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STRATEGIES USED BY FEMALE LARVAL LEPIDOPTERA TO ACCUMULATE NUTRIENT RESERVES

by

Aparna Telang

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Aparna Telang

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and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

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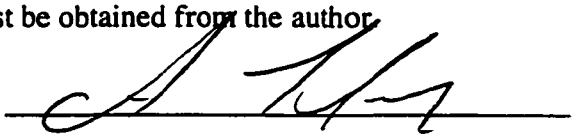
SIGNED: 

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ABSTRACT

Female insects produce eggs that are nutrient-rich. For most Lepidoptera, protein is acquired during larval feeding. Insects use pre-ingestive, post-ingestive and developmental strategies to meet nutritional needs. In this dissertation I examine the importance of these strategies to females of two related lepidopteran species differing in their adult feeding.

Adult*Heliothis virescens* (Family Noctuidae) ingests nectar. The sexes were not distinguished according to their selective feeding behavior, but females accumulated more protein and carbohydrate. When restricted to diets, females ate and accumulated more protein on high protein diets. There were no sexual differences on high carbohydrate diets. Results indicate that female *H. virescens* larvae accumulate protein by regulating both intake and post-ingestive processing on high protein foods.

Adult*Estigmene acrea* (Family Arctiidae) do not feed. The sexes did not differ in their selective feeding behavior. When restricted to diets, female *E. acrea* increased ingestion of diets unbalanced in protein and carbohydrate leading to greater intake of both nutrients but only accumulated more protein. Lastly, *E. acrea* prolong juvenile development if too small at later stages effectively extending their period of ingestion and processing.

As previously shown protein-derived growth in female *H. virescens* progressively increased as dietary protein levels increased. Storage protein, a component of protein-derived growth, was found to similarly increase but was more abundant in females across all pupal stages and diets. Pharate adults retained a portion of total storage protein with females retaining greater levels presumably toward egg provisioning.

Growth of both *H. virescens* and *E. acrea* was highly clustered compared to ingestion values suggesting post-ingestive processing of nutrients to regulate growth. Males and females of both species efficiently utilized carbohydrate except at high ingestion. Females of both species utilized nitrogen more efficiently than did males at all ingestion levels, contributing to their greater protein accumulation. The manner of post-ingestive processing by these two species reflects differences in their larval diet. Clearly, my studies show that female caterpillars regulate both nutrient consumption and post-ingestive physiology to accumulate greater reserves.

CHAPTER 1

INTRODUCTION

Asymmetry in gamete size and associated investment in offspring is a fundamental difference between the sexes in all species of animals. Males produce a large number of small sized gametes, sperm, which are little more than mobile DNA. In contrast, females produce relatively fewer, larger nutrient-rich eggs and can therefore only increase reproductive fitness by turning food into eggs or offspring as quickly as possible (Krebs and Davies, 1993). Female insects produce eggs richly provisioned with nutrients, so that oogenesis is a nutrient-limited process. Clearly, acquiring nutrients from the environment plays a central role in the reproductive biology of females in most insect species (Wheeler, 1996). Insect eggs, with few exceptions, are quite large and contain a great deal of yolk (Chapman, 1998). In addition to lipid and carbohydrate, yolk consists of protein. A female specific protein, or vitellogenin, is commonly produced in the fat body, released into the hemolymph and taken up by the developing oocyte.

Adult and larval holometabolous insects often inhabit different environments during each life stage that results in different nutrients being available to them. Lepidopteran species have been exceptionally useful in examining aspects of female

reproductive biology as they deploy a diverse array of strategies to help meet their nutritional needs for egg provisioning. In general though, Lepidoptera seem to rely more heavily on converting larval nutrient stores to yolk, which may occur before, during, or after adult emergence depending on the taxonomic group. The strategies used by female Lepidoptera for oogenesis fall into three categories. First, some species use up larval reserves and all eggs are mature prior to adult eclosion. The silkworms, *B.mori* and *H.cecropia*, are well-studied examples of this category. Females in the second category emerge as adults with partially complete egg development (many moth species fall into this category). In the third category, females eclose with a stock of nutrient reserves but with immature ovaries (Wheeler, 1996).

Yolk mainly consists of protein and for this reason the relationship between protein and oogenesis has received much attention. Amino acid stores accumulated during larval feeding are utilized during metamorphosis for pupal and adult tissue development, but females must supply their eggs with enough amino acid stores to support embryogenesis. Recent studies support a strong role for storage proteins in adult insects (Wheeler et al., 2000). Insects that do not feed at all during adult stages must allocate protein stores acquired during their larval stages for both metamorphosis and oogenesis. Storage proteins in such insect species may be the main source of amino acids

for vitellogenin synthesis and thus for oogenesis (Wheeler and Buck, 1996).

Given that oogenesis is a nutrient-limited process, how females acquire and utilize nutrients from their environment is an important question. In recent years a good number of studies have examined both behavioral and physiological mechanisms by which insects strive for nutritional homeostasis when faced with nutritional heterogeneity in their environment and the changing needs of their own tissues. Much of these studies have concentrated on compensatory responses to variations in dietary nutrients. There are three ways in which insects have been shown to compensate for such variation. First, the insect may alter consumption rate or consumption time so as to ingest sufficient amounts of various nutrients (Timmins et al., 1988). Second, it may choose to leave an unbalanced food source and select some other food source, termed dietary self-selection (Cohen et al., 1987a; Cohen et al., 1987b; Cohen et al., 1988; Simpson et al., 1988; Simpson et al., 1990). Third, it may be able to alter efficiency of nutrient utilization to make best use of an unbalanced food source. A review by Simpson & Simpson (1990) details such studies on phytophagous insects. Animals require, simultaneously, a wide range of nutrients (such as proteins, carbohydrates, and fats), and more recent work has adopted a view that multiple interactions exist among mechanisms regulating the intake of different nutrients (Simpson and Raubenheimer, 1995).

As I have outlined in the proceeding sections, female insects have larger nutritional needs for provisioning their gametes than their male counterparts. It is commonly thought that female Lepidoptera are heavier than males owing to greater nutrient accumulation as a result of greater food consumption as larvae (Slansky and Scriber, 1985). Some studies have concluded that females attain greater size by ingesting food at a faster rate than males although they develop over a similar time period (Nakano and Monsi, 1968; Van den Berg, 1973; Mathavan and Bhaskaran, 1975; Horie et al., 1976; Bhat and Bhattacharya, 1978). In other cases it is thought that they ingest food over a longer period of juvenile development (Atwal, 1955; Chou et al., 1973; Beckwith, 1976; Mackey, 1978; Lederhouse et al., 1982). All the studies just cited have examined sexual differences but only in very few species of insects and have only measured consumption and utilization of plant foods or have used holidic diets so that specific nutrients could not be measured. The study of female nutritional physiology will benefit a great deal from this multidimensional approach in which consumption and utilization of specific macronutrients will be examined.

This Study

In my dissertation, I will apply the multidimensional framework recently

developed and tested by Simpson and colleagues to study differences in the nutritional physiology between the sexes when females undergo a similar larval life as their male counterparts. I have examined the importance of pre-ingestive, post-ingestive and developmental strategies to females of two related lepidopteran species that differ in their larval diet and in their adult feeding behavior.

Explanation of Dissertation Format

The chapters in this dissertation are included as four appendices. All four appendices represent work that I conducted and papers that I produced. The other authors served either an advisory (Drs. Reginald F. Chapman and Diana E. Wheeler) or a technical role (Norman A. Buck and Valerie Booton); thus, they are co-authors on these papers.

I applied the multidimensional experimental design to two closely related lepidopteran species that differ in their adult feeding strategies. The first species, the subject of Appendix A, is the tobacco budworm, *Heliothis virescens* (Family Noctuidae), and a generalist feeder feeding on many economically important plants as larvae and ingesting nectar as a source of carbohydrate as adults. The second species, the subject of Appendix B, is the salt marsh caterpillar, *Estigmene acrea* (Family Arctiidae, Subfamily Arctiinae). The family Arctiidae is closely related to the family Noctuidae, but unlike

many noctuids, does not generally feed as adults. Consequently, female *E. acrea* larvae should tightly regulate acquisition of both protein and carbohydrate but by which mechanisms?

As detailed in Appendix A, female *H. virescens* larvae increased consumption of high protein foods leading to greater protein intake, and, consequently, to greater protein-derived growth. The experimental design outlined in Appendix A allowed me to manipulate the nutritional environment of larvae. In Appendix C, I examined the dynamics of storage protein levels (as a distinct quantity of overall protein-derived growth) in response to variation in nutritional environment, especially with respect to dietary protein levels.

In Appendices A and B, I reported that caterpillars of *H. virescens* and *E. acrea*, when restricted to one of seven diets varying in levels of protein and carbohydrate ingested different amounts of both nutrients. In contrast to widely varying ingestion values, growth derived from both nutrients was highly clustered indicating that both sexes were relying on post-ingestive processing of nutrients to regulate growth. In Appendix D, I present data on the post-ingestive partitioning of these nutrients into pre- and post-absorptive components to determine the extent of their contributions toward regulating larval growth.

CHAPTER 2

PRESENT STUDY

The methods, results, and conclusions of this study are presented in the papers appended to this dissertation. The following is a summary of the major findings in these papers.

In Appendix A, I examined the importance of consumption and post-ingestive processing as mechanisms for female *H. virescens* larvae to accumulate protein and carbohydrate. In experiments in which caterpillars had a choice of diets, enabling them to select an appropriate protein and carbohydrate intake, females caterpillars ate more carbohydrate than males, but only on the heavily carbohydrate biased treatment. Overall, the sexes were not distinguished according to selective feeding behavior, but females accumulated more protein and carbohydrate over the whole instar than did males. Additionally, when given no choice, females ate more than males and accumulated more protein provided the diet contained a high proportion of protein. If they were reared on a high carbohydrate diet, there were no differences between the sexes. My results indicate that female *H.virescens* larvae accumulate protein by regulating both intake and post-ingestive processing on high protein foods. In the field, late instar *H.virescens* feed on

anthers, which are protein-rich and have the highest amino acid content relative to other cotton floral tissues.

