaca into the circulating hemolymph (Rafaeli and Jurenka, 2003). From there, PBAN is transported to the gland, where it

binds to a G protein-coupled receptor on the membrane of pheromone gland cells, eliciting a cellular response through second messengers, such as cyclic AMP (Choi et al., 2003). This response is thought to exert its control on pheromone biosynthesis by influencing the activity of one or more specific biosynthetic enzymes (Tang et al., 1989). PBAN has been found to affect the activities of different biosynthetic enzymes in different species of moths. In species such as *Argyrotaenia velutinana* (Tang et al., 1989), *Helicoverpa zea* (Jurenka et al., 1991), and *Mamestra brassicae* (Jacquin et al., 1994), PBAN affects substrate availability for synthesis of fatty acids, most likely through activating the enzyme acetyl-CoA carboxylase (Jurenka and Reolofs, 1993), the enzyme that typically regulates fatty acid synthesis in organisms (Stanley-Samuelson et al., 1988). In other species, such as *Spodoptera littoralis* (Martinez et al., 1990; Marco et al., 1997) and *Bombyx mori* (Arima et al., 1991), PBAN controls a later step in pheromone synthesis, probably fatty acid reduction, which reduces a fatty acid to an alcohol. For Type 2 compounds, PBAN appears to regulate production of epoxides in the pheromone gland and not the biosynthesis of the hydrocarbons in oenocytes (e.g., Jurenka et al., 2003).

In addition to sex pheromone, the sex pheromone gland typically contains large amounts of other lipids (Feng and Roelofs, 1977), especially triacylglycerols (TGs), and other neutral glycerolipids (mono- and di-acylglycerols), as well as phosphatidylcholines and phosphatidylethanolamines (Bjostad et al., 1981; Bjostad and Roelofs, 1984; Fang et al., 1995; Foster, 2001, 2004, 2005). These glandular lipids contain substantial amounts of fatty acids that are structurally related to the pheromone components and their precursors (Bjostad et al., 1987). The role of these stored glycerolipids in pheromone biosynthesis varies from providing a large reservoir of fatty acids or acetyl CoA for biosynthesis of pheromone through to being a repository for surplus fatty acids that, if free, as thioesters, would inhibit biosynthetic enzyme

activities and hence disrupt pheromone biosynthesis (e.g., Bjostad et al., 1981; Foster, 2001, 2004). In *Bombyx mori*, triacylglycerols containing pheromone precursor acids are stored in lipid droplets. The number and size of lipid droplets in the gland fluctuates according to stimulation by PBAN and the biosynthesis of pheromone (Fonagy et al., 2000). Stimulation by PBAN presumably facilitates hydrolysis of stored fatty acids in the lipid droplet making them available for reduction to the pheromone (Fonagy et al., 2001). By contrast, fatty acids stored in triacylglycerols in *Heliothis virescens* are primarily synthesized when pheromone is also being synthesized, but at a much slower rate. Thus, this pool represents surplus de novo-synthesized fatty acids that are not reduced to pheromone, presumably because of flux limitations of the fatty acid reductase system (Foster and Anderson, 2012). Therefore, in *H. virescens* glycerolipids act primarily as a 'dead end' storage pool, presumably to maintain fatty acyl CoA concentrations at an appropriate level that is not inhibitory to optimal pheromone biosynthesis.

**Pheromone Production and Nutrient Stores**

Most probably that all the carbon for pheromone production was derived from larval feeding, regardless of whether a species fed as an adult or not. This led to the view of the pheromone gland as a 'closed system', with finite pools of metabolites for pheromone production. A closed system view of the gland was consistent with the general decline in pheromone produced by a female as she aged (Schal et al., 1987; Tang et al., 1992; Foster and Johnson, 2010), presumably as metabolites were exhausted.

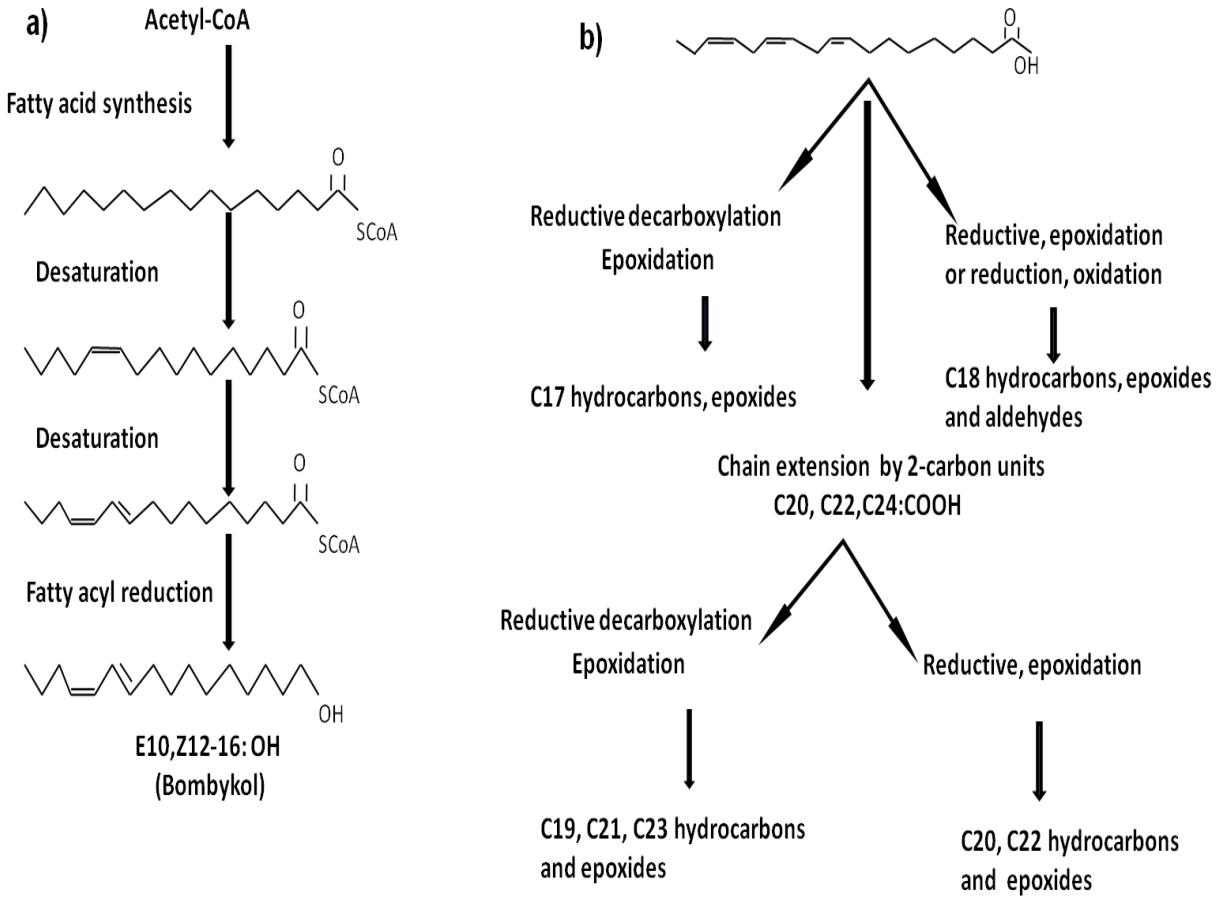



**Figure 2**. Biosynthesis of pheromone compounds of **a)** Type 1, bombykol, in *Bombyx mori* (after Matsumoto, 2010) and **b)** Type 2, polyene hydrocarbons and their epoxides, from linoleic or linolenic acid precursors (after Millar, 2000).

Recently, it was shown that adult sugar (nectar) feeding influences pheromone production in the polyandrous moth *H. virescens*. Both mated and virgin females that fed on sugar produced greater quantities of pheromone than females that were starved (Foster, 2009; Foster and Johnson, 2010). This effect occurred in females that were sugar stressed, either through mating or through prolonged starvation. The sugar stress caused a reduction in concentration of hemolymph trehalose, the major blood sugar in most insects (Thompson, 2003), which was restored quickly through feeding on sugar. Thus, sugar ingested by adults was rapidly circulated and glycolyzed in the pheromone gland to generate cytosolic acetyl CoA for synthesis of fatty

acids (Foster and Anderson, 2012) and pheromone (Foster and Anderson, 2011). Because all Type 1 pheromone components are biosynthesized de novo from the same precursor (acetyl-CoA), this effect of adult sugar feeding on pheromone production should be widespread among species of Lepidoptera. The acquisition of this adult carbon should only influence pheromone quantity (i.e., the amount of total pheromone produced) and not pheromone quality (i.e. types or ratios of components).

This finding, that pheromone quantity produced by adult female moths is influenced by adult-acquired carbon, emphasizes that quantitative aspects of pheromone production has been largely ignored in favor of studying the qualitative aspects (i.e., how different pheromone components are produced and how ratios of components are maintained). Importantly, little is known about how the quantity of pheromone produced by adult female moths is influenced by larval acquisition of carbon metabolites. Such an understanding is important, because the same nutrients used for pheromone production are also used to enhance fecundity (see above) and fuel activities such as flight. Therefore, in order to optimize mating strategies (e.g., at what age or how often to mate, foraging for ovipositional hosts versus mating) with fecundity, females must make choices about allocating carbon.

The carbon nutrients used by an adult moth consist largely of carbohydrates stored in the hemolymph and carbohydrates and fats stored in the fat body (Becker et al., 1996; Thompson, 2003; Arrese and Soulages, 2010). A significant proportion of these stores is carried over from larval feeding, although adult feeding can contribute substantially (see above). The most abundant store of carbon in hemolymph of most insects is trehalose, a non-reducing disaccharide (Becker et al., 1996). Hemolymph trehalose concentration (HTC) is typically regulated in

insects, but not to the same degree as blood glucose concentrations are in mammals (Thompson, 2003). HTC shows relatively large variation according to the physiological and nutritional state of an insect (Thompson, 2003). Friedman et al. (1991) showed that nutrient ratios in diet affect HTC in *Helicoverpa zea* larvae. When larvae were fed on a 10:0 ratio of protein: carbohydrate, they had a very low HTC, whereas larvae fed on a 2:8 ratio had a much higher HTC. Thus, it is conceivable that larval diets causing higher HTC in larvae may also result in higher HTC in newly eclosed adults. For moths that do not feed as adults, these higher reserves could sustain more reproductive activity (e.g., pheromone production, foraging) and increase fecundity throughout their lives. Even in species in which adults feed on nectar, and hence are able to compensate to some degree for larval dietary shortcomings, females could benefit from these greater reserves by producing pheromone in greater quantities shortly after eclosion and hence mating earlier than females with lower larval-acquired reserves.

Thompson et al. (2003) discussed the effect of diet composition on the rate of nutrient (protein and carbohydrate) consumption for growth, metabolism and HTC in larval *Manduca sexta*. Protein consumption is critical for larval growth, whereas the amount of carbohydrate consumed had little effect on growth or weight gain. They suggested that protein-rich diets were able to support both growth and energy production on all diets, including those without carbohydrate. However, when the amount of carbohydrate consumption increased, fat content increased significantly, due to increased lipogenesis. This stored fat is an important metabolite source for adult development during the pupal stage, and possibly for egg production in females. It is not known whether these greater stores of fats in the fat body (and perhaps in the pheromone gland) can contribute to greater production of pheromone throughout the life of an adult female.

In this thesis, I explore the hypothesis that larval nutrition influences adult carbon resources and consequently will quantitatively affect pheromone production. Specifically, I postulate that carbon (in carbohydrate, fat or protein) ratios in diets affect metabolic stores of insects, specifically the stores of hemolymph trehalose and total lipid content (stored primarily in the fat body; Arrese and Soulages, 2010). To address this hypothesis, I prepared larval diets with different carbohydrate, protein and fat levels (i.e., yielding diets with different C: N ratios) and determined their effect on HTC and lipid content of adults and $5^{th}$ instar larvae.

The aim of my research is to better understand how nutrient content of larval diet affects the initial carbon stores of eclosing adults, and its consequences for adult reproductive physiology/behavior. This work creates a foundation for further work exploring how both larval and nutritional stores are allocated to pheromone and egg production throughout the life of a female moth. From a practical viewpoint, this work will help us understand how manipulating larval nutrition (e.g., through nutritional content of crop host plants) could reduce the mating success of moth species that are pests in agriculture.

**Study Insect**

The tobacco budworm, *Heliothis virescens* (Lepidoptera; Noctuidae), is one of the most important pests of field crops, including tobacco, cotton, soybean, sunflower, chickpea and tomato, in the United States (Fitt, 1989; Williams, 2008). In the field, female *H. virescens* can lay 300 to 500 eggs, although 1,000 to 1,500 eggs/female have been recorded on artificial diets (Fye and McAda, 1972). Eggs are spherical, with a flattened base and are deposited on blossoms, fruit, and the terminal growth of plants. Like other species of Lepidoptera, there are five to seven larval instars (Nadgauda and Pitre, 1983). Larvae prefer to feed on plant structures that are high

in nitrogen (Hardwick, 1965), mainly the reproductive and growing parts of the plant, like cotton buds and balls, and tobacco buds (Fitt, 1989). Late instars may diapause, induced by either low temperatures or short day length (Henneberry et al., 1993; Henneberry, 1994). Pupation occurs in the soil. Adults have a siphoning type of mouthpart and feed on plant nectar, like many other species of Lepidoptera (Ramaswamy et al., 1997; Wackers et al., 2007). Females of *H. virescens* are polyandrous (Lamunyon, 2000), with a lifetime average of 2.6 matings (Raulston et al., 1975). Multiple mating of females generally results in greater fecundity (Torresi-Vila et al., 2004). Juvenile hormone (JH) is important for the development of eggs in *H. virescens* (Ramaswamy et al., 1997). It has been postulated that nectar feeding causes increases in JH production and consequently increased egg maturation (Ramaswamy et al., 1997).

*Heliothis virescens* is a highly damaging agricultural pest, primarily due to four characteristics. First, the larvae are polyphagous, feeding on 37 plant species from 14 different families, including a number of important agricultural crops (Blanco et al., 2007). Second, the larvae can diapause, enabling them to survive in a wide range of environmental temperatures (Schneider, 2003). Third, the adults are highly mobile and can move over great distances, enabling them to reach areas that they cannot survive in over winter (Raulston et al., 1986; Roehrdanz et al. 1994). Finally, females are highly fecund and can mature their eggs over a long period so as to take advantage of the availability of nectar and suitable ovipositional sites (Fitt, 1989).

Various methods, including application of insecticides, cultural techniques (Bell and Hayes, 1994; Snodgrass and Stadelbacher, 1994), biological control, use of the microbial insecticide *Bacillus thuringiensis* (Bt; Johnson, 1974; Stone and Sims, 1993), especially in genetically engineered crops (e.g., transgenic Bt cotton; Benedict et al., 1996), and monitoring

populations with sex pheromone (Hartstack et al., 1979; Lopez et al., 1994), are used in the management of this pest in agricultural crops. However, there is a need to develop other, more sustainable methods for continued or improved control of this pest, particularly should resistance to insecticides or Bt toxins become widespread in the field. Intensive insecticide applications for control of *H. virescens* have given rise to the development of resistance to a wide range of synthetic insecticides (Sparks, 1981; Luttrell et al., 1987; Hardee et al., 2001; Teran-Vargas et al., 2005). While the development of transgenic *Bt* cotton expressing Cry1Ac toxin, the most active *Bt* toxin against *H. virescens*, has proven effective for control of *H. virescens* (Blanco et al., 2009), however laboratory selection experiments have demonstrated the potential for development of resistance to this toxin (Ferre´and Van Rie, 2002).

As mentioned previously, one of the major reasons *H. virescens* is a major pests is its high reproductive capacity. It follows that a strategy that reduces its reproductive capacity around agricultural crops should lessen its impact on a crop. Thus its reproductive physiology is a viable target for developing new control methods. One approach is the use of sex pheromones is against the insect, either by using them to monitor populations or by disrupting mating (Cardé and Minks, 1995; Adamczyk and Hubbard, 2006). Sex pheromones are used to monitor *H. virescens* populations around agricultural crops for determining risk and/or monitoring the potential for development of resistance to Bt (Hardee et al., 2001). However, mating disruption is not considered a viable control method for *H. virescens* populations, primarily because of cost (Cardé and Minks, 1995).

*Heliothis virescens* females produce a typical Type I pheromone component blend, consisting of a major component of (*Z*)-11-hexadecenal (Z11-16: Ald), with small amounts of the corresponding alcohol, as well as the aldehydes (*Z*)-9-hexadecenal, (*Z*)-7-hexadecenal, (Z)-9-

tetradecenal, hexadecenal, and tetradecenal (Klun et al., 1980). Pheromone production in the female is diet-regulated by PBAN release from the corpora cardiaca (Groot et al., 2005). Previous work has established that sex pheromone production in *H. virescens* is influenced by both fats in the pheromone gland (Foster, 2005) and HTC (Foster, 2009; Foster and Johnson, 2010, 2011). It is, therefore, an ideal study system to examine the effects of both larval and adult nutrient acquisition on pheromone production, as well as its concomitant effect on egg production (Ramaswamy et al., 1997).

# CHAPTER 2. MATERIALS & METHODS

## Colony Maintenance

*Heliothis virescens* were originally obtained as eggs from the laboratory of Dr. W.L. Roelofs (Cornell University, Geneva, NY) and from the USDA-ARS (Fargo, ND). The colony was maintained as follows. Neonate larvae were placed individually in one ounce plastic cups along with a portion of a casein-wheat germ diet (see later) and held at 25±0.5ºC and a 16:8 (light:dark) photoperiod for approximately two weeks until pupation. After pupation, the sex of insect were checked to separate them as a male and a female, and males and females placed in separate containers. Newly eclosed adults were collected daily and placed in plastic containers along with a 10% sucrose solution (w/v) absorbed onto cotton for feeding *ad libitum*. The next day, males and females were placed in the same container and allowed to mate. Mated females were allowed to oviposit on the walls of the plastic containers over the next few days. Neonate larvae were collected and reared as described.

## Experimental Insects

For experiments, insects were handled similarly as for colony maintenance. For each replicate of an experiment, approximately 30–40 neonate larvae were placed individually on each diet (see below). The progress of larvae was first checked 10–15 days later. Depending upon the experiment, larvae were either left to continue to pupation or sampled when they were 5th instars.

Fifth instars analyzed for either percentage body fat or HTC in different groups in each. Sampling for percentage body fat during the $5^{th}$ instar was destructive. Therefore, separate

groups of insects were sampled for the larval and adult analyses of percentage body fat. However, following hemolymph trehalose sampling (see below), larvae were placed back in their diet-containing cups for sampling HTC of the same adults. These larvae were checked over the next few days to record if and when they had pupated. Larvae that pupated were left for a further day to sclerotize, after which they were sexed and weighed. Following this, pupae were placed in clean containers under the same conditions until they emerged. Adults that emerged were collected each day and analyzed for hemolymph trehalose concentration when 1 to 2 days old. Larvae that had not been sampled for HTC were also checked for pupation, and then sexed, as above. Adults that emerged from these pupae were collected daily and subjected to either hemolymph trehalose or percentage body fat analyses when 1 to 2 days old. Similar numbers of males and females were sampled in all experiments.

**Diets and Experiments**

Four sets of diet were tested in four separate experiments, with each set consisting of a control diet (standard for each set, and the same as used for colony maintenance) and two other diets with changes in the amount of one of the control diet ingredients and/or the addition of another ingredient (Table 1). The control diet was a mixture of wheat germ (obtained from a local health food store), casein, USDA Vitamin Premix, Wesson Salt, agar (all BioServ, Frenchtown, NJ), *p*-hydroxybenzoic acid (Sigma Chemical, Milwaukee, WI), sorbic acid (TCI Chemicals, Montgomeryville, PA) and water (Table 1). Vegetable oil (Our Family, MN) or sucrose (American Crystal Sugar, Moorhead, MN) was added to some of the diets. Each set of diets in an experiment was prepared at the same time (i.e., as a block). Blocks of diets for an

experiment were replicated (see actual numbers in Results) until sufficient insects were available for the various analyses.

Each experiment had three diet treatments, including a control. It should be noted that manipulation of one of the major dietary nutrients, carbohydrate, fat, and protein, influences the carbon to nitrogen (protein) ratio in each experiment. Moreover, an increase in one component (e.g., carbohydrate) necessarily decreases the proportions of the others. In some experiments, decreases in carbon content of one component (e.g., wheat germ) was in part compensated for by addition of another (e.g., carbohydrate). The approximate carbohydrate, fat and protein contents, as well as the caloric value of the each of the diets (Table 2), were calculated using values from http://nutritiondata.self.com.

Experiment 1

Experiment 1 tested insects reared on diets that had different percentages of sucrose (0, 4.2, 12.5%; see Table 1) added to the control diet. The three diets were replicated in nine blocks each. The proportion of insects that survived to $5^{th}$ instar was recorded for each block of diets.

For the hemolymph trehalose analyses, I sampled two sets of insects: one set in which hemolymph was sampled non- destructively at the $5^{th}$ instar and then again at the adult stage for the same insect, and the other in which hemolymph was only sampled for adults. This allowed observation of any effect of larval hemolymph sampling on adult hemolymph trehalose concentration. In addition to hemolymph trehalose analyses, I also recorded the weight of pupae that had been sampled as 5th instars, and the proportion of adults that emerged from the sampled $5^{th}$ instar larvae. For the percentage lipid analyses, insects were sampled as either $5^{th}$ instars or

adults. In addition, the wet weights of dissected larvae (minus gut) and adults (minus wings and antennae) were recorded.

Experiment 2

Experiment 2 tested insects reared on diets with reduced wheat germ (relative to the control diet) which in one case was compensated for by added sucrose (Table 1). Two of the diets were replicated ten times, while one diet (because of high survivorship) was replicated only three times. Insects were analyzed for hemolymph trehalose, percentage lipid, and other parameters, as in Experiment 1.

Experiment 3

Experiment 3 tested insects reared on diets that had normal (relative to the control) or reduced amounts of wheat germ with added fat (vegetable oil) (Table 1). The three diets were each replicated in nine blocks. With the exception of one treatment, insects were analyzed for hemolymph trehalose, percentage lipid, and other parameters, as in Experiment 1. In one treatment (C3+Oil), hemolymph trehalose could not be sampled from adults that had previously been sampled as $5^{th}$ instars, because very few adults (3 adults: one male and two females) emerged from this treatment. Consequently, for this diet, adult hemolymph trehalose data are only available from insects that were not also sampled as larvae.

Experiment 4

Experiment 4 tested insects reared on diets that had reduced protein (casein) which in one diet was compensated for by the addition of sucrose (Table 1). The three diets were each

replicated in four blocks each. The insects were sampled for hemolymph trehalose, percentage lipid, and other parameters, as in Experiment 1.

Table 3 shows the treatments for each of the experiments and the number of insects sampled for the various analyses.

**Hemolymph Trehalose Concentration Analyses**

Hemolymph was sampled and analyzed similarly to the method reported by Foster (2009). Briefly, hemolymph was collected from the insect (adult or larva) by gently holding the insect between the thumb and forefinger, and then making a small puncture in the cuticle of the abdomen using a minute pin. Gentle pressure to the abdomen forced out a small droplet of hemolymph, which was collected with a 5 ml calibrated glass capillary tube (VWR International, Radnor, PA). The hemolymph (ca. 1–10 µl) was expelled into a 2 ml glass vial, and 25 µg (in distilled water) of sorbitol (Sigma Chemicals, St. Louis, MO) added as internal standard. The water was removed from the sample by vacuum in a Labconco vacuum concentrator system (Kansas City, MO), before the sugars in the sample (predominantly trehalose; Foster 2009) were acetylated. Sugars were acetylated by addition of 25 µl of acetic anhydride (Alfa Aesar, Ward Hill, MA) and 25 µl of pyridine (Mallinckrodt, Phillispsburg, NJ) to the dehydrated sample, then heating at 100$^{o}$ C for one hour. Following this, 25 µl of toulene was added to the reaction mixture to facilitate removal of surplus reagents (toluene forms an azeotrope with acetic anhydride) by a gentle stream of nitrogen.

The acetylated sugars were dissolved in 200 µl of distilled dichloromethane, and 1 ml of this injected splitlessly into a Varian 3800 gas chromatograph (GC) with flame ionization detection. The GC was equipped with a 30 m × 0.25 mm i d., ZB-5 column (Phenomenex,

Torrance, CA), with helium as carrier gas at 30 $cm.sec^{-1}$. The GC oven temperature program was 200–280 at 10°$C.min^{-1}$; following an initial delay of 1 min. Peak areas were integrated using Syntech (Hilversum, Netherlands) Gas Chromatography-Electroantennogram Detection software. The amount of trehalose in the original sample was determined by first determining the mass of trehalose octaacetate, relative to the mass of sorbitol hexaacetate internal standard, in the derivatized sample, and then calculating the number of moles of trehalose (number of moles of trehalose octaacetate = number of moles of trehalose). The number for moles of trehalose in the sample was divided by the volume of hemolymph to calculate HTC.

**Percentage Body Fat (Lipid) Analyses**

Percentage lipid was determined using the lipid extraction method of Folch et al. (1957). A larva or adult was first immobilized on crushed ice. For a larva, the head was removed, and the body cuticle transected, dorsally. The gut was removed using fine forceps, and the rest of the insect placed in a pre-labeled and pre-weighed glass vial. The vial was weighed again to obtain the mass of the dissected insect. The vial was placed in an oven at 56° C for approximately 72 hours until the sample was completely dry. The dried sample was homogenized in a glass homogenizer and shaken with 10 ml of dichloromethane: methanol (2:1). The liquid was filtered through a Whatman filter paper (number 3) into a vial. Next, 2 ml of 0.73% sodium chloride solution was added to the filtrate and left to sit for at least 30 min. The upper, clear layer (aqueous) was decanted using a Pasteur pipet. Next, 1 ml of a mixture of 120 ml of methanol, 240 ml of dichloromethane, and 90 ml of 0.034% magnesium chloride was used to wash the sample three times (total of 3 ml of solvent). After each wash, the upper phase was decanted and discarded. Methanol was added to bring the volume back to 10 ml and the solvent allowed to

evaporate overnight so that the residue was solvent free. The residue was dissolved in 3 aliquots of dichloromethane (total 1 ml) and transferred into pre-weighed and labeled glass tubes. The solvent was allowed to evaporate overnight before the dried sample was weighed, yielding the mass of lipid in the insect.

**Data Analyses**

Data for all experiments were analyzed by a two way ANOVA, with an interaction effect, for effects of diet and sex. These interactions were examined to determine whether there were any patterns between sexes among treatments in each experiment. If the diet × sex interaction was not significant ($P > 0.05$; i.e. same pattern between sexes among diets), the data for males and females were combined; if the interaction effect was significant, male and female data were analyzed separately. No significant interactions were observed in the experiments, male and female data were combined. For each of the experiments, data were analyzed as follows:

Proportion of test larvae that survived to $5^{th}$ instar

The proportion of test larvae that survived to $5^{th}$ instar was recorded for each block of diets prepared; the means of these proportions over all the blocks were calculated. The number of blocks of diet prepared in a particular experiment was related to the survival of insects in the batches. If a large proportion of insects survived on a particular diet, then a smaller number of blocks were prepared; if few insects survived, then a relatively large number of blocks were prepared. Consequently, there was a large difference in the number of blocks of diet prepared for the four experiments (see Results), with one experiment requiring only three blocks of each diet. Therefore, the extremely high variation across treatments leads to weak (low power) statistical

comparisons. Hence, survival data are given for information only and were not statistically compared.

Proportion of emerged adults from sampled 5$^{th}$ instar larvae

The proportion of adults that emerged after having been sampled as 5$^{th}$ instar larvae was recorded for each batch of diets. Mean of these proportions over all blocks of diets were calculated. As for the data describing survival to 5$^{th}$ instar, the number of blocks of diets prepared for a given experiment was highly variable. Consequently, these survival data were not compared statistically.

Pupal weight of hemolymph trehalose-sampled 5$^{th}$ instars

These data were analyzed by a two-way ANOVA. Mean pupal weights were compared by a Tukey-Kramer test with a = 0.05.

Dissected wet weight of larvae

The mean wet weights of larvae dissected for the percentage lipid analyses on the different diets (within an experiment) were compared by one-way ANOVA. Note that the sex of the larvae was not determined. The linear relationship between dissected wet weight of a larva and its weight of lipid was tested for all four experiments.

Dissected wet weight of adults

The mean wet weights of adults (for the percentage lipid analyses) for a given set of diets (within an experiment) were compared by two-way ANOVA. Means of treatments compared by

Tukey-Kramer tests at a = 0.05. The linear relationship between dissected wet weight of adult and its total lipid weight was tested for all four experiments.

Hemolymph trehalose concentration analyses

The calculation of HTC had the capacity to introduce large errors, especially if small volumes of hemolymph were sampled. Therefore, prior to statistical comparisons, I examined the data for outliers, using the program JMP (SAS Institute, Carey, NC). Data points that lie outside two standard deviations from the treatment mean were designated outliers and excluded.

For each experiment, I obtained two sets of HTC data. One set was from the group of insects in which the same insect was sampled both as a larva and as an adult. These data were compared separately for larvae and adults by a two-way ANOVA. Means of treatments were compared by Tukey-Kramer tests at a = 0.05. Additionally, with this set of data, I tested the linear correlation between larval and adult HTCs, and the linear relationship between pupal weight and larval and adult HTCs, across treatments in all four experiments.

The second set of HTC data was from analyses of adults that had not been sampled as larvae. This set served as a control for any effects of larval hemolymph sampling on adult hemolymph trehalose concentration for the various treatments. Data from this set were analyzed as for the first data set.