In Appendix B, I applied the same experimental design to study the importance of these same mechanisms to *E. acrea*, which does not feed as an adult. Consequently, female larvae should tightly regulate acquisition of both protein and carbohydrate but by which mechanism? In similar choice experiments, *E. acrea* larvae adjusted their total food intake and achieved an intake of protein and carbohydrate to meet their nutritional needs. As was seen for *H. virescens*, the sexes did not differ in their selective feeding behavior. When given no choice, *E. acrea* females responded to an inadequate diet (with respect to both nutrient levels) by increasing consumption and this led to greater protein and carbohydrate intakes. These same experiments indicated that females relied on post-ingestive processing of nutrients and accumulated more protein than did males (again similar to *H. virescens* females). Lastly, I found that *E. acrea* prolongs juvenile development if it is too small as a late stage larva. This longer development time effectively extended their period of food intake, thus enabling initially small larvae to catch up and even surpass those that had started off bigger.

In Appendix A, I reported that female *H. virescens* larvae increased consumption of high protein foods leading to greater protein intake, and, consequently, to greater

protein-derived growth. In Appendix C, I report on a follow-up study in which we examined the dynamics of storage protein levels in response to variation in nutritional environment, especially with respect to dietary protein levels. Storage proteins have been found to play a major role in insect metamorphosis and egg production and are accumulated during the actively feeding larval stage. Yet, few studies have focused on how nutrition affects storage protein levels. Three storage proteins were identified in male and female *Heliothis virescens* pupae, one arylphorin and two high-methionine hexamers. Storage proteins were quantified in early pupae and in pharate adults. Storage protein levels peaked in 48-hrs-old pupae and were more abundant in females across all stages. Both male and female pharate adults retained a portion of total storage protein levels with females retaining greater levels. For females, post-eclosion protein reserves will undoubtedly be used toward egg manufacturing, while the role of protein reserves in males remains speculative. In my previous study of *H. virescens* larvae, I found that protein-derived growth in females progressively increased as dietary protein levels increased. Present data show that levels of storage protein also progressively increased along with dietary protein levels. This suggests that females allocated protein, in excess of adult tissue formation, toward storage protein needs. This study is the first to

demonstrate how dynamic storage protein levels can be in face of varying levels of dietary protein.

Previously, I reported (in Appendices A and B) that when larval *H. virescens* and *E. acrea* were restricted to one of four completely artificial diets varying in protein and carbohydrate levels, growth of both sexes was highly clustered compared to ingestion values. A pattern of tightly clustered growth in comparison to widely ranging ingestion values is taken to indicate that animals are relying on post-ingestive processing of nutrients to regulate their growth. In Appendix D, I present utilization and nutrient budget data on the post-ingestive partitioning of these nutrients into pre- and post-absorptive components to determine the extent of their contributions toward regulating larval growth. Both species were found to efficiently utilize carbohydrate except at very high ingestion rates. Of the carbohydrate utilized, more was unaccounted for as intake increased and we assume the unaccounted material served as a respiratory substrate, as suggested by other studies. In contrast to carbohydrate use, the pattern of nitrogen utilization differed between the species. Larval *H. virescens* were highly efficient at retaining nitrogen except at very high ingestion rates whereas *E. acrea* showed a progressive increase in nitrogen excretion in response to increased nitrogen ingestion. Regardless, females of both species utilized nitrogen more efficiently than did males at

all ingestion levels, thus contributing to their greater protein-derived growth. Results for *H. virescens* also showed that protein and amino acids accounted for a small proportion of fecal nitrogen and that most must be of post-absorptive nature. In contrast, nitrogen excreted by *E. acrea* could be partitioned into both pre- and post-absorptive components. The manner of post-ingestive processing by these two species reflects differences in their larval diet.

In summary, a review of studies regarding insect nutritional physiology concludes that females are heavier than males due to greater nutrient accumulation as a result of greater food consumption. Clearly, results of my studies indicate that female caterpillars regulate both nutrient consumption and post-ingestive physiology to achieve greater growth. Additionally, my study, in using chemically defined artificial diets, explains the nutritional physiology of female caterpillars in terms of specific macronutrients important to them.

APPENDIX A

HOW FEMALE CATERPILLARS ACCUMULATE THEIR NUTRIENT RESERVES

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How female caterpillars accumulate their nutrient reserves

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Abstract

Female Lepidoptera are often heavier than males. We examined the importance of consumption and post-ingestive processing as mechanisms for female *Heliothis virescens* larvae to meet the protein and carbohydrate requirements. In experiments in which caterpillars had a choice of diets, enabling them to select an appropriate protein and carbohydrate intake, females caterpillars ate more carbohydrate than males, but only on the heavily carbohydrate biased treatment. Overall, the sexes were not distinguished according to the selective feeding behavior, but females accumulated more protein and carbohydrate over the whole instar than the males did. Additionally, when given no choice, females ate more than males and accumulated more protein provided the diet contained a high proportion of protein. If they were reared on a high carbohydrate diet, there were no differences between the sexes. Our results indicate that female *H. virescens* larvae accumulate protein by regulating both intake and post-ingestive processing on high protein foods. In the field, late instar *H. virescens* feed on anthers, which are protein-rich and have the highest amino acid content relative to other cotton floral tissues.

Keywords: Larval diet; Female nutrition; Growth regulation; Protein accumulation; *Heliothis virescens*

1. Introduction

Female insects are known to produce eggs richly provisioned with nutrients, and oogenesis is regarded as a nutrient-limited process (Wheeler, 1996). Most lepidopteran species obtain nutrients for egg provisioning during their larval stage and supplement with nectar feeding as adults. However, the lack of substantial amounts of amino acids in nectar (Slansky and Scriber, 1985), in addition to the high protein demand of egg provisioning, suggests that females rely on their larval diet to obtain this protein.

It is commonly thought that female Lepidoptera are heavier than males owing to greater nutrient accumulation as a result of greater food consumption as larvae (Slansky and Scriber, 1985). In some species this is accomplished by females undergoing an additional larval stage; such is the case with the gypsy moth, *Lymantria dispar*. Studies on other species, in which the two sexes have the same number of larval stadia, have concluded that females attain greater size by ingesting food at a greater rate (Nakano and Monsi, 1968; Van den Berg, 1973; Mathavan and Bhaskaran, 1975; Horie et al., 1976; Bhat and Bhattacharya, 1978). In these last studies caterpillars were reared on hostplants; the biochemical nature of plants changes both temporally and spatially thus making measurement and manipulation of nutritional composition impossible.

Recently, an experimental framework has been developed in which multiple requirements of specific nutrients important to animals can be examined, with the use of strict, chemically defined diets (Raubenheimer and Simpson, 1993; Simpson and Raubenheimer, 1993). Each nutrient is represented by a single axis in a multidimensional plot. Ingested nutrients must meet an insect's requirement for growth and other metabolic processes. In this relationship, the level of nutrients allocated to tissue growth and storage represents the growth target, and the intake target is the amount the animal must consume in order to meet its growth and metabolic needs (Simpson and Raubenheimer, 1995). This framework allows us to determine how female larvae acquire nutrient stores that are qualitatively and quantitatively differ-

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ent than males. Do they do so by increasing the ingestion of specific nutrients or do differences between the sexes arise post-ingestively? Our study animal, the tobacco budworm, *Heliothis virescens* (F.), is a generalist feeder feeding on many economically important plants as larvae and ingests nectar as a source of carbohydrate as adults.

2. Materials and methods

2.1. Animals

Egg masses containing *H. virescens* were obtained from the Western Cotton Research Laboratories in Phoenix, Arizona and maintained in incubators at 25°C and a 12:12 h light:dark cycle. Upon hatching, neonates were placed individually in 30 ml plastic rearing cups fitted with plastic lids to maintain moisture and were maintained at the same temperature and photoperiod as above. Larvae were reared on experimental diets fortified with wheatgerm to promote good growth and development. We determined from our previous experiments that larvae were unable to develop properly when reared on strict chemically defined diets from hatching. However, from these same experiments we determine that the addition of wheatgerm to the diet in the early stages enabled the later stages on chemically defined diets to develop normally. On entering their fifth, and last instar, insects were weighed, transferred to 150 ml plastic cups fitted with plastic lids and randomly assigned to diet treatments.

2.2. Synthetic diets

Artificial diets were based on those for *Spodoptera littoralis* (Simpson et al., 1988). The protein source consisted of vitamin-free casein and the digestible carbohydrate was sucrose. All diets were agar based (3% solution wt/vol) and contained 54% cellulose (non-nutritive bulking agent) and 4% essential micronutrients (vitamins, cholesterol, salts and linoleic acid). The remaining 42% consisted of protein and carbohydrate at various levels. Each insect was given a fresh, preweighed block of diet daily in all experiments.

2.3. Experiment 1

This experiment was designed to determine if males and females differed in their consumption and/or performance when they had no choice of diet, and whether similar effects occurred with different relative amounts of protein and carbohydrate. Two replicates of each experiment were run. Fifth-instar caterpillars were restricted to one of seven diets with the following dry weight percentages of protein (P) to carbohydrate (C): 7P:35C; 14P:28C; 21P:21C; 24P:17C; 28P:14C; 32P:10C; 35P:7C. These insects were essentially confined to a 'rail' in protein vs. carbohydrate space (Raubenheimer and Simpson, 1993), and, since they were not given a choice of alternative foods, their 'intake target' was unattainable on most diets. However, the 'growth target' may still be approached by selectively altering the efficiency with which each nutrient is utilized through post-ingestive processing. Consumption and performance, based on growth and stadium duration, were measured for each insect. Chemical analysis of insect carcasses was used to quantify growth derived from both the major nutrients.

2.4. Experiment 2

This experiment was designed to determine the ability of males and females to feed selectively when given a choice between two diets differing in the protein:carbohydrate ratio. Two replicates of each experiment were run. Four of the seven diets from the first experiment on which larvae performed similarly well were chosen: 7P:35C; 21P:21C; 28P:14C; and 35P:7C. The four diets were paired in the following manner with each pairing assigned a treatment number: treatment 1=7P:35C vs. 28P:14C; treatment 2=21P:21C vs. 35P:7C; treatment 3=7P:35C vs. 35P:7C; and treatment 4=21P:21C vs. 28P:14C. Portions of each diet of approximately equal fresh mass were provided and changed daily. Diet portions were placed opposite each other within the rearing cups, and trays of cups were rotated daily at 180°, to avoid any left side–right side bias. The location of the trays in the incubator were also changed daily.

2.5. Sample preparation and chemical analyses

For both experiments, the following methods of sample collection and analyses were employed. Uneaten diet and frass from each insect were collected daily and stored at −80°C. All samples were later oven dried at 50°C to constant weight, after which frass was prepared for nitrogen and carbohydrate analyses. Upon pupation, insects were sexed, killed by freezing and were later oven dried and weighed. All measurements were carried out on a dry weight basis. Dry mass of food eaten was calculated as the difference between the initial dry weight of diet given and the uneaten portions. Initial dry weights of diet portions were estimated using a regression analysis on fresh diet portions of various mass values. Protein and carbohydrate intake was measured by multiplying the amount of food eaten by percent dry weight of dietary protein and carbohydrate in each diet type.

For experiment one, insects from only four of the seven diet treatments, those chosen to include in experiment two, were included for chemical analyses. Eight males and females from each treatment were chosen,

based on growth performance, for chemical analyses (a total of 16 insects from each treatment giving a total of 64 insects for each experimental replicate). Dried pupae were individually ground using a mortar and pestle, and cooled with liquid nitrogen. Pupae were subsequently dried again to remove any water that may have condensed onto the samples during grinding. Dried frass was ground using a Teflon pestle on a rotating shaft attached to a variable speed motor.

Both insect carcasses and frass were analyzed for total nitrogen using a flash combustion method on a Model 440 CHN/O/S Elemental Analyzer. Dry weight growth over the course of the fifth larval stage was obtained by first estimating the initial dry weights which were estimated from a prepared regression equation of dry weight on wet weight based on another group of larvae killed upon molting into the fifth stage (Raubenheimer and Simpson, 1993). These same insects were also analyzed for total nitrogen to give an estimate of the initial nitrogen content. Nitrogen values were multiplied by the conversion factor of 6.25 to get protein values for all samples (Raubenheimer and Simpson, 1993). Growth derived from carbohydrate (which also includes lipid growth) was estimated as the difference between the protein growth and the total dry weight growth, as, presumably, all dry weight growth is derived from the dietary protein and carbohydrate.

Carbohydrate content of frass was determined using a slightly modified anthrone method (Wheeler and Buck, 1992). Samples of frass, 40–50 mg, were homogenized in 1.5 ml microcentrifuge tubes in 800 µl of 1.0 M NaOH. Samples were vortexed, placed on an automated shaker for 15 min and centrifuged at 12,000*g* at 4°C for 10 min. A 100 µl aliquot of supernatant was diluted with 900 µl of 1.0 M NaOH. According to the percent dry weight of sucrose in a particular diet, either 10, 25 or 50 µl aliquot of the 1:10 dilutions were brought up to a total of 100 µl with 1.0 M NaOH and included in the anthrone assay. To all 100 µl sample aliquot, 2 ml of anthrone reagent was added and color was developed at 100°C for 12 min. Samples were cooled to room temperature and absorbance was read at 625 nm using a Beckman DU-62 spectrophotometer. A standard curve ranging from 0 to 50 µg glucose was run for each batch of samples, and absorbance values were linear over this range. Sample values were then read from this standard curve.

2.6. Data analysis

Intake and growth data from both experiments were interpreted according to the methodology of the geometrical framework (Simpson and Raubenheimer, 1995). Intake data were analyzed using both analysis of covariance (ANCOVA) (Raubenheimer and Simpson, 1992) and bi-coordinate plots (Raubenheimer and Simpson, 1992, 1994) with initial fresh mass as a covariate. Protein and carbohydrate intake data from experiment 2 (selective feeding) were analyzed independently using ANCOVA. The occurrence and extent of selective feeding was gauged by analyzing consumption of the paired foods in a paired *t*-test. Insects that consumed the two foods in equal amounts might not be selectively feeding. Performance data were also analyzed using ANCOVA. Mean relative growth rate (hereafter abbreviated as MRGR) was calculated for the entire fifth stadium (Adams and van Emden, 1972; Kogan and Cope, 1974). Stadium duration and MRGR were statistically analyzed using the initial mass as a covariate, and the growth derived from protein and carbohydrate were analyzed using protein and carbohydrate intake as covariates, respectively. Data were statistically analyzed using JMP IN (version 3.2.1, SAS Institute Inc.). A *P*-value within the range of $0.01 \leq 0.05$ is reported as moderate evidence for a significant difference between means, while a *P*-value ranging from $0.05 \leq 0.09$ is reported as suggestive, but an inconclusive evidence of a difference. Adjusted mean values (±standard errors of mean) were obtained from statistical models and used in all graphic illustrations, unless indicated otherwise.

3. Results

3.1. Experiment 1 (restricted to one diet)

3.1.1. Protein and carbohydrate consumption

In our analyses, the covariate, initial fresh mass and replicate effects strongly influenced the total food eaten, protein consumption and carbohydrate consumption (Table 1). Total dry mass of food eaten, by both males and females, was strongly influenced by dietary composition, independent of covariate and replicate effects, although no significant differences were detected between the sexes (Table 1). However, females ate more food than males on the diets where the amount of protein equaled or was greater than the amount of carbohydrate in the diet, with the exception of diet 24P:17C (Fig. 1). Overall, females ate 12.46 mg more total dry weight of food compared to males (95% confidence interval (CI): 3.29–28.21 mg more total dry food). Protein intake was also influenced by the dietary composition and there was moderate evidence that it differed between the sexes (Table 1, Fig. 2). Females maintained greater protein consumption on the diets where the amount of protein equaled or exceeded the amount of carbohydrate, with the exception of diet 24P:17C. On the whole, female larvae consumed 3.92 mg more dry weight protein compared to males (95% CI: 0.37–7.47 mg more protein). Carbohydrate intake was also strongly influenced by

Table 1
F ratios and *P* values from ANCOVAs for consumption data in experiment 1

Response variables	Total food eaten		Protein eaten		Carbohydrate eaten	
Effects (df)	*F*	*P*	*F*	*P*	*F*	*P*
Diet (6)	52.4	0.000	398.3	0.000	908.7	0.000
Sex (1)	2.4	0.121	11.3	0.030	0.5	0.481
Dietxsex (6)	1.2	0.324	0.9	0.240	0.9	0.509
Replicate (1)	18.1	0.000	11.7	0.000	13.9	0.000
Covariate (1)	52.8	0.000	58.8	0.000	32.7	0.000
Error (233)						
Total (248)						

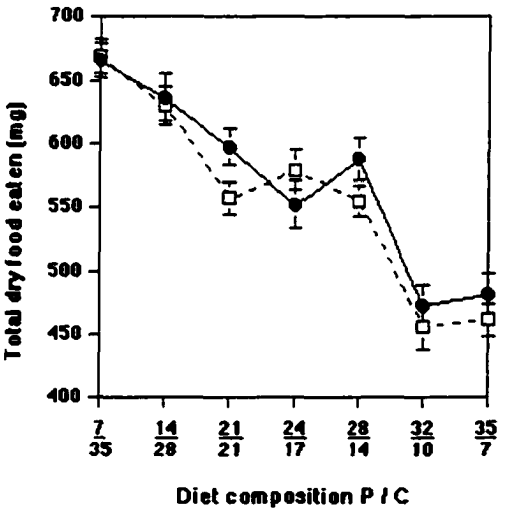

Fig. 1. Total dry weight of food eaten by females (●) and males (□) on all seven diets. Diets are labeled according to the percent dry weight of protein (top numbers) and percent dry weight of carbohydrate (bottom numbers).

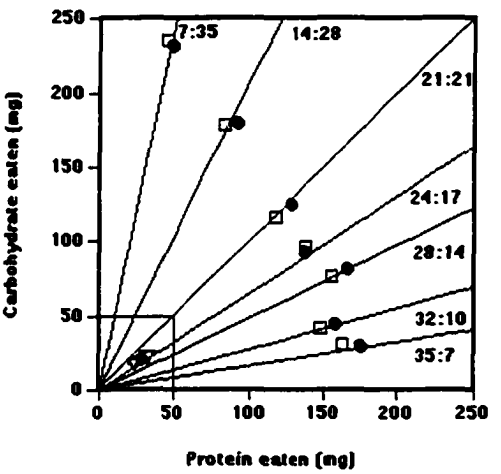

Fig. 2. Bicoordinate plot of the cumulative intake of protein and carbohydrate across the fifth stadium for females (●) and males (□). The boxed insert within overall plot shows the protein- and carbohydrate-derived growth for four of the seven diets over the fifth stadium drawn to the same scale (see Fig. 3 for an enlarged view). The numbers at the end of each rail indicate the ratio of percent dry weight protein to carbohydrate, respectively, for each diet. Lack of error bars indicate s.e. less than the size of the symbol.

dietary composition but no evidence that this differed between males and females was found (Table 1, Fig. 2).

3.1.2. Performance

All the response variables related to performance were either moderately or strongly influenced by covariate and replicate effects (Table 2). Independent of those effects there was convincing evidence that the duration of time larvae spent in the fifth stadium was influenced by the dietary composition but no significant differences were found between males and females (Table 2). Stadium duration was the shortest for both male and female larvae on diets 14P:28C and 21P:21C (adjusted means of 6.87 and 6.77 days, respectively) and increased as the dietary imbalance increased with respect to both protein and carbohydrate. Larvae spent an average of 7.62 days as fifth instars on diet 35P:7C.

The initial fresh mass differed between the sexes (Table 2). Males weighed, on average, 2.78 mg more at the start of their fifth instar compared to females (95% CI: 0.32–5.26 mg more). After accounting for initial weight, there was convincing evidence that the mean relative growth rate of caterpillars differed among all the diets and between the sexes (Table 2). It was estimated that female larvae grew on average 0.011 mg/mg/day faster than males on all diets (95% CI: 0.007–0.15 mg/mg/day faster). The largest difference between the sexes was observed on diet 14P:28C with females growing 0.015 mg/mg/day faster than males.