Percentage body fat (lipid) analyses

For each set of diets, data on percentage lipid of larvae were obtained and compared by one-way ANOVA, whereas the percentage lipid data of adults were compared by two-way ANOVA. Means of treatments compared by Tukey-Kramer tests at a = 0.05.

**Table 1.** Composition of diets used in the four experiments. Changes in diet components, relative to control diets, are shown in bold.

| **Experiment/Diet** | **Wheat germ (g)** | **Casein (g)** | **Vitamin Premix (g)** | **Salt Mix (g)** | ***p*-hydroxy benzoic acid** | **Sorbic acid (g)** | **Agar (g)** | **Sucrose (g)** | **vegetable oil (g)** | **Water (ml)** |
|---|---|---|---|---|---|---|---|---|---|---|
| **Experiment 1 - added sucrose.** | | | | | | | | | | |
| Control 1 (C1) | 80.0 | 17.0 | 6.67 | 5.34 | 0.67 | 1.34 | 10 | | | 590 |
| C1+4.2%Sug | 80.0 | 17.0 | 6.67 | 5.34 | 0.67 | 1.34 | 10 | **3.34** | | 590 |
| C1+12.5%Sug | 80.0 | 17.0 | 6.67 | 5.34 | 0.67 | 1.34 | 10 | **10.0** | | 590 |
| **Experiment 2 – decreased wheat germ plus sucrose.** | | | | | | | | | | |
| Control 2 (C2) | 80.0 | 17.0 | 6.67 | 5.34 | 0.67 | 1.34 | 10 | | | 590 |
| C2-Wg | **70.0** | 17.0 | 6.67 | 5.34 | 0.67 | 1.34 | 10 | | | 590 |
| C2-Wg+14.3%Sug | **70.0** | 17.0 | 6.67 | 5.34 | 0.67 | 1.34 | 10 | **10.0** | | 590 |
| **Experiment 3 - decreased wheat germ plus oil.** | | | | | | | | | | |
| Control 3 (C3) | 80.0 | 17.0 | 6.67 | 5.34 | 0.67 | 1.34 | 10 | | | 590 |
| C3-Wg+Oil | **70.0** | 17.0 | 6.67 | 5.34 | 0.67 | 1.34 | 10 | | **10.0** | 590 |
| C3+Oil | 80.0 | 17.0 | 6.67 | 5.34 | 0.67 | 1.34 | 10 | | **10.0** | 590 |
| **Experiment 4 - decreased protein plus sucrose.** | | | | | | | | | | |
| Control 4 (C4) | 80.0 | 17.0 | 6.67 | 5.34 | 0.67 | 1.34 | 10 | | | 590 |
| C4-Ca | 80.0 | **10.0** | 6.67 | 5.34 | 0.67 | 1.34 | 10 | | | 590 |
| C4-Ca+12.5%Sug | 80.0 | **10.0** | 6.67 | 5.34 | 0.67 | 1.34 | 10 | **10.0** | | 590 |

**Table 2.** Calculated percentages of carbohydrate, fat, protein, and caloric values of the diets used in Experiments 1–4. See Table 1 for a list of dietary ingredients.

| | % Carbohydrate | % Fat | % Protein | Calories/gram diet |
|---|---|---|---|---|
| **Experiment 1 - added sucrose.** | | | | |
| Control 1 (C1) | 45.3 | 16.3 | 26.9 | 2.88 |
| C1+4.2%Sug | 46.8 | 15.8 | 26.1 | 2.95 |
| C1+12.5%Sug | 49.5 | 15 | 24.8 | 3.08 |
| **Experiment 2 – decreased wheat germ plus sucrose.** | | | | |
| Control 2 (C2) | 45.3 | 16.3 | 26.9 | 2.88 |
| C2-Wg | 44.5 | 15.7 | 27.2 | 2.81 |
| C2-Wg+14.3%Sug | 49.1 | 14.4 | 25 | 3.04 |
| **Experiment 3 - decreased wheat germ plus fat.** | | | | |
| Control 3 (C3) | 45.3 | 16.3 | 26.9 | 2.88 |
| C3-Wg+Oil | 40.8 | 22.6 | 25 | 3.33 |
| C3+Oil | 41.8 | 22.7 | 24.8 | 3.31 |
| **Experiment 4 - decreased protein plus sucrose.** | | | | |
| Control 4 (C4) | 45.3 | 16.3 | 26.9 | 2.88 |
| C4-Ca | 47.3 | 16.8 | 23.6 | 2.85 |
| C4-Ca+12.5%Sug | 51.5 | 15.5 | 21.7 | 3.06 |

**Table 3**. Analyzed insects in Experiments 1–4**.** Larvae (1) Tre, Adults (1) Tre, and Adults (2) Tre were the insects that were sampled for hemolymph trehalose. Suffix (1) indicates insects that were sampled both as larvae and adults, while suffix (2) indicates insects that were only sampled as adults. And, Larvae Lipid and Adults lipids were the insects tested for body fat analysis. N shows the number of replicates for the tested insects. See Table 1 for a list of dietary ingredients.

| **Experiment/Experimental insects stages** | **Larvae (1) Tre (N)** | **Adults (1) Tre (N)** | **Adults (2) Tre (N)** | **Larave Lipid (N)** | **Adults Lipid (N)** |
|---|---|---|---|---|---|
| **Experiment 1 - added sucrose** | | | | | |
| Control 1 (C1) | 14 | 14 | 10 | 12 | 14 |
| C1+4.2%Sug | 17 | 17 | 18 | 12 | 18 |
| C1+12.5%Sug | 17 | 17 | 16 | 9 | 17 |
| **Experiment 2 – decreased wheat germ plus sucrose** | | | | | |
| Control 2 (C2) | 16 | 16 | 18 | 12 | 20 |
| C2-Wg | 16 | 16 | 18 | 12 | 21 |
| C2-Wg+14.3%Sug | 16 | 16 | 21 | 11 | 21 |
| **Experiment 3 - decreased wheat germ plus oil** | | | | | |
| Control 3 (C3) | 17 | 17 | 18 | 11 | 22 |
| C3-Wg+Oil | 13 | 13 | 18 | 11 | 22 |
| C3+Oil | 12 | N/A | 18 | 11 | 22 |
| **Experiment 4 - decreased protein plus sucrose** | | | | | |
| Control 4 (C4) | 19 | 19 | N/A | 12 | 24 |
| C4-Ca | 14 | 14 | N/A | 12 | 24 |
| C4-Ca+12.5%Sug | 14 | 14 | N/A | 12 | 23 |

# CHAPTER 3. RESULTS

## Proportion of Larvae that Survived to 5th Instar

Table 3 shows the survival rate (to the time of 5th instar sampling) of insects on the different diets in the four experiments. Control survival was highly variable across the four experiments but, notably, increased over the four experiments from a mean of 44–71%. As the experiments were carried out in sequential order, this suggests that my diet preparation and handling of insects improved with experience. Typically, survival percentages were fairly similar between the control diet and the experimental diets within an experiment, although the mean survival percentage of insects on the C1+12.5%Sug diet in Experiment 1 was substantially lower than for the other two diets, while the mean survival of insects on the C2-Wg diet in Experiment 2 was substantially higher than that on the control diet.

## Percentage of Emerged Adults from Sampled 5th Instar Larvae

Table 4 shows the mean percentages of adults, sampled for hemolymph when 5th instars, that emerged as adults for all four experiments. For the first three experiments, mean percentage emergence was generally low, usually < 50%. In the fourth experiment, however, the mean percentage emergence was roughly 70% for all three diets. This suggests, again, that my handling of insects improved with experience.

## Pupal Weight of Trehalose-Sampled 5th Instars

Mean weights of pupae that were sampled for hemolymph as 5th instars are shown in Figure 3. The interaction between diet and sex was not significant ($P > 0.05$) for any of the experiments, so pupal weight data of males and females were combined for treatments. In Experiments 1, 2, & 4 (Fig. 3 a, b, d), there were no differences ($P > 0.05$, Tukey-Kramer HSD test) in pupal weights among any of the treatments. However, in Experiment 3 (Fig. 3c) the two

added fat treatments were different from each other; the mean weight of pupae on the diet with reduced wheat germ plus fat (C3-Wg+Oil) was greater ($P > 0.05$, Tukey-Kramer HSD test) than the mean weight of pupae on the diet with added fat (C3+Oil). Pupae that had been reared on either of these diets did not have a different mean weight from pupae that had fed on the control diet.

**Dissected Wet Weight of Larvae**

There were no differences ($P > 0.05$) in mean dissected wet weights of larvae among treatments in all four experiments (Fig. 4).

**Dissected Wet Weight of Adults**

In all four experiments, there were no significant interactions between diet and sex. Hence, male and female data were combined. There were no differences ($P > 0.05$) in mean dissected wet weight of adults among treatments in all four experiments (Fig. 5).

**Hemolymph Trehalose Concentration**

The mean HTCs of insects analyzed both as larvae and adults, and as adults only, are given in Figures 6, 7, 8, & 9. Note that in Experiment 3, I did not obtain any adults from insects that had been sampled as larvae for one of the diets (C3+oil) and, consequently, adult HTC data are not available for this treatment. In all four experiments, there was no significant interaction between diet and sex for either larvae or adult HTC. Thus, data for both males and females (both larvae and adults) were combined.

In Experiment 1 (added sucrose), there were no differences in mean HTC of larvae, previously sampled adults, or adults that had not been sampled as larvae among any of the diets (Fig. 6 a, b, & c). In Experiment 2 (reduced wheat germ) there were no differences in mean HTC

of larvae on the different diets (Fig. 7a). However, for adults that had previously been sampled (Fig. 7b), insects reared on the decreased wheat germ diet (C2-Wg) had a lower mean HTC than did those on the other two diets (i.e., control and reduced wheat germ plus sucrose). For adults that had not been sampled previously, the trend was similar, although the difference between C2-Wg and C2-Wg+14.3%Sug was not significant (Fig.7c). In Experiment 3 (added oil), there was an effect of diet on larval HTC. In particular, addition of oil to the diet appeared to result in a decrease in larval HTC. This effect was significant between insects fed on the control diet and those fed on the reduced wheat germ diet with added oil. Diet did not affect mean adult HTCs (Fig. 8 a, b, & c). In Experiment 4 (reduced protein), diet had no effect on larval or adult HTC (Fig. 9a & b). There was an experimental problem with the analyses for the adults that had not been previously sampled, so these data are not available.

There was no significant linear relationship between pupal weight and larval HTC in Experiments 1, 2 and 3 (Figs 6d, 7d, & 8d), but there was in Experiment 4 (Fig. 9c). The pupal weight and adult HTC were not significantly correlated in all four experiments (Figs 6e, 7e, 8e, & 9d). Across all the experiments, there was a significant positive correlation ($P < 0.0001$) between HTCs of larva and adult, indicating that the greater the HTC in a larva the greater HTC in the resulting adult (Fig. 10).

**Table 4.** Mean percentage survival to 5th instar of first instar larvae on different diets. C1, C2, C3, and C4 = Control diets from Experiments 1, 2, 3 and 4, respectively. C1+4.2%Sug = 4.2% sucrose, of total wheat germ, added to control diet; C1+12.5%Sug =12.5% sucrose, of total wheat germ, added to control diet; C2-Wg = wheat germ content was decreased relative to the control diet; C2-Wg+14.3%Sug =14.3% sucrose, of total wheat germ, added to the decreased wheat germ diet; C3-Wg+Oil = vegetable oil added to the decreased wheat germ diet; C3+Oil= vegetable oil added to the control diet; C4-Ca= decreased casein content relative to the control diet; C4-Ca+12.5%Sug = 12.5% sucrose, relative to the amount of wheat germ, was added to the casein-reduced diet. N = Number of blocks made of each diet, each block consisting of diet for 30–50 insects.

| **Mean %survival of 5th instar larvae (N)** | **Diets** | | |
|---|---|---|---|
| **Experiment 1** | **Control 1**<br>44.1±5.9 (9) | **C1+4.2%Sug**<br>45.1±5.9 (9) | **C1+12.5%Sug**<br>33.0±5.9 (9) |
| **Experiment 2** | **Control 2**<br>54.7±6.5 (10) | **C2-Wg**<br>82.4±11.9 (3) | **C2-Wg+14.3%Sug**<br>51.7±6.5 (10) |
| **Experiment 3** | **Control 3**<br>68.9±6.5 (9) | **C3-Wg+Oil**<br>52.2±6.5 (9) | **C3+Oil**<br>58.4±6.5 (9) |
| **Experiment 4** | **Control 4**<br>70.9±16.3 (4) | **C4-Ca**<br>77.2±16.3 (4) | **C4-Ca+12.5%Sug**<br>76.8±16.3 (4) |

**Table 5.** Mean percentage survival to adult of insects sampled for hemolymph at 5$^{th}$ instar. C1, C2, C3, and C4 = Control diets from experiments 1, 2, 3 and 4, respectively. C1+4.2%Sug = 4.2% sucrose, of total wheat germ, added to control diet; C1+12.5%Sug =12.5% sucrose, of total wheat germ, added to control diet; C2-Wg = wheat germ content decreased relative to control diet; C2-Wg+14.3%Sug =14.3% sucrose, of total wheat germ, added to the decreased wheat germ diet; C3-Wg+Oil = vegetable oil added to the decreased wheat germ diet; C3+Oil = vegetable oil added to the control diet; C4-Ca = casein amount decreased relative to the control diet; C4-Ca+12.5%Sug = 12.5% sucrose, relative to the amount of wheat germ, added to the casein-reduced diet. N = Number of blocks made of each diet, each block consisting of diet for 30–50 insects.

| **Mean % survival to adult of larvae sampled for hemolymphat 5$^{th}$ instar (N)** | **Diets** | | |
|---|---|---|---|
| **Experiment 1** | **Control 1**<br>36.1±9.9 (8) | **C1+4.2%Sug**<br>39.9±9.9 (8) | **C1+12.5%Sug**<br>23.1±9.9 (8) |
| **Experiment 2** | **Control 2**<br>44.3±11.8 (9) | **C2-Wg**<br>70.3±20.4 (3) | **C2-Wg+14.3%Sug**<br>47.3±11.8 (9) |
| **Experiment 3** | **Control 3**<br>28.5±8.7 (7) | **C3-Wg+Oil**<br>24.2±8.1 (8) | **C3+Oil**<br>5.5±8.7 (7) |
| **Experiment 4** | **Control 4**<br>64.2±20.2 (4) | **C4-Ca**<br>70.1±20.2 (4) | **C4-Ca+12.5%Sug**<br>77.1±20.2 (4) |