Table 2
F ratios and *P* values from ANCOVAs for performance data in experiment 1

Response variables	Stadium duration		Initial fresh mass		MRGR		Protein growth (corrected)[a]		Protein growth		Carbohydrate growth	
Effects (df)	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Diet (6)[b]	16.7	0.000	0.8	0.507	40.4	0.000	2.8	0.048	23.9	0.000	63.4	0.000
Sex (1)	1.7	0.190	4.9	0.027	24.4	0.000	3.1	0.082	5.7	0.018	0.0	0.963
Dietxsex (6)[b]	0.7	0.7			0.6	0.690	0.3	0.825	0.8	0.483	0.5	0.709
Replicate (1)	9.6	0.002	65.5	0.000	61.1	0.000	19.4	0.000	4.2	0.044	46.9	0.000
Covariate (1)[c]	28.6	0.000			11.1	0.001	25.1	0.000				
Error (233)[d]												
Total (248)[e]												

[a] Protein-derived growth is corrected for protein intake (covariate).
[b] For all three response variables regarding nutrient growth, main effects diet and dietxsex were associated with 3 degrees of freedom.
[c] The covariate for protein growth (corrected) was protein eaten. Protein and carbohydrate growth was analyzed without a covariate. For all other response variables, initial fresh mass served as the covariate.
[d] For response variables initial fresh mass, protein growth (corrected), protein and carbohydrate growth. Error was associated with 240, 76, 77 and 77 degrees of freedom, respectively.
[e] For all three response variables regarding nutrient growth, total degrees of freedom was 85.

We found moderate evidence that growth derived from dietary protein, on correcting for protein intake, differed among diets but found inconclusive evidence that the sexes differed in this response (Table 2). However, protein intake explained a substantial amount of variation in our statistical model, thus leaving little variation to explain the differences between the sexes. With our ANOVA model, we found convincing evidence that growth derived from protein differed among diets and moderate evidence that mean protein-derived growth differed between males and females (Table 2, Fig. 3). Mean protein-derived growth was estimated to be 1.89 mg greater in female larvae than that in males (95% CI: 0.32–3.46 mg greater). Mean protein-derived growth for females was 30.04 mg±0.58 s.e. Growth derived from both nutrients, represented in Fig. 3, was replotted as a function of the ratio of dietary protein to carbohydrate (Fig. 4). Growth derived from dietary carbohydrate was similar in the two sexes, but females exhibited greater growth derived from protein as the proportion of dietary protein increased (Fig. 4) and the difference was significant on diet 35P:7C (*t*-test=2.52, *P*=0.021). On this diet, mean protein-derived growth was estimated to be 3.69 mg greater in female larvae (95% CI: 0.63–6.76 mg greater). Mean protein-derived growth for females was 33.87 mg±1.2 s.e. Growth derived from the dietary carbohydrate was also analyzed as both ANCOVA and ANOVA models. Regardless of whether the carbohydrate intake was accounted for or not, no evidence was found for differences between the sexes (Table 2).

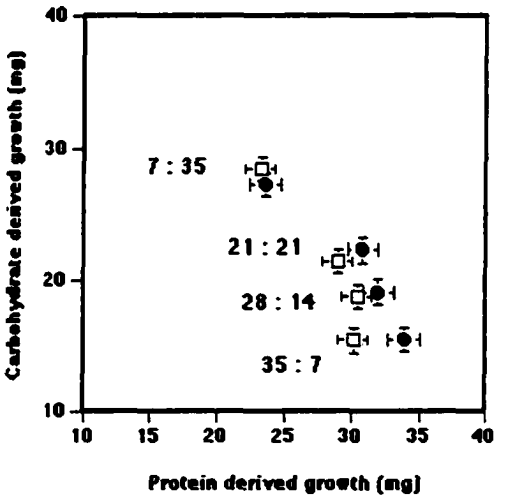

Fig. 3. Bicoordinate plot of growth derived from dietary protein and carbohydrate across the fifth stadium for females (●) and males (□) on four of the seven diets. Error bars represent standard errors. Lack of bar indicates s.e. less than the size of symbol.

3.2. Experiment 2 (choice of two diets)

3.2.1. Protein and carbohydrate consumption

The intake of both the protein and carbohydrate differed between replicates but neither was influenced by the covariate, initial fresh mass (Table 3). On accounting for these effects, there was moderate evidence that both protein and carbohydrate consumption differed among treatment groups. Both males and females consumed similar amounts of protein but there was moderate evidence that the sexes ate different amounts of carbohydrate (Table 3). It was estimated that female larvae consumed 10.46 mg more carbohydrate, over all the treatments studied, than did the males (95% CI: 0.328–20.60 mg more carbohydrate). The largest difference

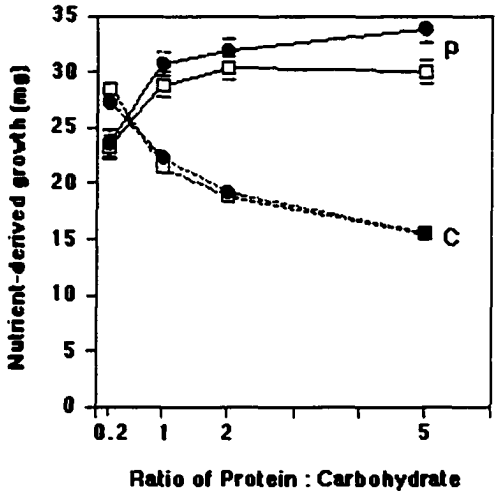

Fig. 4. Growth derived from both nutrients as a function of the ratio of dietary protein to carbohydrate for females (●) and males (□) on four of the seven diets. Dashed lines (C) represent carbohydrate-derived growth for both sexes. Solid lines (P) represent protein-derived growth for both sexes. Error bars represent standard errors. Lack of bars indicates s.e. less than the size of the symbol.

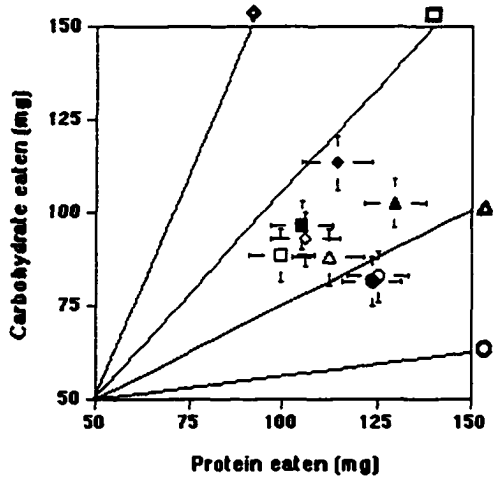

Fig. 5. Carbohydrate and protein intake by female (◆●■▲) and male (◇○□△) caterpillars allowed to selectively feed on two different diets. Solid lines represent the expected intake if equal quantities of the two diets were eaten within each treatment. Symbols, external to plot, at the end of each line denote treatment. (1) ◇: 7P35C+28P14C; (2) ○: 21P21C+35P7C; (3) □: 7P35C+35P7C; (4) △: 21P21C+28P14C.

Table 3
F ratios and *P* values from ANCOVAs for performance data in experiment 1

Response variables	Protein eaten		Carbohydrate eaten		Stadium duration		MRGR		Protein growth		Carbohydrate growth	
Effects (df)	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Group (3)	2.8	0.039	3.2	0.027	1.1	0.355	5.5	0.002	6.7	0.001	2.9	0.042
Sex (1)	1.3	0.253	4.2	0.043	2.0	0.155	1.2	0.268	5.3	0.025	7.1	0.010
Group×sex (3)	0.4	0.736	0.9	0.427	1.2	0.296	0.3	0.797	0.1	0.976	0.3	0.845
Replicate (1)	10.6	0.001	16.9	0.000	8.8	0.004	7.4	0.007	1.5	0.223	3.8	0.057
Covariate (1)[a]	0.3	0.566	0.0	0.865	12.3	0.001	0.1	0.726				
Error (125)[b]												
Total (134)[c]												

[a] The covariate, when included, was initial fresh mass.
[b] For response variables protein- and carbohydrate-derived growth, error was associated with 54 degrees of freedom.
[c] For response variables protein- and carbohydrate-derived growth, total degrees of freedom was 62.

between the sexes was observed in treatment 1 (35C:7P vs. 28P:14C) on which females ingested 20.48 mg more carbohydrate compared to male larvae.

This experiment also examined the extent of selective feeding by *H. virescens* caterpillars. Larvae ate equal amounts of the two diets in treatment 4 (△) (two-sided *P*-value=0.187, from a paired *t*-test) but ate selectively between food pairs in treatments 1 (◇) and 2 (○) (two-sided *P*-value <0.000 in both cases) and in treatment 3 (□) (two-sided *P*-value=0.002) (Fig. 5).

3.2.2. Performance

The initial fresh weight of males was higher than that of females (two-sided *P*-value=0.062, ANCOVA *F*-test) (estimate of difference=3.9 mg, 95% CI: 0.19–8.0 mg higher). On accounting for the influences of both the covariate and replicate effects, duration of time spent in the fifth stadium was found to be similar among the four groups and between the sexes (Table 3). There was strong evidence that MRGR of larvae differed among the groups with no evidence of gender differences (Table

3). Further analyses using a posteriori multiple comparison of means revealed that MRGR of larvae in treatment 3 (diet 7P:35C vs. 35P:7C) were significantly lower than those of larvae on the other treatments (rejection at α=0.05, Tukey–Kramer HSD).