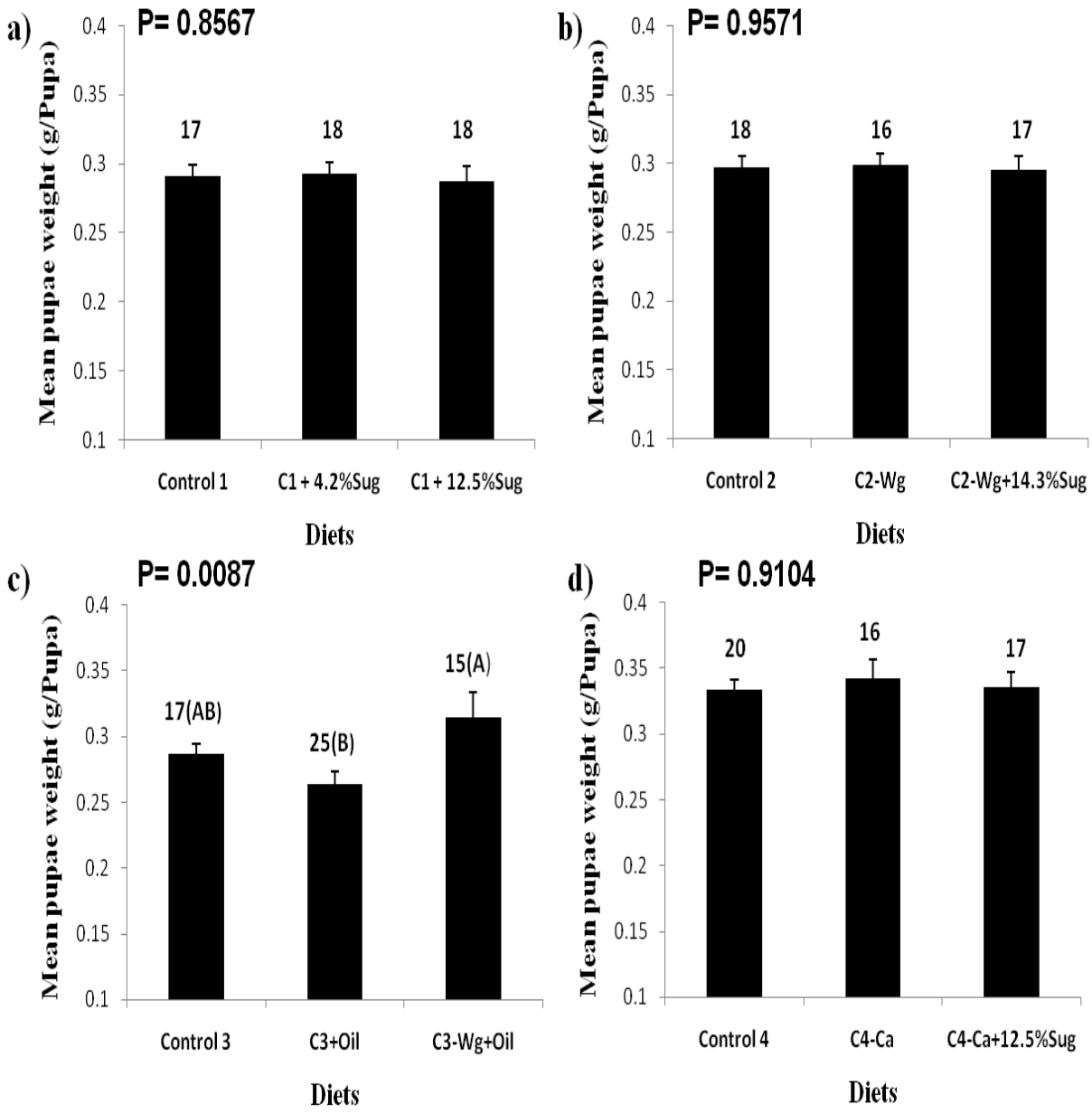



**Figure 3.** Mean pupal weight of hemolymph-sampled 5$^{th}$ instars from (a) Experiment 1, (b) Experiment 2, (c) Experiment 3, and (d) Experiment 4. C1, C2, C3, and C4 = Control diets in Experiments 1, 2, 3 and 4, respectively. C1+4.2%Sug = 4.2% sucrose, of total wheat germ, added to control diet; C1+12.5%Sug =12.5% sucrose added to control diet; C2-Wg = wheat germ content decreased relative to control diet; C2-Wg+14.3%Sug =14.3% sucrose, of total wheat germ, added to decreased wheat germ diet; C3-Wg+Oil = vegetable oil added to the decreased wheat germ diet; C3+Oil = vegetable oil added to the control diet; C4-Ca = amount of casein decreased relative to control diet; C4-Ca+12.5%Sug = 12.5% sucrose, relative to the amount of wheat germ, added to the casein-reduced diet. See Table 1 for composition of diets. N is shown above standard errors. Different letters inside parentheses atop bars indicate means that are different ($P < 0.05$) by Tukey-Kramer tests. P-values from two-way ANOVAs are given.

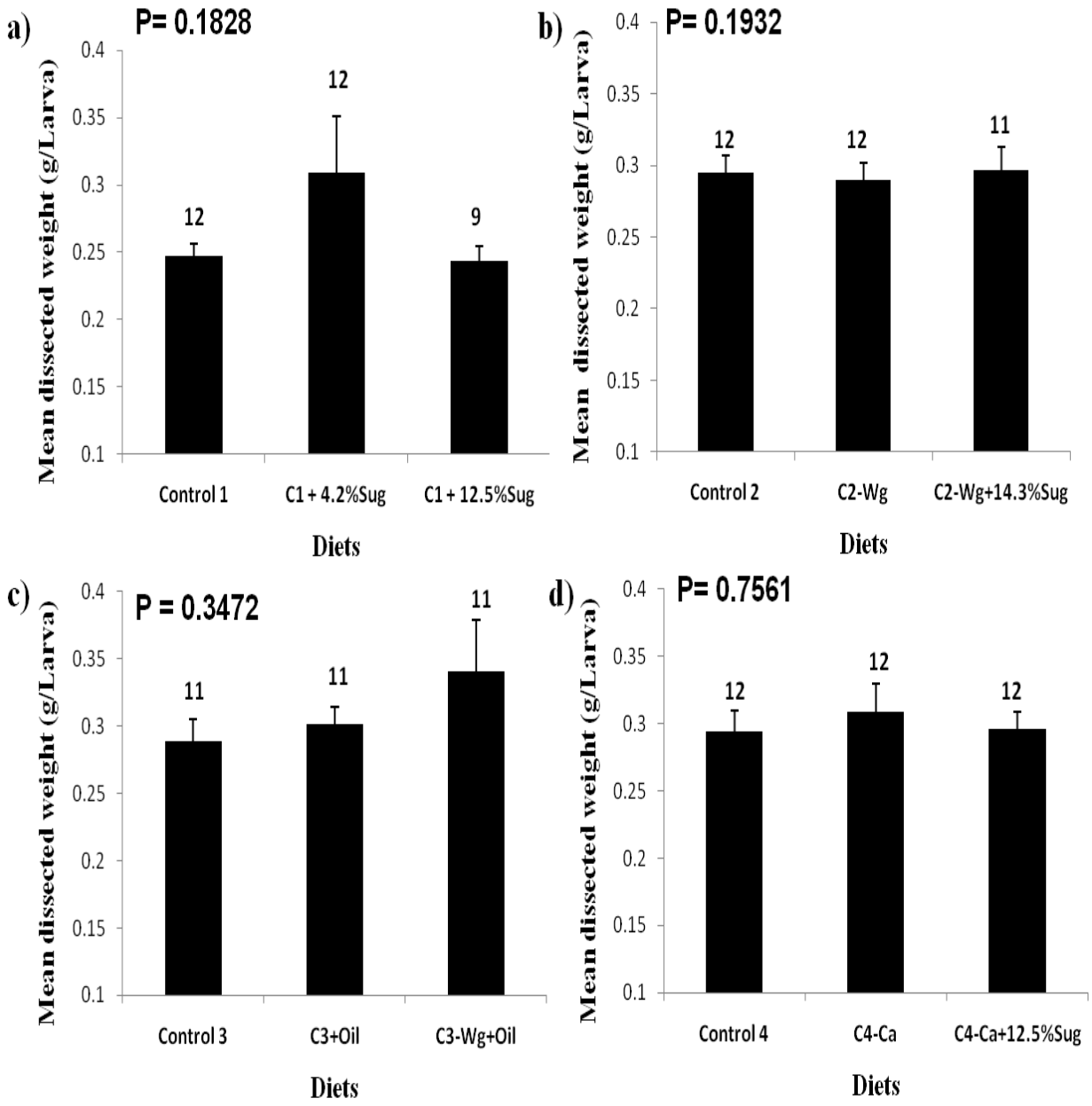



**Figure 4.** Mean dissected (minus gut) weights of larvae in (a) Experiment 1, (b) Experiment 2, (c) Experiment 3, and (d) Experiment 4. C1, C2, C3, and C4 = Control diets in Experiments 1, 2, 3 and 4, respectively. C1+4.2%Sug = 4.2% sucrose, of total wheat germ, added to control diet; C1+12.5%Sug =12.5% sucrose added to control diet; C2-Wg = wheat germ content decreased relative to control diet; C2-Wg+14.3%Sug =14.3% sucrose, of total wheat germ, added to decreased wheat germ diet; C3-Wg+Oil = vegetable oil added to decreased wheat germ diet; C3+Oil = vegetable oil added to control diet; C4-Ca = amount of casein decreased relative to control diet; C4-Ca+12.5%Sug = 12.5% sucrose, relative to the amount of wheat germ, added to casein-reduced diet. See Table 1 for composition of diets. N is above the standard errors. P-values from one-way ANOVAs are given.

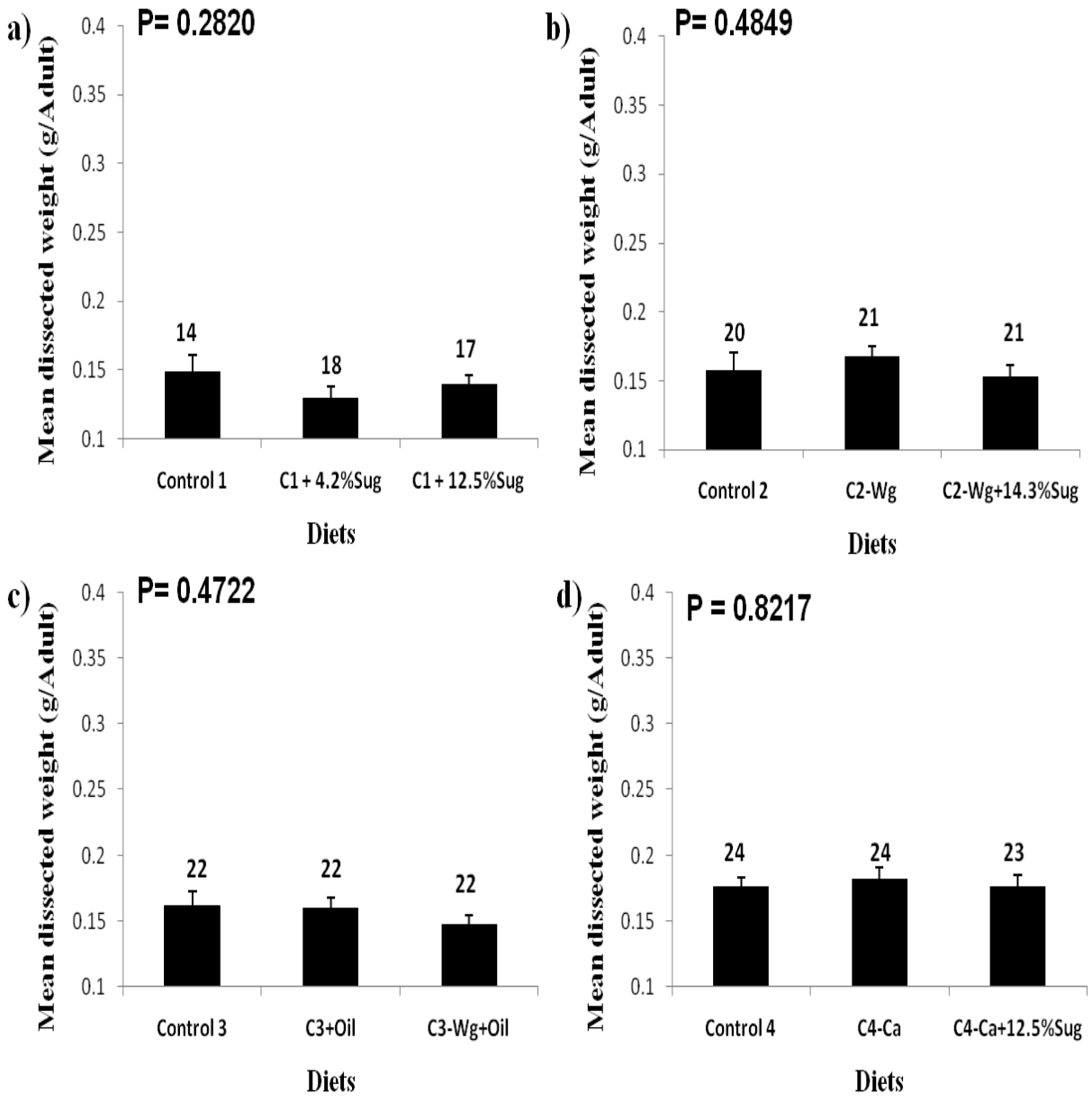



**Figure 5.** Mean dissected (minus antennae and wings ) weights of adults in (a) Experiment 1, (b) Experiment 2, (c) Experiment 3, and (d) Experiment 4. C1, C2, C3, and C4 = Control diets in Experiments 1, 2, 3 and 4, respectively. C1+4.2%Sug = 4.2% sucrose, of total wheat germ, added to control diet; C1+12.5%Sug =12.5% sucrose added to control diet; C2-Wg = wheat germ content decreased relative to control diet; C2-Wg+14.3%Sug =14.3% sucrose, of total wheat germ, added to decreased wheat germ diet; C3-Wg+Oil = vegetable oil added to decreased wheat germ diet; C3+Oil = vegetable oil added to control diet; C4-Ca = amount of casein decreased relative to control diet; C4-Ca+12.5%Sug = 12.5% sucrose, relative to amount of wheat germ, added to casein-reduced diet. See Table 1 for composition of diets. N is above the standard error bars. P-values from two-way ANOVAs are given.

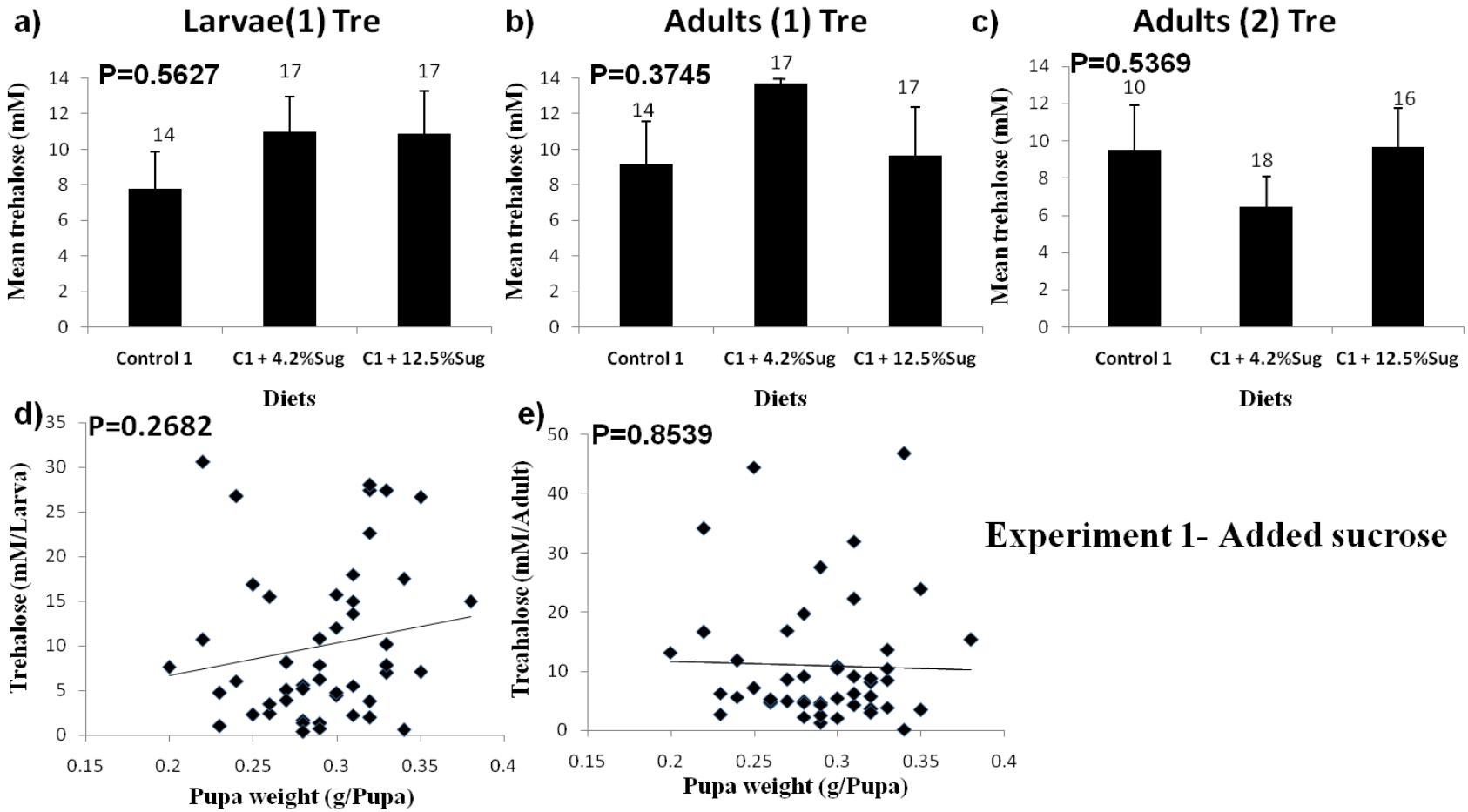



**Figure 6.** Hemolymph trehalose concentration of insects in Experiment 1**.** Mean hemolymph trehalose concentrations (mM) of insects fed on different diets. (a) Larvae, (b) adults, and (c) adults not sampled as larvae. Suffix (1) indicates insects that were sampled both as larvae and adults, while suffix (2) indicates insects that were only sampled as adults. C1 = control diet; C1+4.2%Sug = 4.2% sucrose, of total wheat germ, added to control diet; C1+12.5%Sug =12.5% sucrose added to control diet. There were no significant differences in hemolymph trehalose concentrations among insects on any of the diets. P-values from two-way ANOVAs are given. N is shown above standard error. Relationships between pupal weight and (d) larval or (e) adult hemolymph trehalose concentrations across all diets in the experiment. A line of best fit and p-values are given for both graphs.

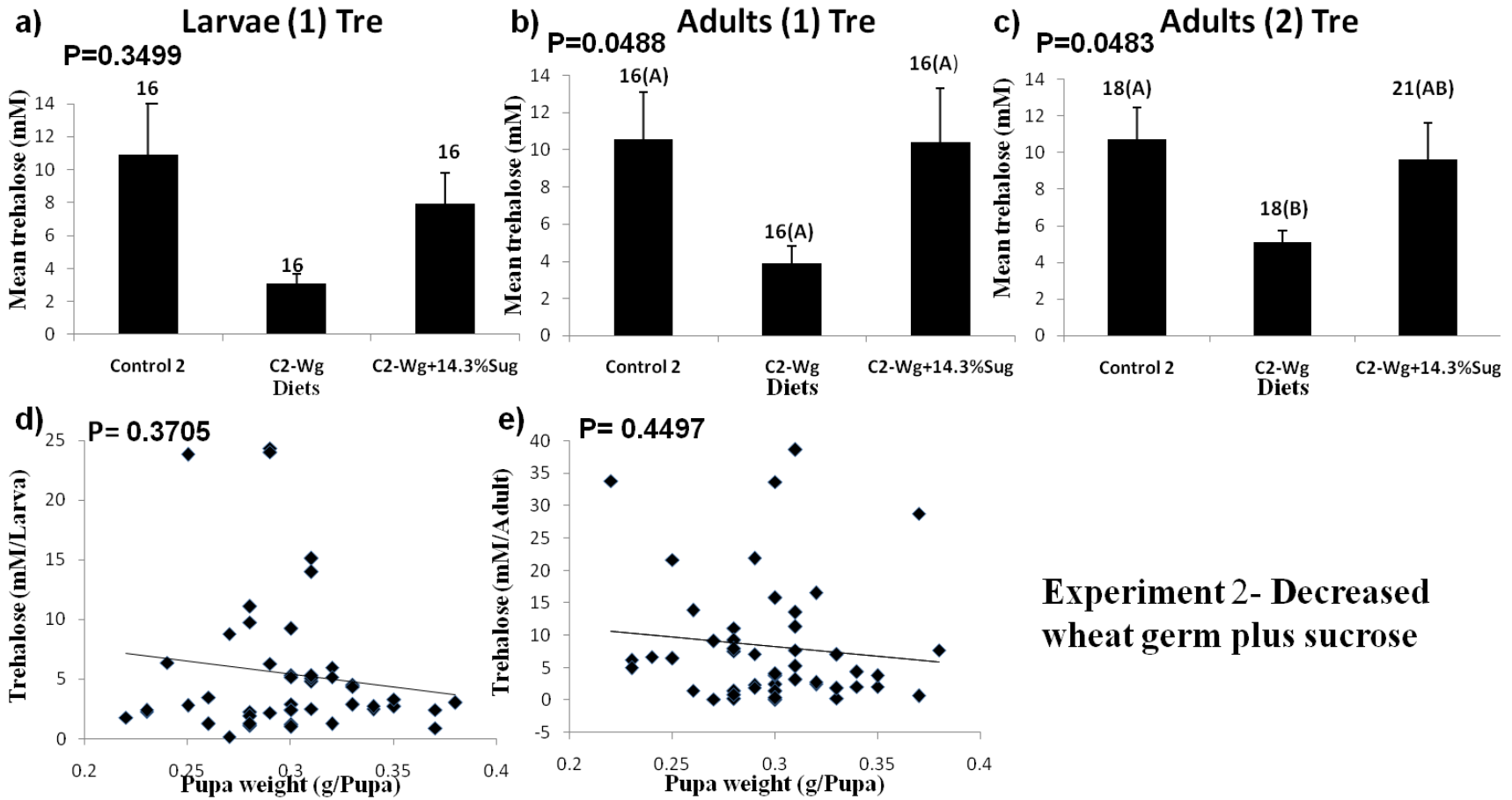



**Figure 7.** Hemolymph trehalose concentration of insects in Experiment 2**.** Mean hemolymph trehalose concentrations (mM) of insects fed on different diets**.** (a) Larvae, (b) adults, and (c) adults not sampled as larvae. The suffix (1) indicates insects sampled both as larvae and adults, while the suffix (2) indicates insects that were sampled only as adults. C2 = control diet; C2-Wg = wheat germ content decreased relative to control diet; C2-Wg+14.3%Sug =14.3% sucrose, of total wheat germ, added to the decreased wheat germ diet. N is shown above standard error. P-values from two-way ANOVAs are given. Different letters, in parentheses, indicate means that are different ($P < 0.05$) by Tukey-Kramer tests. Relationships between pupal weight and (d) larval or (e) adult hemolymph trehalose concentrations across all diets in the experiment. A line of best fit and p-values are given for both graphs.

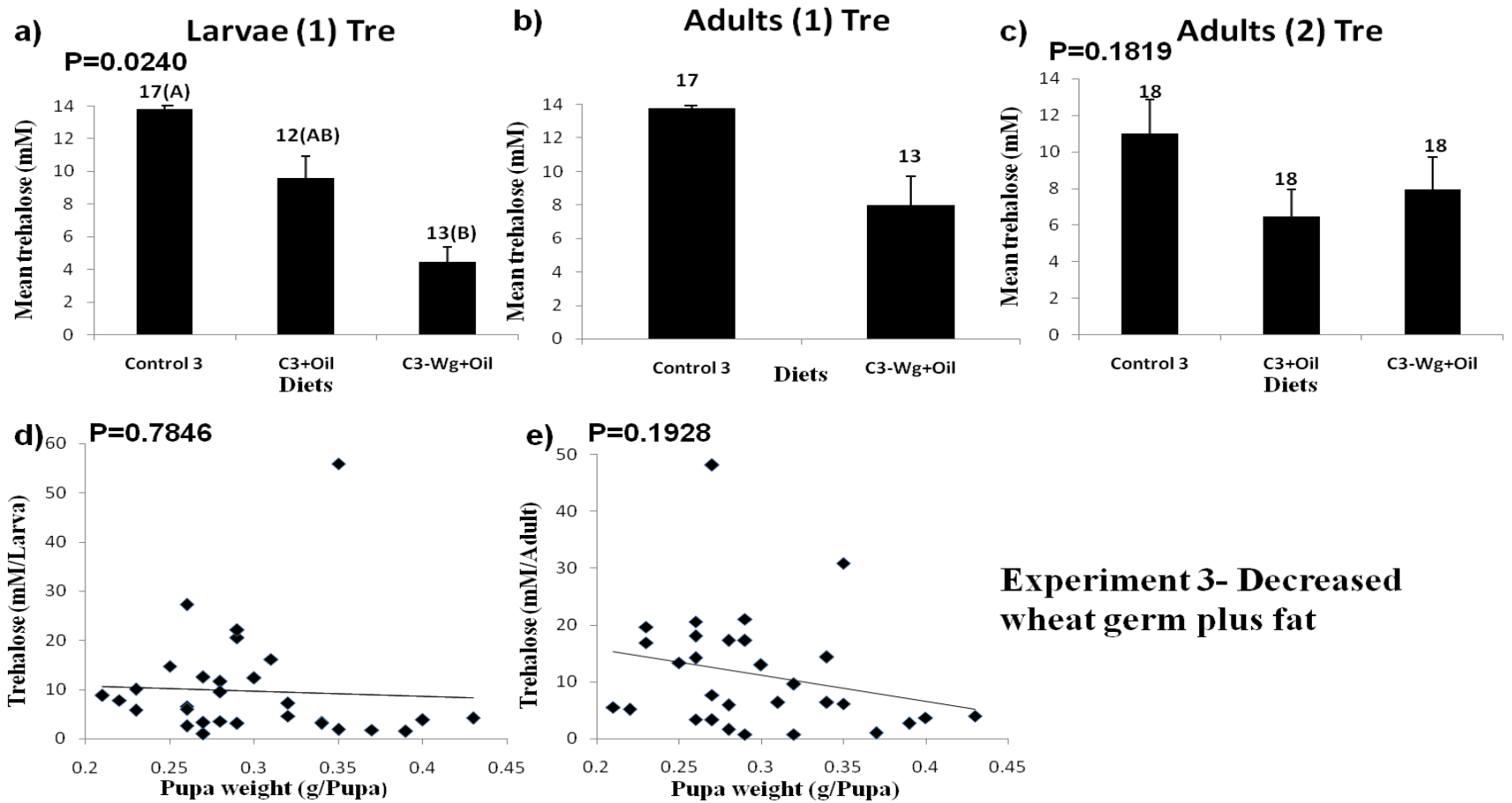
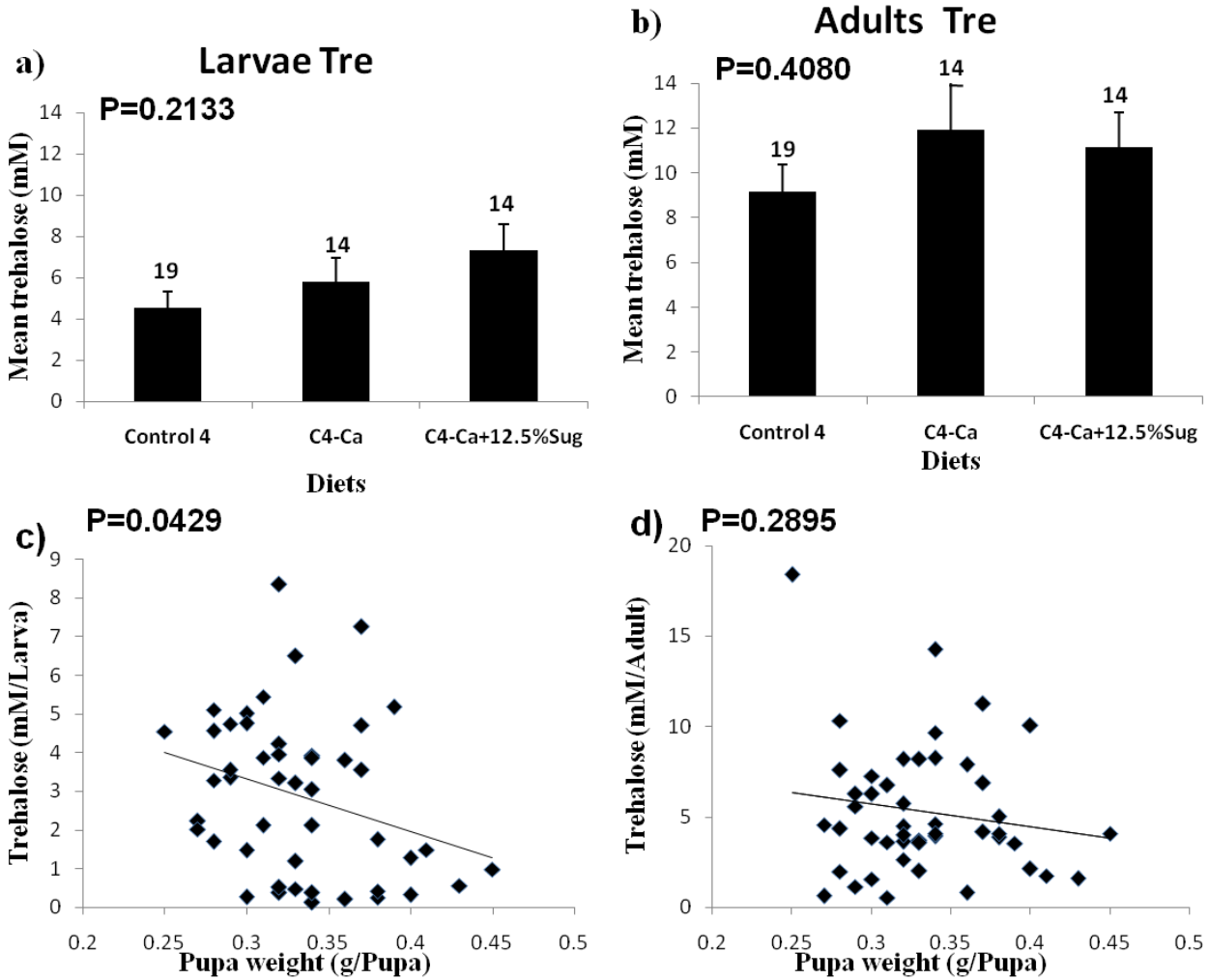



**Figure 8.** Hemolymph trehalose concentration in insects for Experiment 3**.** Mean hemolymph trehalose concentrations (mM) of insects fed on different diets. (a) Larvae, (b) adults, and (c) adults not sampled as larvae. The suffix (1) indicates insects that sampled both as larvae and adults, while suffix (2) indicates insects that sampled only as adults.C3 = control diet; C3+Oil = vegetable oil added to control diet; C3-Wg+Oil = vegetable oil added to decreased wheat germ diet. N is shown above standard error. P-values from one-way ANOVA for larvae (1) and two-way ANOVA for adults (2) are given. Different letters, in parentheses, indicate means that are different ($P < 0.05$) by Tukey-Kramer tests. Relationships between pupal weight and (d) larval or (e) adult hemolymph trehalose concentrations across all diets in the experiment. A line of best fit and p-values are given for both graphs.