There was suggestive evidence that the sexes differed in growth derived from both nutrients, after removing the effects of intake (protein-derived growth, ANCOVA P-value=0.063; carbohydrate-derived growth, ANCOVA P-value=0.071). However, growth derived from both nutrients was also analyzed as an ANOVA model to compare the growth between self-selecting and non-selecting larvae. From our ANOVA model, we found convincing evidence that protein-derived growth differed among treatment groups and moderate evidence for differences between males and females (Table 3, Fig. 6). Mean protein-derived growth was estimated to be 2.60 mg greater in females than in males (96% CI: 0.33–4.86 mg greater). Female larvae accumulated the greatest amount of protein in treatments 2 and 4 (mean±s.e. protein-derived growth was 36.56±1.58 and 35.48±1.58, respectively). In contrast to the non-selecting larvae in experiment 1, we obtained moderate evidence that growth derived from dietary carbohydrate differed among treatments and between the sexes (Table 3, Fig. 6). Mean carbohydrate-derived growth was estimated to be 2.32 mg greater in females compared to males (95% CI: 0.58–4.06 mg greater). Self-selecting females derived their highest carbohydrate growth in treatments 1 and 2 (mean±s.e. carbohydrate-derived growth was 23.67±1.30 and 22.22±1.21, respectively).

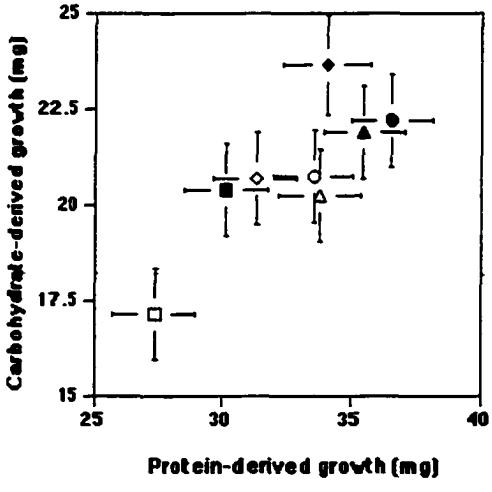

Fig. 6. Bicoordinate plot of growth derived from the dietary protein and carbohydrate for both female (◆●■▲) and male (◇○□△) caterpillars allowed to selectively feed on two different diets. Symbols denote treatment (see the caption to Fig. 5 for description of food pairings within each treatment). Note that scales on the two axes are different.

4. Discussion

Throughout most of the life cycle in Lepidoptera, females tend to be heavier than males, a feature that has been attributed to the role of producing eggs (Slansky and Scriber, 1985). However, few studies have examined the nutritional physiology of caterpillars separately for males and females. Some of these studies have concluded that females achieve their greater size by feeding and developing for a longer period of time (Atwal, 1955; Chou et al., 1973; Beckwith, 1976; Mackey, 1978; Lederhouse et al., 1982). Some studies on Lepidoptera, in which the sexes develop for the same period of time, have concluded that females attain greater size by ingesting food at a greater rate (Nakano and Monsi, 1968; Van den Berg, 1973; Mathavan and Bhaskaran, 1975; Horie et al., 1976; Bhat and Bhattacharya, 1978). Our study, in using chemically defined artificial diets, explained the nutritional physiology of female caterpillars in terms of specific macronutrients important to them. We were highly interested in how females acquire protein, given the importance of this nutrient to their role as egg producers and because protein is typically acquired only during larval feeding. In general, insects can meet their nutritional needs by selecting among suitable foods, increasing the consumption of an inadequate diet to compensate for the nutrient deficiencies and/or through post-ingestive processing of such nutrients. The experimental framework designed by Simpson and Raubenheimer allows one to study and evaluate these processes independently. Our results revealed three important features regarding mechanisms used by female caterpillars to accumulate nutrients.

Firstly, we found that the caterpillars varied their food intake according to the balance of nutrients in the diet. When restricted to diets, caterpillars adjusted their total intake and ate less as the proportion of dietary protein increased (Fig. 1). When given a choice of foods, *H. virescens* larvae also adjusted their total intake to achieve an appropriate intake of protein and carbohydrate to meet their nutrient needs. When allowed to feed selectively between a carbohydrate or protein biased food, larvae fed more on the diet in which protein content was relatively higher (data points fall to the right of both treatments 1 and 3 lines, Fig. 5). In contrast, when given the opportunity to choose between a balanced and protein biased diet, caterpillars fed more on the balanced diet (data points fall to the left of both treatments 2 and 4 lines, Fig. 5) but this was significant only in treatment 2. Larvae ate equal amounts of both foods in treatment 4 in which both protein and carbohydrate was present in roughly equal proportions. Thus, the ability of caterpil-

lars to self-select was more apparent when they must choose between foods that are biased with respect to one nutrient over the other (treatments 1 and 2). Caterpillars fed more or less randomly in treatments 3 and 4 suggesting that selective feeding is not necessary and perhaps not possible when nutrients are encountered at intermediate levels and that they may be approaching their intake target. Overall, with the exception of females ingesting more carbohydrate compared to males in treatment 1, males and females were not distinguished according to the selective feeding behavior. The behavioral capacity of caterpillars to self-select has been previously demonstrated in caterpillars of *Heliothis zea* (Waldbauer et al., 1984) and *S. littoralis* (Simpson et al., 1988).

Secondly, our results showed that females eat more than males but only under certain dietary conditions. When restricted to a diet containing low levels of protein relative to carbohydrate (7P:35C and 14P:28C) the two sexes ate similar amounts, but on diets with a greater proportion of protein, the females ate more than males leading to a greater intake of protein (Figs. 1 and 2). When allowed to choose between foods, females tend to eat more (female date points fall above and to the right of male data points in Fig. 5), except when the combination of foods resulted in a heavily protein biased condition (treatment 2). In treatment 2, protein and carbohydrate intakes were similar between males and females (Fig. 5). Although values of protein intake for females generally fell to the right of the protein intake values for males, differences were not statistically significant (Table 3). Nonetheless, females ate more of both foods in treatment 4 (less protein biased than that of treatment 2) resulting in the greatest intake of protein compared to all other diet treatments. In treatment 4 we combined diets 21P:21C with 28P:14C, the only diets on which females increased the total food consumption over that of males under no-choice conditions (Fig. 1).

Thirdly, results of both the choice and no-choice experiments support the importance of post-ingestive processing of nutrients in allowing females to accumulate their greater nutrient reserves. When we restricted insects to one of the seven diets varying in levels of protein and carbohydrate, caterpillars ingested different amounts of both nutrients, but growth of both sexes was highly clustered compared to the ingestion values. This pattern is evidence for the post-ingestive compensatory responses that have been reported in Lepidoptera and a number of other insect species (Simpson and Simpson, 1990; Raubenheimer and Simpson, 1993; Slansky, 1993; Zanotto et al., 1993). Under no-choice conditions, females accumulated more protein than the males when dietary protein levels were high. Increased protein-derived growth largely reflected increased protein intake on such diets; however, suggestive evidence for post-ingestive regulation was observed when the effect of intake was removed (Table 2).

Although the choice experiment was designed to determine the importance of selective feeding, it also lent substantial support to the importance of post-ingestive processing. Females surpassed males with respect to protein-derived growth in treatment 4 but attained their greatest protein- and carbohydrate-derived growth in treatment 2 where they selectively fed between a balanced and protein biased diet (Fig. 6). When restricted to diet 21P:21C, females accumulated carbohydrate at a similar level to self-selecting females in treatment 2 but fell short of their protein–growth level. Likewise, females restricted to diet 35P:7C attained a similar protein-derived growth to self-selecting females of treatment 2 but fell short of their carbohydrate–growth level. Only when females fed selectively between these diets in treatment 2 did they accumulate significantly greater levels of both protein and carbohydrate than the males. This necessitated reliance of post-ingestive processing as intake of both the nutrients was similar between the sexes in treatment 2 (Fig. 5). Further, female larvae attained greater protein- and carbohydrate-derived growth compared to males even when intake of both nutrients was accounted for, thus providing strong evidence for a post-ingestive effect favoring females.

In summary, we have shown that female *H. virescens* larvae accumulate protein by regulating both intake and post-ingestive processing of dietary protein, especially on high protein foods. On a heavily protein biased food, intake of both nutrients were similar between the sexes, though females accumulated greater amounts of nutrients compared to males. This suggests that, on very high protein foods, intake and post-ingestive processing is adequately meeting the tissue and storage needs. However, on a diet that is lower in protein, females increase consumption, thereby increasing the protein intake, to meet the nutrient demands, and still effectively accumulate more protein than the males do. This response to dietary protein level is consistent with what has been observed in the field. Female moths oviposit on the terminals of the cotton plant, *Gossypium hirsutum* (L.). Neonates migrate to the terminal area and then to squares (floral buds), on which they feed. Later instars burrow through the calyx or petals of the developing squares and feed on the anthers, on which they grow and develop quickly (Shaver et al., 1977; Hedin et al., 1991). Cotton anthers contain, on average, 27% protein with an additional 3–5% free amino acids (Hedin and McCarty, 1990), the highest total amino acid content compared to the other cotton floral tissues (Hedin et al., 1991). A separate chemical analysis of cotton found that the total carbohydrate, not including cellulose, content of anthers (on average 45%) was also high relative to other cotton floral tissues (Hedin and McCarty, 1990). Therefore, late instar larvae migrate to and grow well on the plant tissue

of highest nutritional value. Our results indicate that female *H. virescens* larvae easily reach their optimal carbohydrate-derived growth (which includes lipid growth) and accumulate the required protein by regulating both intake and post-ingestive processing of protein.

H. virescens is typical of the majority of Lepidoptera, in which adults regularly obtain carbohydrate but little protein from nectar. Protein stores acquired from larval feeding has recently been found to play an important role for egg production by *Plutella xylostella*, a species that undergoes vitellogenesis during the adult stage (Wheeler et al., 2000). Like *P. xylostella*, vitellogenesis and oocyte production in *H. virescens* occurs soon after adult eclosion (Zeng et al., 1997). As a consequence, protein acquired during larval feeding must play a similarly important role in egg production in *H. virescens* females.