**Experiment 4 - Decreased protein plus sucrose**

**Figure 9.** Hemolymph trehalose concentration of insects in Experiment 4**.** Mean hemolymph trehalose concentrations (mM) of insects fed on different diets.(a) Larvae and (b) adults, C4 = control diet; C4-Ca = decreased (relative to control) casein; C4-Ca+12.5%Sug = 12.5% sucrose, relative to the amount of wheat germ, added to the casein-reduced diet. N is shown above the standard error. P-values from two-way ANOVAs are given. Relationships between pupal weight and (c) larval or (d) adult hemolymph trehalose concentrations across all diets in the experiment. A line of best fit and p-values are given for both graphs.

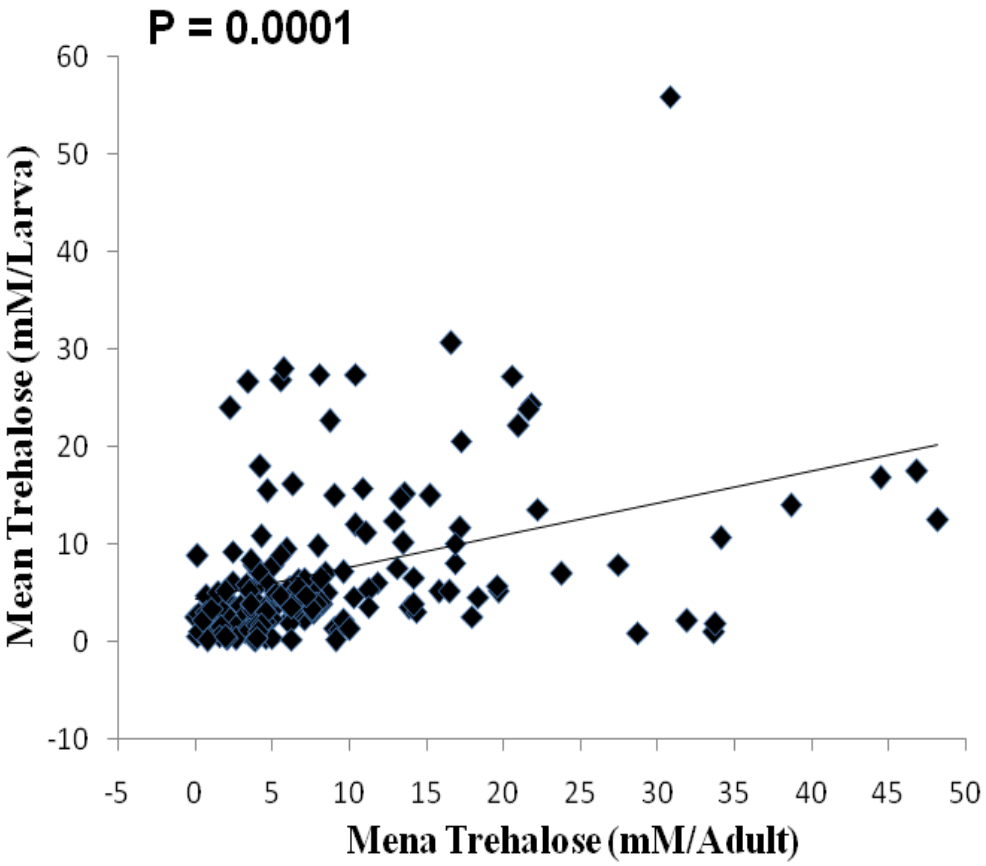


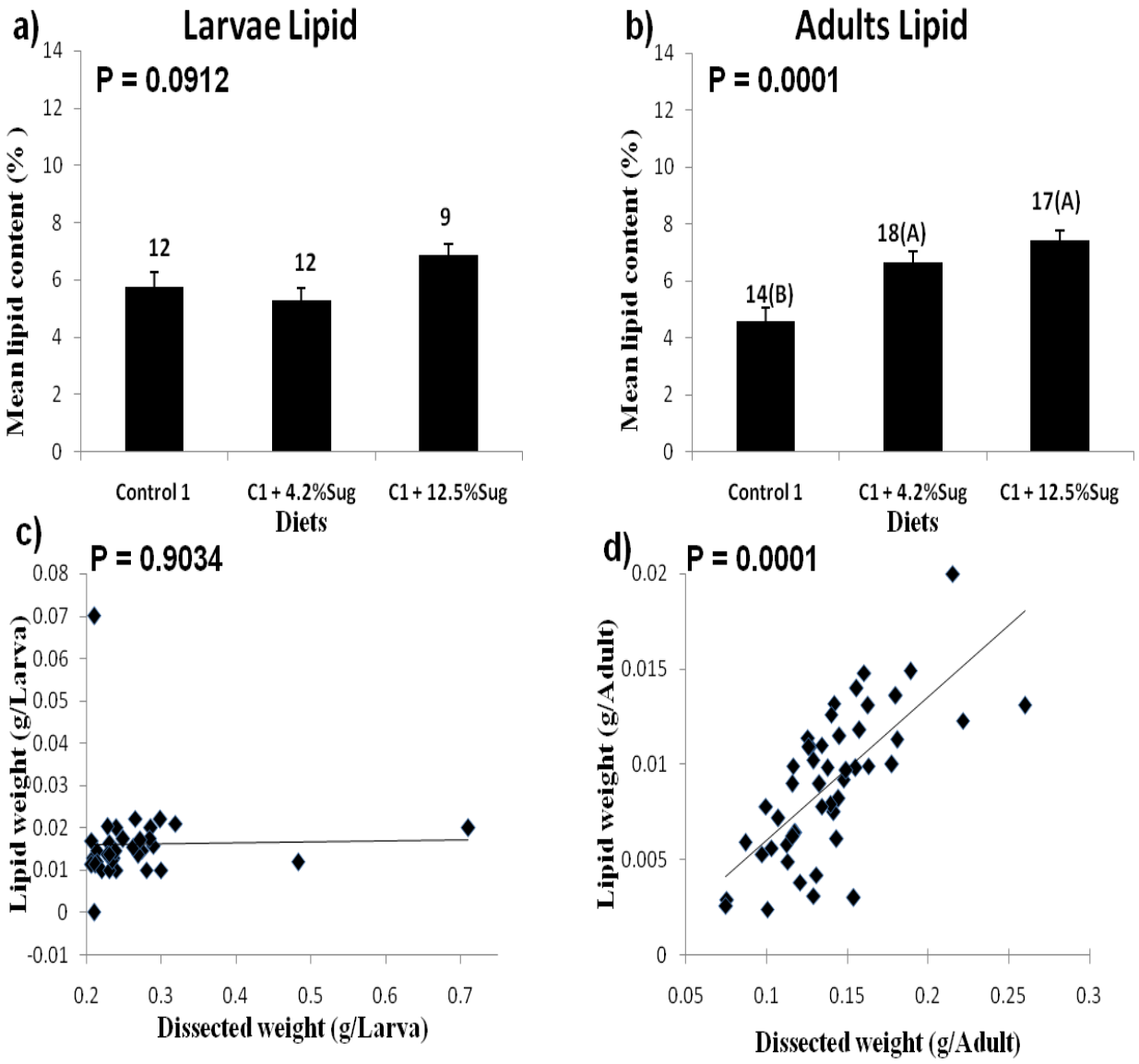

**Figure 10.** Correlation between larval and adult hemolymph trehalose concentrations of insects across all the diets tested in the four experiments. A line of best fit and p-value are given in the graph. N = 172; $R^2 = 0.085246$, $P < 0.0001$.

**Percentage Body Fat (Lipid)**

The mean percentage body lipid analyses for larvae and adults on the different diets of Experiments 1–4 are given in Figures 11, 12, 13, & 14. In all four experiments, there was no significant interaction between diet and sex for adults, so the male and female data were combined. Sex of larvae was not determined.

In Experiment 1 (added sucrose), there was no difference in mean percentage body lipid of larvae among treatments. By contrast, adults that fed on diets with added sucrose (4.2% or 12.5%) had a greater mean percentage of body lipid than insects that fed on the control diet (Fig. 11). In Experiment 2 (reduced wheat germ), there was no difference in mean percentage body lipid of larvae among the diets. Adults that had fed on the two reduced wheat germ content diets tended to have greater mean body lipid percentages than adults that had fed on the control diet. However, the effect was only significant for the diet with added sucrose (C2-Wg+14.3%Sug). There was no difference in mean body lipid percentage between insects that had fed on either of the two diets with decreased wheat germ (Fig. 12). In both Experiments 3 (added fat) and 4 (decreased protein), there were no differences in mean percentage body lipid for either larvae or adults among diets (Figs 13 & 14).

The dissected weight and larval lipid weight were significantly correlated in Experiments 2, 3, and 4, with larval lipid weight increasing with increasing dissected body weight (Figs 12c, 13c, & 14c). However, there was no correlation between these two parameters in Experiment 1 (Fig. 11c). The dissected weight and adult lipid weight were significantly correlated, with adult lipid weight increasing with increasing dissected adult body weight, for all four experiments (Figs 11d, 12d, 13d, &14d).




**Experiment 1- Added sucrose**

**Figure 11.** Lipid analyses of insects from Experiment 1. Mean percent lipid of larvae (a) and adults (b) fed on different diets. C1 = control diet; C1+4.2%Sug = 4.2% sucrose, of total wheat germ, added to control diet; C1+12.5%Sug =12.5% sucrose added to control diet. N is shown above standard error. P-values from one-way ANOVA for larvae and two-way ANOVA for adults are given. Different letters (in parentheses) atop bars indicate means that are different ($P < 0.05$) by Tukey-Kramer tests. Correlation between dissected weight and lipid weight for (c) larvae and (d) adults. A line of best fit and p-values are given for both graphs.