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APPENDIX B

FEMALE *ESTIGMENE ACREA* LARVAE RELY ON PRE-INGESTIVE, POST-INGESTIVE AND DEVELOPMENTAL STRATEGIES TO ACCUMULATE NUTRIENTS

Female *Estigmene acrea* larvae rely on pre-ingestive, post-ingestive and developmental strategies to accumulate nutrients

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Abstract

Female Lepidoptera are often heavier than males. In a previous study we found that female *Heliothis virescens* (Family Noctuidae) larvae relied on both consumption and post-ingestive processing as mechanisms to acquire protein. We conducted a comparative study with *Estigmene acrea* (Family Arctiidae) and examined similar mechanisms. In choice experiments, E. acrea larvae adjusted their total food intake and achieved an intake of protein and carbohydrate to meet their nutritional needs. As was seen for *H. virescens*, the sexes did not differ in their selective feeding behavior. When given no choice, *E. acrea* females responded to an inadequate diet (with respect to both nutrient levels) by increasing consumption and this led to greater protein and carbohydrate intakes. These same experiments indicated that females relied on post-ingestive processing of nutrients and accumulated more protein than did males (again similar to *H. virescens* females). Lastly, we found that *E. acrea* prolongs juvenile development if it is too small as a late stage larva. This longer development time

effectively extended their period of food intake, thus enabling initially small larvae to catch up and even surpass those that had started off bigger.

Keywords: Larval diet; Female nutrition; Growth regulation; Protein accumulation; Developmental polyphenism.

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1. Introduction

Female insects produce eggs richly provisioned with nutrients. Therefore, an important aspect of the reproductive biology of females in most insect species is the strategy she uses to accumulate such nutrients. The larval and adult stages of lepidopteran species, like most other holometabolous groups, rely on different nutritional resources. Yet, a fundamental challenge for female Lepidoptera is that they provision eggs with a great deal of protein but their adult diet of nectar, while rich in sugars, is a poor source of amino acids (Slansky and Scriber, 1985). This suggests that females rely on their larval diet to accumulate greater stores of protein and other specific nutrients than do males, but how the necessary protein and other nutrients are acquired from their environment and utilized is still largely an unanswered question.

Some studies have concluded that females attain greater size by ingesting food at a faster rate over a similar time period (Nakano and Monsi, 1968; Van den Berg, 1973; Mathavan and Bhaskaran, 1975; Horie et al., 1976; Bhat and Bhattacharya, 1978). In other cases it is thought that they ingest food over a longer period of juvenile development (Atwal, 1955; Chou et al., 1973; Beckwith, 1976; Mackey, 1978; Lederhouse et al., 1982), which may include females undergoing an extra instar. In a previous study we found that female *H. virescens* larvae accumulated the needed protein by regulating post-ingestive processing of protein, but, in addition, by eating more of a diet that was high in protein (Telang et al., 2001). Thus, an individual insect may adjust its strategy depending on the available nutrients. In the present paper, we report the results of our experiment on *Estigmene acrea*, the salt marsh caterpillar (Fam. Arctiidae, Subfamily Arctiinae). The family Arctiidae is closely related to the family Noctuidae, to which *Heliothis* belongs, but unlike many noctuids, does not generally feed as adults.

Consequently, female *E. acrea* larvae should tightly regulate acquisition of both protein and carbohydrate but by which mechanism?

2. Materials and methods

2.1. Animals

Estigmene acrea caterpillars were originally collected from the field in Arizona and were maintained in the laboratory on a synthetic, wheatgerm-enhanced diet. Eclosed adults were allowed to mate in large plastic boxes decorated with wax paper, which served as the egg laying substrate. Eggs were collected from several females since several individuals of both sexes were housed together in one box. Egg masses were maintained at LD 16:8 h, 25°C until hatching. Upon hatching, neonates were placed individually in 30 ml plastic cups fitted with plastic lids and were maintained in an environmental chamber at LD 16:8 h, 28:25°C. Larvae were reared on wheatgerm-enhanced diet to maintain normal growth and development (Telang et al., 2001). Larvae to be used in experiments were removed from this diet at the end of their fifth larval stadium, and then reared on experimental diet. *Estigmene acrea* undergo 6-7 larval instars; subsequently, on entering their sixth instar, insects were weighed, transferred to 150 ml plastic cups and randomly assigned to diet treatments.

2.2 Synthetic diets

Artificial diets were modified from diets previously described for *Heliothis virescens* (Telang et al., 2001). Earlier experiments with *E.acrea* larvae on synthetic diets, designed for *H.virescens*, resulted in pupae with poorly developed cuticle, a condition subsequently remedied upon adding phenylalanine to experimental diets (E. A. Bernays, pers. comm.). All diets were agar based (3% solution wt/vol) and contained 53.2% cellulose (non-nutritive bulking agent), 0.8% phenylalanine and 4% essential

micronutrients (vitamins, cholesterol, salts and linoleic acid). The remaining 42% consisted of protein and carbohydrate at various levels. Each insect was given a fresh, pre-weighed block of diet daily in all experiments.

2.3 Experiment 1

In our first experiment we sought to determine if the sexes differed in their consumption and/or performance when they were restricted to one of seven diets, and whether their behavior changed in response to varying levels of dietary protein and carbohydrate. Our experimental protocol was comparable to that applied to *H.virescens* (Telang et al., 2001) except that both sixth and seventh-instar *E.acrea* larvae were experimental subjects. Our experiment was repeated twice and used the following diets varying in dry weight percentages of protein (P) to carbohydrate (C): 7P:35C; 14P:28C; 21P:21C; 24P:17C; 28P:14C; 32P:10C; 35P:7C. Performance (based on growth and stadium duration) and consumption were measured for each insect. Insect carcasses were chemically analyzed to quantify growth derived from both macronutrients.

Insects included in each replicate of experiment one displayed different developmental patterns, some pupating at the end of their sixth and others after the seventh-instar. In replicated two, all caterpillars pupated at the end of their sixth-instar. For experiment one, replicate one, two to three males and females from four of the seven diets, that underwent six larval instars, were chosen for chemical analyses (a total of 6 insects from each treatment giving a total of 24 insects from replicate one). In addition, twelve insects on the same four diets that pupated at the end of their seventh instar were also included for chemical analyses. For experiment one, replicate two, a total of ten males and females from each treatment were chosen for chemical analyses (a total of 20 insects from each treatment giving a total of 80 insects from replicate two).

2.4 Experiment 2

This experiment was designed to measure the ability of male and female *E.acrea* larvae to select an appropriately balanced diet when allowed to choose between two diets differing in the protein:carbohydrate ratio. One replicate of this experiment was run. Four of the seven diets from the first experiment on which larvae performed similarly well were chosen: 7P:35C; 21P:21C; 28P:14C; and 35P:7C. The four diets were paired in the following manner with each pairing assigned a treatment number: treatment 1 = 7P:35C vs. 28P:14C; treatment 2 = 21P:21C vs. 35P:7C; treatment 3 = 7P:35C vs. 35P:7C; and treatment 4 = 21P:21C vs. 28P:14C. All other details of this protocol are as previously described (Telang et al., 2001).

2.5 Sample Preparation and chemical analyses

Dry mass of food eaten was calculated as the difference between the initial dry weight of diet given and the uneaten portions. Initial dry weights of diet were estimated from a regression analysis on fresh diet portions of various mass values. Protein and carbohydrate intake was measured by multiplying the amount of food eaten by percent dry weight of dietary protein and carbohydrate in each diet type. Dried pupae were prepared and analyzed for total nitrogen as described previously (Telang et al., 2001). However, dry weight growth over the course of the sixth and seventh instar was calculated by first estimating initial dry weights from a prepared regression equation of dry weight on wet weight based on a separate group of larvae killed upon molting into the sixth instar. These insects were also analyzed for total nitrogen to estimate initial nitrogen content. Nitrogen values were converted to protein by multiplying by a factor of 6.25 (Raubenheimer and Simpson, 1993). Growth derived from carbohydrate (which also includes lipid growth) was calculated by subtracting protein growth from total dry

weight growth, since all dry weight growth was derived from dietary protein and carbohydrate, there being no other major nutrients in the diet, and assuming cellulose is not digested.

2.6 Data Analysis

As in our earlier work, intake and growth data from both experiments were interpreted according to the geometrical framework developed by Simpson and Raubenheimer (1995). Intake data from both experiments were analyzed using both analysis of covariance (ANCOVA) (Raubenheimer and Simpson, 1992) and bi-coordinate plots (Raubenheimer and Simpson, 1992, 1994, 1995) with initial fresh mass as a covariate. For experiment one, insects from replicate 1 and 2 were grouped and analyzed according to the pattern of their development. One set of analyses compared insects from both replicates 1 and 2 in which all individuals pupated at the end of their 6^{th} instar stage. A second set of analyses consisted only of replicate 1 insects in which some larvae pupated at the end of their 6^{th} instar stage (referred to as 6^{th} instar cohort) and some at the end of their 7^{th} instar stage (referred to as 7^{th} instar cohort).

For experiment two, the occurrence and extent of selective feeding was gauged by analyzing consumption of the paired foods in a paired t-test. Insects that consumed the two foods in equal amounts were interpreted as feeding non-selectively. Performance data were also analyzed using ANCOVA. Mean relative growth rate (MRGR) was calculated separately for both the sixth and seventh stadia (Adams and van Emden, 1972; Kogan and Cope, 1974). Stadium duration and MRGR were statistically analyzed using initial mass as a covariate, and growth derived from protein and carbohydrate were analyzed using protein and carbohydrate intake as covariates, respectively. Data were statistically analyzed using JMP IN (version 3.2.1, SAS Institute Inc.). A P-value within

the range of $0.01 \leq x \leq 0.05$ is reported as moderate evidence for a significant difference between means, while a P-value ranging from $0.05 < x \leq 0.09$ is reported as suggestive, but inconclusive, evidence of a difference.

3. Results

3.1 Developmental polyphenism

Caterpillars of *Estigmene acrea* on wheatgerm-enhanced diet undergo either 6 or 7 larval instars (pers. observ), and this occurred in experiment 1, replicate 1. In our current experiments we found that larvae varied in the size they attained upon entering their 6th larval stage (Figs. 1A). Both males and females entering the 6th instar with an initial dry weight of less than 200 mg invariably underwent an additional instar, pupating after the seventh larval instar. Above this nearly all individuals, and all those initially weighing more than 250 mg, had only six instars. Seventh instar cohort females weighing 200 – 400 mg still underwent an additional instar (Fig. 1A) although a few 6th instar cohort females in this same range underwent six (data not shown). Most of the 6th instar cohort weighing 200 – 400 mg consisted of males (data not shown). In replicate 2 all insects were initially heavier than this and all completed development in 6 instars.

When comparing the 6th larval stage between cohorts, stadium duration did not differ between the sexes and the 6th instar cohort developed over a longer time period than did the 7th instar cohort (Fig. 1B). Consequently, MRGR did not differ between the sexes but the 7th instar cohort grew significantly faster over their 6th larval stage. On the other hand, stadium duration differed between the sexes when comparing the last larval stages of the 6th and 7th instar cohorts, as well as between the cohorts (Table 4). Overall, females spent three days longer as last instar larvae than did the males and the 7th instar cohort spent ten days longer in their 7th instar stage than did the 6th instar cohort during

their 6th instar stage (Fig. 1B). Males and females of both cohorts displayed similar MRGR during their last larval stages with the 6th instar cohort growing faster compared to the 7th instar cohort. This is not surprising, however, given that the 7th instar cohort developed over a longer period of time during their last larval stage (Table 4). The developmental trajectory an individual followed consequently dictated both their feeding and performance responses. We were interested in how these responses varied among different diet regimes and whether the sexes responded differently.

3.2 Consumption

3.2.1. Differences between the sexes in total food eaten

Total dry mass of food eaten was strongly influenced by dietary composition and significant differences were found between the sexes (Table 1). The extent of differences between the sexes in how much food was eaten varied among the different diets (indicated by a significant interaction between the two variables) (Table 1, Fig. 2). There were no differences between the sexes on the balanced (21P:21C) diet but females ate more of the carbohydrate biased diets and on the extreme protein biased diet (35P:7C) in both replicates (Fig. 2).

3.2.2. Differences during development in total food eaten

Overall, 6th instars from replicate 2 ate more total dry weight of food than did replicate 1 6th instar cohort (Fig. 2). However, differences between replicate 1 and 2 insects reflect differences in initial fresh mass, as replicate 2 insects were larger at the start of their 6th instar (Fig. 1A). The 6th instar cohort ate more food during their 6th larval stage than did the 7th instar cohort, but, overall, the 7th instar cohort ate more food during their 7th larval stage than did the 6th instar cohort during their 6th larval stage (Fig. 2). Total dry mass of food eaten was not influenced by dietary composition but females ate

significantly more food, with non-significant interactions between the two variables (Table 3).

3.2.3. Differences in protein and carbohydrate intake

Protein-intake was influenced by dietary composition and there was strong evidence that it differed between the sexes as well as between replicates (Table 1, Figs. 3A and 3B, Fig. 4). Overall, female larvae ate more dry weight protein compared to males and replicate 2 insects consumed more dry weight protein than did replicate 1 6th instar cohort. However, on diet 35P:7C, females of the replicate 1 6th instar cohort consumed more dry weight protein compared to replicate 2 females (Figs. 3A and 4). Lastly, we found that dietary composition influenced carbohydrate intake but differences between the sexes were slight (Table 1). Overall, females consumed more dry weight carbohydrate than did males with the largest difference between the sexes found on diet 7P:35C (compare Figs. 3A and 3B, Fig. 4). As was the case for protein intake, replicate 2 insects ate more carbohydrate compared to the replicate 1 6th instar cohort. *Estigmene acrea* ingested widely varying levels of nutrients but growth derived from these nutrients was clustered, suggesting reliance on post-ingestive processing (Fig. 4).

The amount of protein eaten by both 6th and 7th instar cohorts during their 6th larval stage varied among diets but no significant differences were found between the sexes (Table 3, Figs. 3A and 3B). There was moderate evidence that protein intake differed overall between these insect groups and, although differences between the sexes in both groups were minimal on diet 21P:21C, females of both groups ate more protein than did the males on the other diets (Table 3, Figs. 3A and 3B). Overall, the 6th instar cohort ate more protein during their 6th larval stage than did the 7th instar cohort. The amount of protein eaten during the final stages by the 6th and 7th instar cohorts was

strongly influenced by dietary composition with females clearly eating more protein compared to males (Table 3, Figs. 3A and 3B). On average, females of the 7th instar cohort ate more protein during their 7th larval stage than did the 6th instar cohort during their 6th larval stage. The two larval groups differed in their protein intake with the 7th instar cohort consuming more protein than did the 6th instar cohort. Differences in protein intake between the sexes in the 7th instar cohort were significantly greater than were between the sexes in the 6th instar cohort (data not given).

As was the case for protein intake, the intake of carbohydrate by both 6th and 7th instar cohorts during their 6th larval stage was strongly influenced by dietary composition but no significant differences were found between the sexes (Table 3, Figs. 3A and 3B). The 6th instar cohort ate more carbohydrate than did the 7th instar cohort over the course of their 6th larval stage. The amount of carbohydrate by both cohorts over their final larval stages was strongly influenced by dietary composition but, unlike the case for protein intake, males and females consumed similar amounts of carbohydrate (Table 3). Cohorts consumed different amounts of carbohydrate with the 7th instar cohort eating more carbohydrate over their 7th larval stage than did the 6th instar cohort over their 6th larval stage.

3.2.4. Selective feeding by E. acrea caterpillars

When insects were given a choice of two unbalanced diets (experiment 2), neither protein nor carbohydrate intake differed among treatment groups ($P = 0.337$ and $P = 0.118$ for protein and carbohydrate, respectively). Males and females consumed similar amounts of protein ($P = 0.262$) but there was suggestive evidence that the sexes ate different amounts of carbohydrate ($P = 0.045$). Larvae adjusted intake and selectively fed between food pairs in treatments 1 (◊) ($P = 0.004$) and 3 ( ) ($P = 0.009$), treatments in

which the combined P:C ratio was carbohydrate biased (Fig. 5). On the other hand, larvae ate equal amounts of the two diets in treatments 2 (O) (P= 0.395) and 4 (Δ) (P= 0.987), treatments in which the combined P:C ratio was protein biased (Fig. 5).

3.3 Performance

3.3.1 Differences between the sexes

We found strong evidence that growth derived from dietary protein, corrected for protein intake, differed among diets and between the sexes (Table 2, Figs. 6 and 7). On most diets, females were able to convert ingested protein into growth with relatively high efficiency (values fall to the right of protein-derived growth values for males) (Figs. 6 and 7). However, on diets 21P:21C and 35P:7C, values were similar between males and females of replicate 1 and 2, respectively (Fig. 6). Mean protein-derived growth across all diets was greater in female larvae than in males. We also investigated how efficiently ingested carbohydrate was converted into growth, which includes lipid growth. However, our ANCOVA model revealed significant interactions between the covariate, carbohydrate intake, and diet (P= 0.015), which required the fitting of separate regressions for each level of dietary treatment. There was no significant interaction term between the covariate and sex. Unlike the case of protein-derived growth, females seemed to convert ingested carbohydrate into growth less efficiently than did males, at least at the lower carbohydrate intakes which took place on protein-biased diets (Fig. 6). Male larvae accumulated higher levels of carbohydrate than did females on most diets (values lay above carbohydrate-derived growth values for females) (Fig. 6).

3.3.2 Differences during development

Protein-derived growth, corrected for protein intake, differed substantially between replicate 1 6th instar cohort and replicate 2 insects (Table 2). It was estimated

that replicate 2 insects accumulated more protein compared to replicate 1 6th instar cohort (Fig. 6). Growth derived from dietary protein and carbohydrate was compared between 6th and 7th instar cohort of replicate 1. Neither protein- nor carbohydrate-derived growth were influenced by dietary composition but there was suggestive evidence that protein-derived growth differed between the sexes (Table 5). On average, female replicate 1 larvae accumulated more protein than did males (Fig. 7). There was no evidence that the 6th and 7th instar cohorts accumulated different amounts of protein during their 6th and 7th larval stages, respectively (Table 5, Fig. 7). To the contrary, we found no evidence that the sexes accumulated different amounts of carbohydrate over their last larval stage but found convincing evidence that the cohorts accumulated different amounts of carbohydrate (Table 5, Fig. 7). The 7th instar cohort accumulated more carbohydrate over their 7th larval stage than did larvae in the 6th instar cohort over their 6th larval stage.

3.3.3 Final weight of rep 1 7th instar cohort compared to others.

Final dry weights of pupae in both the 6th and 7th instar cohorts were significantly influenced by dietary composition ($P= 0.0001$). We found significant differences between the cohorts ($P= 0.005$), with the 7th instar cohort weighing 18.56 mg more compared to the 6th instar cohort (95% CI: 5.84 – 31.28 mg more). Overall, females outweighed males ($P= 0.015$), by 7.06 mg (95% CI: 3.36 – 30.74 mg more).

4. Discussion

Larval and adult stages of lepidopteran species, like other holometabolous insects, often consume different nutritional resources. Female insects produce eggs that contain large supplies of nutrients to support embryonic development and the strategy she uses to acquire nutrient plays a central role in her reproductive biology. In general, insects can

meet their nutritional needs by selecting among suitable foods, increasing consumption of an inadequate food to compensate for the nutritional deficiency and/or through post-ingestive processing of specific nutrients. In addition, an adjustment of a life history trait, such as an extended development time, also allows an individual to take advantage of the above three strategies over a longer period of time, consequently becoming a strategy in itself. Our present results indicate that female *E. acrea* larvae rely on all four strategies to accumulate both protein and carbohydrate.