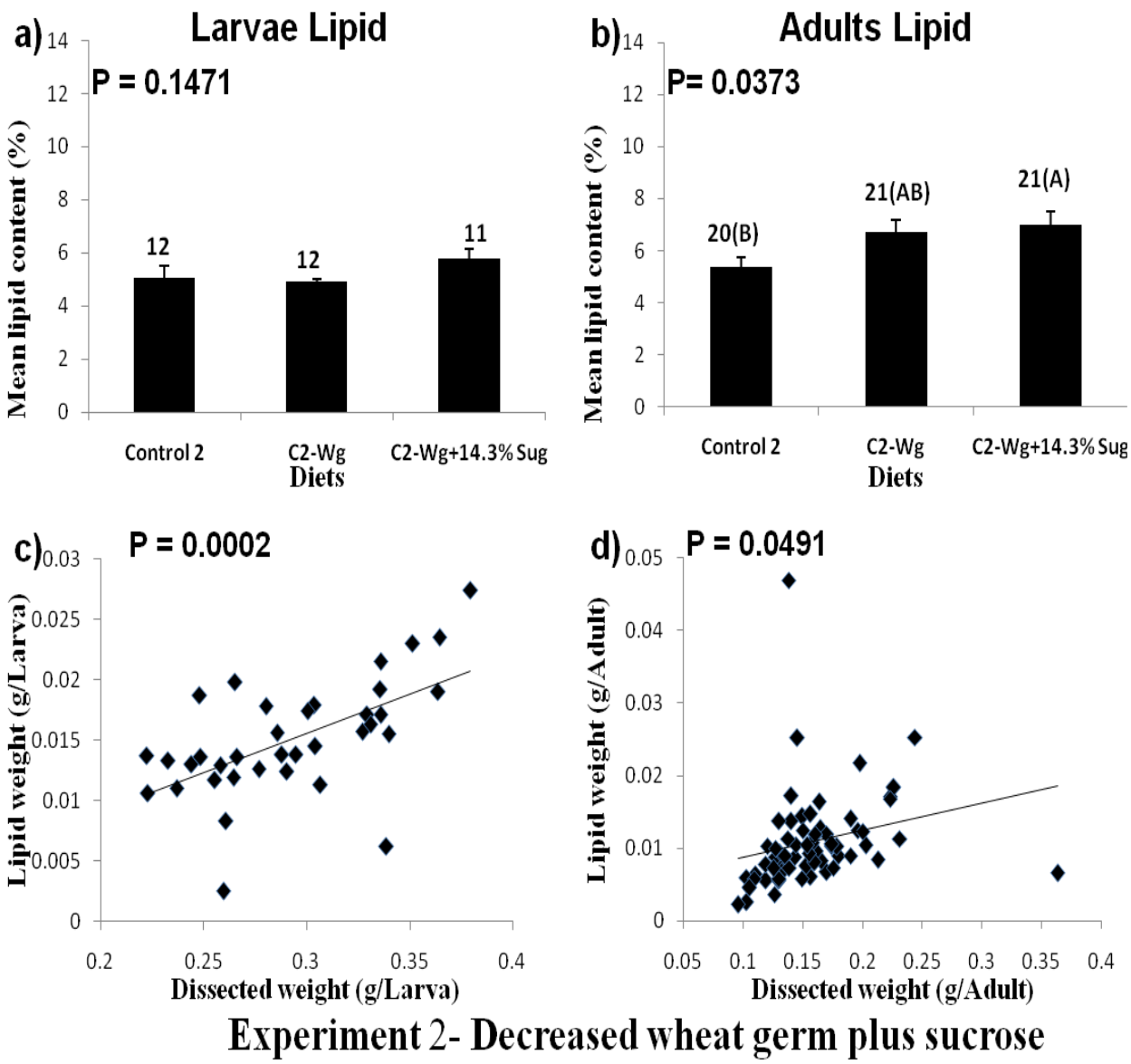
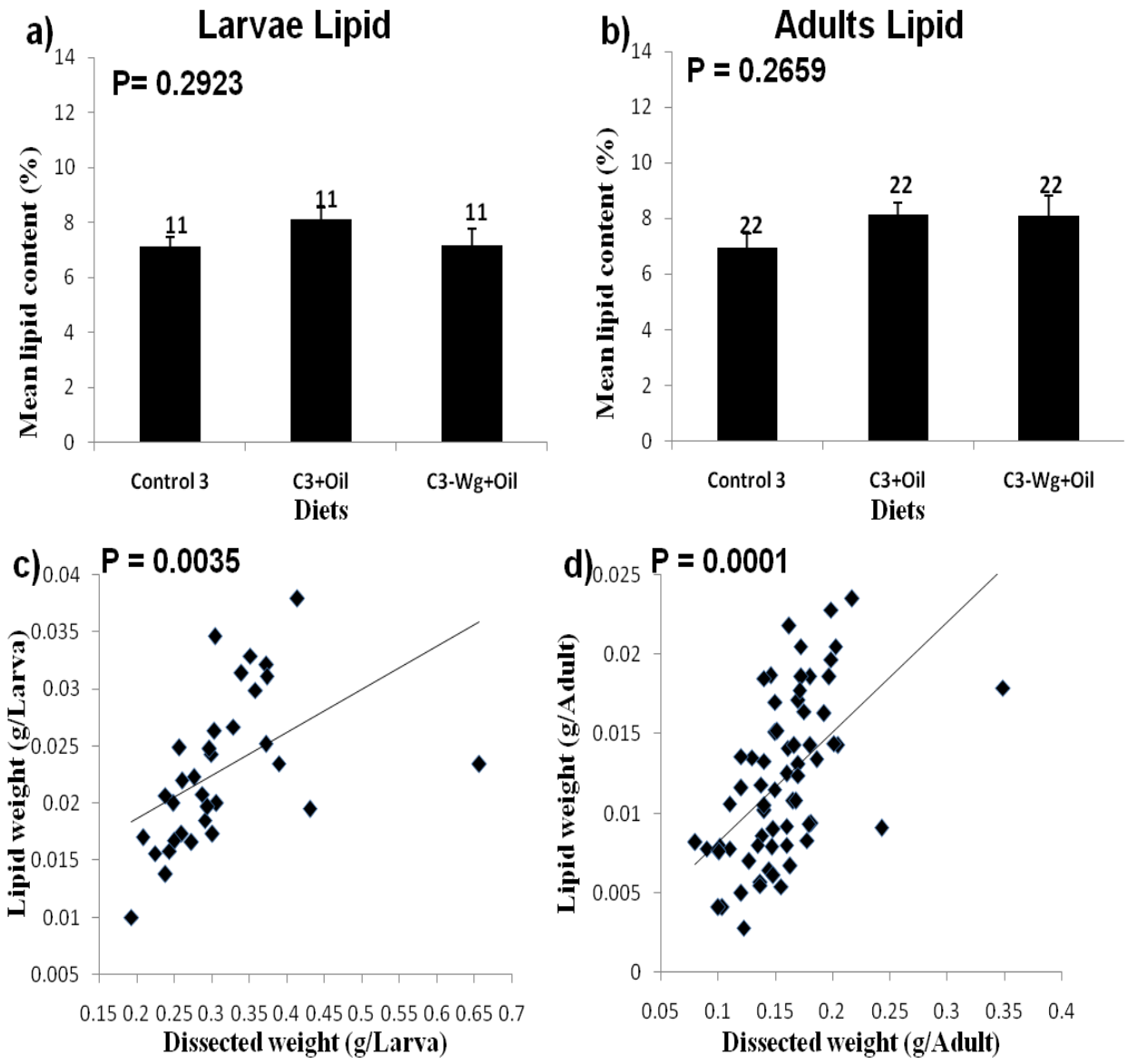



**Figure 12.** Lipid analyses of insects from Experiment 2. Mean percent lipid of larvae (a) and adults (b) fed on different diets. C2 = control diet in experiment 2; C2-Wg = wheat germ content decreased relative to control diet; C2-Wg+14.3%Sug =14.3% sucrose, of total wheat germ, added to decreased wheat germ diet. N is shown above the standard error. P-values from one-way ANOVA for larvae and two-way ANOVA for adults are given. Different letters (in parentheses) atop bars indicate means that are different ($P < 0.05$) by Tukey-Kramer tests. Correlation between dissected weight and lipid weight for (c) larvae and (d) adults. A line of best fit and p-values are given for both graphs.




# Experiment 3- Decreased wheat germ plus fat

**Figure 13.** Lipid analyses of insects from Experiment 3. Mean percent lipid of larvae (a) and adults (b) fed on different diets. C3 = control diet; C3+Oil = vegetable oil added to control diet; C3-Wg+Oil = vegetable oil added to decreased wheat germ diet. N is given above the standard error. P-values from one-way ANOVA for larvae and two-way ANOVA for adults are given. Correlation between dissected weight and lipid weight for (c) larvae and (d) adults. A line of best fit and p-values are given for both graphs.

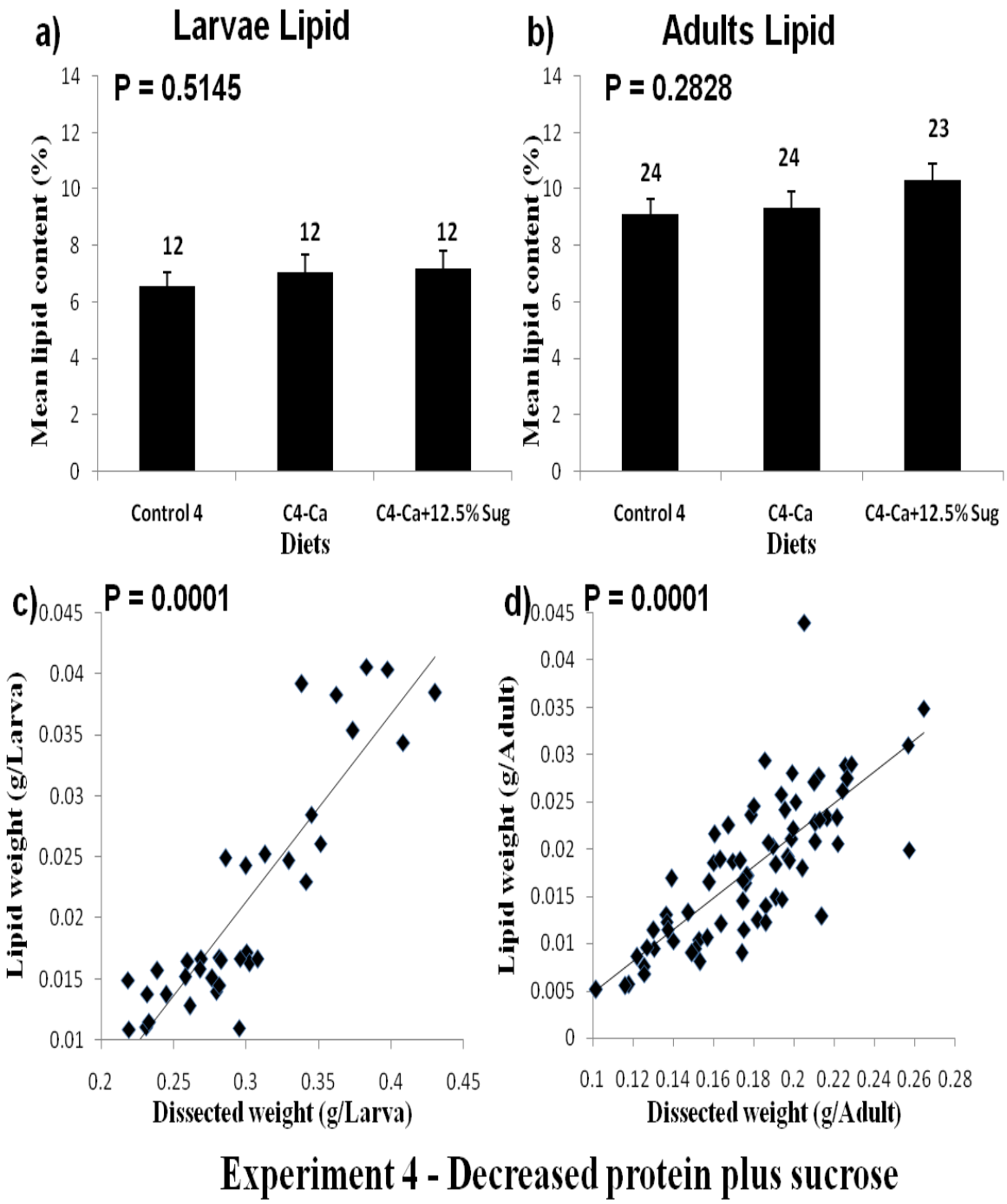



**Figure 14.** Lipid analyses of insects from Experiment 4. Mean percent lipid of larvae (a) and adults (b) fed on different diets. C4= control diet; C4-Ca = decreased (relative to the control diet) casein diet; and C4-Ca+12.5%Sug = 12.5% sucrose, relative to the amount of wheat germ, added to the casein-reduced diet. N is given above the standard error. P-values from one-way ANOVA for larvae and two-way ANOVA for adults are given. Correlation between dissected weight and lipid weight for (c) larvae and (d) adults. A line of best fit and p-values are given for both graphs.

# CHAPTER 4. DISCUSSION

The moth *H. virescens* acquires all of its dietary nitrogen and much of its dietary carbon that will eventually be used for reproduction during the larval stage. Adults of this moth are capable of supplementing larval-acquired carbon through feeding on plant nectar (carbohydrates) throughout adult life (Wheeler, 1996; Ramaswamy et al., 1997). Given that adult females have a limited time in which to mate and lay their eggs (Zeng et al., 1997), and that delays in initial egg laying can be deleterious to female fitness (Wheeler, 1996; Ramaswamy et al., 1997; Jiménez-Pérez and Wang, 2003), it follows that the effect of adult feeding on reproduction may vary according to both the quantity of carbon acquired by the larva and the timing of mating and/or adult feeding. For instance, for females that acquire low amounts of carbon during the larval stage, adult feeding and mating shortly after eclosion may be more critical than for females that eclose with an abundance of larval-acquired carbon.

In this study, I manipulated larval dietary carbon components (carbohydrate, fat and protein) to determine the effect on certain larval and adult carbon pools, specifically, HTC and body lipid. My aim was to produce insects that varied in these parameters as late instar larvae and after adult eclosion (i.e., with carbon derived only from larval acquisition) so that I could test the effect of these parameters on adult female reproduction (pheromone production, egg production), as well as compare any differences occurring through pupation. Unfortunately, these experiments proved to be more time-consuming than predicted originally, and I ran out of time before I could test the effects of variation in these initial (i.e., larval-acquired) adult carbon pools on adult reproduction. However, the results from my work will provide a basis for future studies examining the effect of initial (larval-acquired) adult carbon pools on reproduction and the effect of adult carbon feeding on supplementing these pools.

To test whether larval dietary components influenced larval and initial adult (both female and male) carbon pools, I conducted four experiments including with each including a control diet (standard among experiments) and two other diets that had different levels or sugar, fat or casein (protein). Table 6 shows the effect of diet manipulation on larval & adult HTC, as well as larval and adult lipid analyses for all four experiments. In Experiment 1, the test diets contained added sucrose. Overall, this had the effect of increasing the percentage of carbohydrate and total carbon content of the diet relative to the amount of protein. This carbon manipulation had no effect on mean HTC of both larvae and adults with respect to diet or pupal weight, and also had no effect on the mean percentage of body lipid or of mean total body lipid of larvae. However, it did affect the mean percentage of body lipid in adults and, specifically, the total amount of body lipid among adult females and males. Both adult females and males had a trend of increasing total body lipid with increasing adult weight. I interpret the results of this experiment as indicating that, in spite of a high carbohydrate diet, HTC in both larvae and adults is regulated to a homeostatic level. In insects, HTC is controlled in a homeostatic way in certain developmental stages (Becker et al., 1996), but overall concentrations change dramatically during molting and metamorphosis (Sakamoto and Horie, 1979; Hirano and Yamashita, 1980; Siegert, 1987). Consequently, it is unlikely that adults on these high carbohydrate diets will produce greater levels of pheromone through increased glycolytic flux (from trehalose) after eclosion (Friedman, 1978). The surplus carbohydrate acquired by a larva from these added sucrose diets was, therefore, allocated to increased levels of stored lipids, presumably fats in the fat body (Inagaki and Yamashita, 1986). These fat stores remained (in large part) through to the newly eclosed adult and, therefore, become available for reproductive activities, such as egg production. The conversion of carbohydrates to lipid in fat body in insects is supported by several studies (Hines

and Smith, 1963; Bailey, 1975; Venkatesh and Morrison, 1980; Inagaki and Yamashita, 1986; Briegel, 1990). For example, Lee et al. (2002) showed that carbohydrate-biased diets affect lipid contents of pupal *Spodoptera littoralis*, due to conversion of excess carbohydrate into lipid. Finally, I cannot rule out (since I did not analyze for it) that the increased carbohydrate levels in these diets did not also result in increased levels of stored glycogen in both larvae and adults (e.g., as in *B. Mori*, Inagaki and Yamashita, 1986). Glycogen stores are known to increase prior to pupation, and are used for energy and glucose for chitin synthesis in insects (Steele, 1982).

**Table 6.** Summary of effects of diet manipulation on response variable (larvae and adults tre & larvae and adults lipid) in each experiment. If response variable is; 0 = no effect, + = response variable increased, and − = response variable decreased, in the particular diet. All response variables are compared to the control diet which is 0.

| **Experiment/Effects** | **Larvae Tre** | **Adults Tre** | **Larvae Lipid** | **Adults Lipid** |
|---|---|---|---|---|
| **C1+4.2%Sug** | 0 | 0 | 0 | 0 |
| **C1+12.5%Sug** | 0 | 0 | 0 | + |
| **C2-Wg** | 0 | − | 0 | 0 |
| **C2-Wg+14.3%Sug** | 0 | 0 | 0 | + |
| **C3-Wg+Oil** | − | 0 | 0 | 0 |
| **C3+Oil** | 0 | N/A | 0 | 0 |
| **C4-Ca** | 0 | 0 | 0 | 0 |
| **C4-Ca+12.5%Sug** | 0 | 0 | 0 | 0 |

The second experiment tested diets with reduced amounts of wheat germ (primarily carbohydrate), with one of these diets having added sucrose to compensate for the loss of carbohydrate. Effectively, the diet with reduced wheat germ had a lower carbohydrate: protein ratio than the control diet, while the diet with reduced wheat germ and added sucrose had a slightly higher carbohydrate: protein ratio than the control diet. This manipulation resulted in no difference in mean HTC among larvae. However, adults that fed on the diet with decreased

wheat germ (C2-Wg) as larvae had a lower mean HTC than those that fed on the other two diets. While there was no difference in mean percentage of body lipid of larvae among the diets, adults that fed on the diet with added sucrose had a higher mean percentage of body lipids than did the control insects. This effect was reflected in the total body fat data, with adult females on the diet with added sugar having greater lipid levels than the control insects. As in Experiment 1, the results of this experiment indicate that surplus carbohydrate in the larval diet is stored, at least partly, as lipids. However, it appears that suboptimal (i.e., reduced) levels of carbohydrate can have deleterious effects on adult carbohydrate stores, in particular adult female HTC. This reduced HTC of females at eclosion could affect the glycolytic flux to pheromone production in these females. However, it should be noted that adult feeding (on sugar) would restore HTC levels of these females to those insects that had fed on more optimal larval diets. Similarly, these low HTCs could, potentially, affect egg production in these females, as hemolymph sugar is used in egg production (Katagiri et al., 1998; Thompson, 2003), particularly in resynthesizing non-essential amino acids for vitellogenin production (O'Brien et al., 2004, 2005). Thus, adult feeding is likely more critical for successful reproduction for insects that fed as larvae on low carbohydrate diets than for insects that fed on a better balanced diet. The effect of dietary carbohydrate on HTC of larvae of various Lepidopteran species, including *Helicoverpa zea* (Boddie), *Spodoptera exigua*, and *Manduca sexta*, has been reported (Simpson et al., 1988; Friedman et al., 1991 Ahmed et al, 1993; Thompson 1997, 1998). These studies showed that levels of carbohydrate (sugar) in diets affect HTC substantially. For instance, larvae of *H. zea* had lower HTC on a diet containing no/low sucrose than on a diet with high sucrose (Friedman et al., 1991).

In the third experiment, I tested the effects of diets with added oil (fat); one of these diets also had reduced levels of wheat germ (primarily carbohydrate). Overall, both these diets had increased levels of fat to protein, compared to the control diet, although one of these diets also had a reduced level of carbohydrate (compared to the control). These diet manipulations resulted in reduced HTC in larvae that fed on the diet with reduced wheat germ plus oil compared to the controls. This trend was apparent in adults, although it was not significant, consistent with the results of Experiment 2. There were no differences in mean percentage body lipid or in mass of body lipid for either larvae or adults among diets. However, both larvae and adults (males and females) had significant trends of increasing total lipid content for insects with increasing size. Thus, these data indicate that the additional fat in the diet, at least at the levels tested, had little effect on either HTC or stored fat levels. As excess fats cannot be converted to sugar by insects (Arrese and Soulage, 2010), then a greater amount of fat in the diet cannot be used directly to produce a greater amount of stored carbohydrate. Thus, larvae on the diet with decreased wheat germ (i.e., predominantly carbohydrate) and added oil had lower HTC. This effect was not manifested in adults, presumably because, during pupation, insects used more fat reserves in lieu of carbohydrate reserves (compared to controls etc.) for metamorphosis (Rudolfs, 1926a; Rudolfs, 1926b; Wellington and Maelzer, 1967). As insects on the added fat diet were not greater in size and did not have greater amounts of stored fat, I assume that any excess fat acquired from feeding on the added fat diet was largely excreted.

In the final experiment, I tested the effect of reduced protein in the diet. One of the diets had extra sucrose to compensate for reduced protein. Reduced protein had no effect on larval or adult HTC, percent fat or total fat content. As in the other experiments, there was a trend of increasing

fat content with increasing (dissected) weight. Thus, protein content in the diet (within the range tested) has little effect on carbohydrate or fat levels of the resulting insects.

Overall, my experiments showed that of the three major components, carbohydrate, fat, and protein, of the diet of *H. virescens*, manipulation of carbohydrate content had the greatest effect on the measured carbohydrate and fat stores (i.e., presumably the major stores of metabolic carbon) of adults. An increase (relative to the control diet) in carbohydrate content of the diet resulted in an increase in stored fat in adults, while a decrease in carbohydrate content (relative to the control diet) of the diet resulted in a decrease in HTC. Changes in fat or protein content of the diet had little, if any, effect on adult HTC or fat content at eclosion. Changing levels of dietary protein did not affect HTC of *M. sexta* larvae; however, changes in sucrose content resulted in changes in larval HTC (Thompson and Redak, 2000). Merkx-Jacques et al. (2008) showed that *Spodoptera exigua* caterpillars raised either on extreme protein-biased, optimal protein: carbohydrate ratio, or carbohydrate-poor diets performed similarly with regard to pupal size and lipid reserves. Stockhoff, (1993) showed that the consumption and utilization of protein and fat by gypsy moth, *Lymantria dispar*, is related to the developmental stage of the insect. Early instars showed preferences for high protein/low lipid diet, whereas later instars preferentially consumed low lipid/low protein diets. This suggested that stored fats are likely to be more beneficial for energy use during the non-feeding pupal and adult stages.

Before considering the possible effects of the differences I found in carbon stores on adult female reproduction, it is worth considering the constraints of my experimental approach. Firstly, I made only slight modifications to the control diet, figuring that major modifications might have deleterious effects on larval and adult survival. As it was, survival to pupation and adulthood was similar for all the diets. The only major problem I experienced was the

survivorship of insects after sampling hemolymph on the added oil diet in Experiment 3; very few (not enough for the HTC analysis) of these insects survived after pupation for sampling as adults. It is not clear what caused this problem. Certainly it was not an inherent problem with the diet, as insects on this diet that were not sampled for hemolymph as larvae survived well to the adult stage. Given that survivorship was similar, at roughly 50%, among all treatments, it is reasonable to assume that the insects I analyzed for each of the diets were fairly representative of insects reared on those diets, rather than representing a select few that were capable of surviving overly harsh dietary constitutions. Presumably, the standard diet for *H. virescens* was formulated on the basis that it yielded a high percentage of relatively healthy and normally fit insects, fairly representative of insects in nature that feed on host plants. Therefore, my relatively minor manipulations might be considered typical of insects that in nature that fed on slightly sub-optimal or supra-optimal host plants, which, within an agricultural context, might be representative of different lines. Greater dietary component manipulations might have resulted in more profound effects on carbohydrate and fat stores in adults, but might also have had profound effects on survivorship.

Second, I allowed the insects to satiate on each diet; that is, they were given an abundance of each diet until they pupated. Therefore, even though a diet was abundant or deficient in a particular component, the insects could have compensated for that lack through decreased or increased feeding, e.g., *H. virescens* (Lee et al., 2006); *S. exigua* (Merkx-Jacques et al., 2008), thereby effectively giving them the same nutrient intake as insects on the control diet. Compensatory feeding is especially important for female insects to obtain sufficient protein to mature a suitable number of eggs (Mevi-Schütz and Erhardt, 2005; Beck, 2007, but also see the study Khel and Fischer, 2012). Nevertheless, the results suggest that even when provided with

abundant food to compensate for component deficiencies, which adult carbohydrate and fat reserves can vary with differences in carbohydrate content of the larval diet.

Finally, it should be recognized that I only analyzed a limited number of general metabolite pools in larvae and adults. For carbohydrates, I only analyzed HTC. However, substantial amounts of carbohydrates in insects are stored as glycogen in the fat body (Arrese and Soulages, 2010). Glucose from this glycogen can be mobilized readily to maintain homeostasis of HTC (Friedman, 1978; Thompson, 2003). I did not analyze for glycogen and therefore do not know whether amounts varied among the insects that fed on different diets. For example, a high carbohydrate diet might have resulted in greater stores of glycogen and no change in HTC in adults. Moreover, I only quantified total lipids in the insects. In addition to fats, this pool consists of hydrocarbons, wax esters, fatty alcohols and sterols (Foster and Roelofs, 1990). Of these, fats are probably the most abundant chemical group (Gilby, 1965; Ziegler, 1991) and probably the most important for contributing directly to reproduction through providing substrates for energy (e.g., for flight), pheromone and egg production in adult females (Lockey, 1988; Stanley, 2006; Arrese and Soulages, 2010). The lipid contents that I determined for the insects on different diets are likely to overestimate the amount of fat, although the relative proportions among the different types are likely to reflect the relative amounts of fat.

Taking into account the above constraints, my work shows that variations in larval diet constituents can affect the initial (at eclosion) amounts of metabolic carbon pools in adult females, notably HTC and stored fats. These differences in initial pool amounts may, subsequently, have effects on the reproductive physiologies of females. The most obvious physiologies utilizing these metabolic carbon pools are foraging (especially flight; Beenakkers,

1984; Van der Horst et al., 2001), production of pheromone for attraction of mates (Foster, 2005, 2009; Foster and Johnson, 2010, 2011), fertilization of eggs, and production of eggs themselves. Of these, the most carbon-demanding physiology is egg production. Females must provision their oocytes with abundant protein, lipid and carbohydrate (Kim et al. 1985; Chino et al., 1977; Raikhel and Dhadialla 1992). At eclosion, female *H. virescens* have few, if any, mature occytes but rapidly mature (i.e, uptake of yolk) their oocytes following mating (Ramaswamy et al., 1997). Prior to adult feeding, nutrients for oocyte maturation are derived totally from larval-acquired sources. Thus, deficiencies in these stores could have profound effects on egg maturation rates and lifetime fecundity (Awmack and Leather, 2002). Many studies have shown that delays in egg maturation in female Lepidoptera can have significant effects on fecundity over a female's lifetime (Ramaswamy et al., 1997). While species of Lepidoptera (except certain species of *Heliconius* butterflies Gilbert, 1972) in which the adult feed cannot compensate for lack of protein acquisition by the larva through adult feeding, adult female feeding on carbohydrates is nevertheless important for synthesizing non-essential amino acids for the yolk proteins uptaken by oocytes during maturation. O'Brien et al. (2004; 2005) showed that up to 80% of the carbon in some non-essential amino acids in eggs of *Speyeria mormonia* was derived via adult feeding. This demonstrates the importance of carbohydrates, whether larval- or adult-derived, in egg protein production. Thus, a shortage of (larval-acquired) carbohydrate stores in adults may profoundly affect egg production, especially in environments in which plant nectar for adults is scarce.

While fats for oocytes can be synthesized from carbohydrate in insects (Ziegler and Roth, 1985; Ziegler and Ibrahim, 2001; Arrese and Soulages, 2010) and thus adult nectar feeding could potentially be used to compensate for any shortage of fats (i.e., acquired through larval feeding)

in females at eclosion, fat synthesis from nectar in adult insects appears to be relatively slow (Foster and Anderson, 2012). Thus, adult feeding may actually supply relatively little of the fat provisioned to oocytes by female *H. virescens* (Foster, and Anderson, unpublished results). Consequently, stored fats, acquired through larval feeding, are likely to be much more important for provisioning of eggs by females. Thus nutrient deficits, in my experiments carbohydrate, in larval diets that result in reduced stores of fats in adults at eclosion may actually not be able to be compensated by adult nectar feeding. This is likely to affect egg provisioning and possibly embryonic or larval survival (Arrese and Soulages, 2010).

Pheromone production in moths is derived from carbon stored as carbohydrate (e.g., trehalose; Foster, 2009; Foster and Johnson, 2010, 2011) and fat in the pheromone gland (Foster, 2005). Very little is known about the mechanisms that control the quantity of pheromone produced by female moths. It is likely that biosynthetic enzyme flux capacities control the production of pheromone, but it has also been demonstrated that precursor availability, especially from hemolymph trehalose and stored glandular fatty acids, can also limit the quantity of pheromone a female moth can produce (Foster and Anderson, 2012). *Heliothis virescens* females produce substantial quantities of sex pheromone within a day of eclosion and continue to produce pheromone throughout their lifetime to achieve multiple matings (Foster and Johnson, 2011). This sustained production of sex pheromone throughout the lifetime is achieved within a context of high metabolic carbon demand, especially of hemolymph trehalose, for egg production (see above). Therefore, periodic feeding by adult females is required to sustain HTC [Fig. 2, (g) & (h); Foster and Johnson, 2010]. A recent study (Foster and Anderson, unpublished) has shown that while adult feeding appears to supply much of the carbon for pheromone production early in an adult female's life, production of pheromone later

in the female's life is fueled increasingly by larval carbon, presumably from stored glandular fats. Thus, larval-acquired carbon reserves (glandular fats) may become increasingly important for continued pheromone production and mating. Although I did not actually measure pheromone gland fat amounts, their quantities are strongly correlated with total fat amounts in females (Foster and Anderson, unpublished). Therefore, larval diets (such as the added sucrose diets I tested) that increase female fat content may benefit production of high amounts of pheromone throughout a female's life, ensuring multiple mating, which in turn may result in increased egg production and fecundity of the female (Wheeler, 1996; Ramaswamy et al., 1997). Moreover, pheromone production for the initial mating (i.e., within a day or so of eclosion and perhaps before the adult female has fed) of the female is likely to be (at least largely) from carbon precursors acquired by larval feeding. Therefore, carbohydrate deficient diets (such as those I tested) that result in decreased HTC in newly eclosed females could limit pheromone production and handicap a deficient female in her ability to attract mates over a distance (Foster and Johnson, 2010, 2011). The relationships between the size of larval-acquired carbon pools and pheromone production throughout the life of a female should be tested in future studies.

The other demanding physiological activity a female moth engages in is foraging flight, for both food (nectar) and hosts on which to oviposit. Both carbohydrates and lipids have been implicated as fuels for sustained flight in Lepidoptera. Some studies (Gibo and McCurdy 1993; Malcolm and Zalucki, 1993; Brower et al., 2006; Nation, 2008) have shown that carbohydrates are used initially for flight, but after sustained flight, especially migration (Brower et al. 2006), fats are the primary metabolite used. A study by O'Brien (1999) on the nectivorous sphingid *Amphion floridensis* showed that the fuel used for flight was dependent upon the feeding state of the adult moth. If moths were satiated with nectar, then carbohydrates were used for extended

flight. However, if the moth were starved then fats fueled extended flight. As for the other reproductive physiologies, it is conceivable that larval acquisition of metabolic carbon could have a profound influence on flight of female *H. virescens*, particularly the long migratory flights that occur each year to re-colonize northern areas of the USA where the species cannot survive over winter (Raulston et al., 1986; Westbrook et al., 1994). While we do not know whether carbohydrates or fat primarily fuel sustained flight in *H. virescens*, if we assume that the situation is similar to that for *A. floridensis*, then low fat reserves acquired from an inadequate larval diet (e.g., as in the low carbohydrate diet I tested) could limit migratory distances of this species, especially if nectar resources were scarce. Again, the effects of larval carbon pool sizes on adult fuel use and flight should be tested.

In conclusion, through manipulating key dietary components, especially carbohydrate content, I have demonstrated that it is possible to produce adult *H. virescens* that have different amounts of key carbon pools. These variations in larval-acquired carbon pools could have profound effects on adult reproductive physiologies such as flight, and egg and pheromone production, especially if compensatory adult feeding is constrained. Future work should investigate these effects and determine whether they impact insect fitness in natural and agricultural situations.

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