First, when given a choice of foods, *E. acrea* larvae adapted their total intake of foods and achieved an intake target of protein and carbohydrate to meet their nutritional needs. When given a choice of either protein or carbohydrate biased food, *E. acrea* larvae fed selectively more on the diet with higher protein content (data points fall to the right of both treatments 1 and 3 lines, Fig. 5). In contrast, larvae ate equal amounts of both foods in treatments 2 and 4 in which one diet was balanced and the other was protein biased. Results from both studies indicate that caterpillars in general are able to feed selectively when faced with foods that are unbalanced with respect to one nutrient over the other (treatments 1 and 3). *Estigmene acrea* caterpillars fed randomly on treatments 2 and 4 suggesting that selective feeding is not necessary or is difficult when nutrients are present at intermediate levels and that perhaps caterpillars are approaching their intake target. Overall, *E. acrea* larvae indicated an intake target with a P:C ratio of 1.44 (255 mg protein : 177 mg carbohydrate, Fig. 5). Equal consumption of foods on treatments 2 and 4 would have led to a P:C intake of 2.0 and 1.4, respectively, thus lending more evidence to the possibility that insects on these treatments were approaching their intake target, a pattern similar to that reported for *H. virescens* (Telang

et al., 2001). Overall, males and females did not differ in their selective feeding behavior, again a pattern similar to what we found for *H. virescens* (Telang et al., 2001).

Secondly, *E. acrea* females responded to an inadequate diet by increasing consumption, again a similar situation to that observed for *H. virescens*. Females and males ate similar amounts of food on diet 21P:21C in which protein and carbohydrate were balanced. However, females significantly increased consumption of diets unbalanced with respect to both protein and carbohydrate leading to greater protein and carbohydrate intake (Figs. 3A and 3B, Fig. 4). In contrast, *H. virescens* females ate more than males only on diets with similar and greater proportion of protein that led them to a greater protein intake (Telang et al., 2001).

Thirdly, results of the no-choice experiment suggest that female *E. acrea* larvae also relied on post-ingestive processing of nutrients to accumulate both protein and carbohydrate. These insects were restricted to one of seven diets varying in levels of protein and carbohydrate, so that insects face a situation in which they must consume too much of an overabundant nutrient and consume too little of a deficient nutrient (Simpson and Raubenheimer, 1995). Because insects are restricted to only one diet, individuals can only move up or down a nutritional rail depending on overall consumption of each diet (Fig. 4). *Estigmene acrea* individuals ingested different amounts of both nutrients resulting in widely varying ingestion levels, but growth derived from ingested nutrients was clustered relative to the ingestion values (Fig. 4). Such post-ingestive compensation for an inadequate diet was previously reported for *H. virescens* (Telang et al., 2001), among other Lepidoptera, and a number of other insect groups (Simpson and Simpson, 1990; Raubenheimer and Simpson, 1993; Slansky, 1993; Zanotto et al., 1993). Overall, female *E. acrea* larvae accumulated more protein than did the males (Figs. 6 and 7).

Although much of this increased protein-derived growth is attributed to females consuming more protein on these same diets, we removed the effect of protein intake and obtained strong evidence that post-ingestive processing contributed to protein-derived growth as well (Table 2). Finally, a fourth strategy may entail a life history adjustment such as extended juvenile development if a late stage larva is too small. A longer development time effectively extends an insect's period of food intake.

Our data indicate that female *E. acrea* were better able to accumulate both protein and carbohydrate on diet 7P:35C (Figs. 6 and 7). This contrasts with the situation we observed for *H. virescens* in which female larvae regulated both intake and post-ingestive processing of dietary protein on heavily protein-biased foods. That data indicated that on high protein foods, intake and post-ingestive processing adequately met tissue and storage needs. We interpreted their response to dietary protein level as a reflection of their typical larval diet in the field in which late instar larvae feed and grow well on the anthers of developing cotton floral buds (Shaver et al., 1977; Hedin et al., 1991). Cotton anthers contain the highest total amino acid levels and high carbohydrate levels, not including cellulose, compared to other cotton floral tissues (Shaver et al., 1977; Hedin and McCarty, 1990; Hedin et al., 1991). In contrast, *E. acrea* larvae are highly polyphagous and feed on the leaves of a number of hostplants (M.S. Singer, pers. comm.). Plant leaves are considered to be sources of high carbohydrate but low in nitrogen (Slansky and Scriber, 1985). Therefore, it is not surprising that *E. acrea* females attained their greatest protein- and carbohydrate-derived growth on diet 7P:35C, a P:C ratio closer to their natural larval diet.

Insects included in each replicate of experiment one (restricted to one diet) displayed different developmental patterns. On some diets, replicate one insects pupated

at the end of either their sixth or seventh-instar. In replicate two, all caterpillars pupated at the end of their sixth-instar. Replicate 1 insects consisted of progeny obtained from field collected late instar larvae that pupated and eclosed as adults under laboratory conditions (details given in Material & Methods). Caterpillars not used in the replicate 1 experiment were maintained on our wheatgerm-fortified artificial diet as standard laboratory colony. Replicate 2 insects consisted of progeny from adults obtained from this laboratory colony. Our first set of analyses compared replicate 1 and 2 insects that pupated at the end of their 6th instar stage. Overall, we found that replicate 2 females were larger at the start of their 6th instar larval stage, ate more food and consequently achieved greater protein and carbohydrate intake. Replicate 2 females also spent a shorter length of time during their last larval stage and, consequently, displayed faster MRGR compared to replicate 1 larvae (Table 2). These differences in consumption and performance resulted in replicate 2 females accumulating more protein and carbohydrate compared to replicate 1 female larvae. Differences in *E. acrea* progeny performance may reflect differences in the nutritional environment experienced by their parental stock. Examples of maternal influences on offspring performance are numerous and have been reviewed recently (Mousseau and Dingle, 1991; Reavey, 1992; Rossiter et al., 1993; Braby, 1994; Bernardo, 1996a, b; Fox and Czesak, 2000).

A second set of analyses focused on the developmental polyphenism we observed within replicate 1 insects. Our data suggest that the 6th and 7th instar cohorts may have started out on different developmental trajectories, possibly from time of hatching (although our experiments were not designed to examine this). All neonates used in replicate 1 of experiment 1 supposedly experienced similar environmental and nutritional conditions and only experienced a different nutritional environment upon entering a diet

treatment. We found that insects destined to pupate at the end of their 7th larval stage were significantly smaller upon entering their 6th larval stage, but initial fresh weights of the newly molted 7th instars were similar to initial weights of newly molted 6th instar cohort individuals. Thus, we suspect a critical size/mass threshold, similar to what has been well documented in *Manduca sexta* (Nijhout and Williams, 1974; Nijhout, 1975; Safranek and Williams, 1984), exists for *E. acrea* as well. Once newly molted 7th instars met this critical size/mass threshold, their developmental path enabled them to grow for a longer period of time (Higgins and Rankin, 1996). The 7th instar cohort developed longer during their 7th larval stage with females developing for a longer period of time overall (Table 4). Once on this developmental trajectory, the consumption and performance responses we observed, for the most part, were not surprising.

Overall, we found that the 7th instar cohort ate more food, and differences between the sexes were greater than were seen to occur between the sexes of the 6th instar cohort (Table 3, Fig. 2). This increased food consumption led to females eating more protein during their 7th instar stage than did the 6th instars during their 6th instar stage (Table 3, Figs. 3A and 3B). Furthermore, the 7th instar cohort ate more carbohydrate over their 7th larval stage compared to the 6th instar cohort, but no differences between the sexes were observed (Table 3, Figs. 3A and 3B). For the most part, the 7th instar cohort performed as one would expect given their commitment to an additional larval stage and feeding behavior as described above. We found no evidence that the 6th and 7th instar cohorts accumulated different amounts of protein during each of their last larval stages (Table 5, Fig. 7). This was surprising considering that the 7th instar cohort ate more protein overall (Figs. 3A and 3B). However, the 7th instar cohort accumulated more carbohydrate over their last larval stage than did the 6th instar cohort over their last larval stage (Fig. 7).

Many cases of developmental polyphenism have found a strong link between supernumerary molts by insects and an inadequate nutritional environment (Beck, 1950, 1971; Schmidt and Lauer, 1977; Jones et al., 1980; Klein and Beck, 1980; Weatherby and Hart, 1986; Shafiei et al., 2001). However, given that replicate 1 larvae were reared under the same environmental and nutritional conditions, prior to placement into experimental diet treatments, variation in progeny quality may be a better explanation (Mousseau and Dingle, 1991). Regardless of the reason for the differential developmental response by *E. acrea* progeny, if larvae have to molt into an extra instar, that developmental path enables them to grow for a longer period of time (Higgins and Rankin, 1996), thus affording them the opportunity to feed over a longer time. Consequently, this enables initially small insects to catch up and even surpass those that had started off bigger. This underlies a classic trade-off between the benefits of increased size (namely in increased fecundity) and the costs of a longer developmental time to reach a bigger size (namely an increased risk of predation / mortality) (Nylin and Gotthard, 1998).

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FIGURE CAPTIONS

Figure 1. (A) Frequency histogram of initial fresh weight of replicates 1 and 2 insects at the start of the 6th stadium in experiment 1. Shaded (females) and striped (males) portions indicate initial weight of larvae which completed their development in seven instars, unshaded indicate replicate 1 insects which completed development in six instars and stippled portions indicate replicate 2 insects which completed development in six instars. (B) Overall duration of time spent by replicates 1 and 2 larvae during their six (6th instar cohort and replicate 2) or six + seven instars (7th instar cohort). Stippled bars indicate stadium duration of 6th instar cohort males and females and unshaded indicate stadium duration of replicate 2 males and females. Striped bars indicate stadium duration of 7th instar cohort males and females (lower portion displays time spent during the 6th larval stage and upper portion displays time spent during the 7th larval stage).

Figure 2. Total dry weight of food eaten by 6th instar pupal groups of replicate 1 [females (■) and males ( )] and replicate 2 [females (●) and males (○)] insects. Total dry weight of food eaten by both males and females in replicate 1 7th instar pupal group is also shown (✱). Diets are labeled according to the % dry weight of protein (top numbers) and % dry weight of carbohydrate (bottom numbers).

Figure 3. (A) Bicoordinate plot of cumulative intake of protein and carbohydrate across the 6th stadium by replicate 1 6th instar cohort females (■), across the 6th stadium by replicate 1 7th instar cohort females (◆) and across the 7th stadium by replicate 1 7th instar cohort females (▲). (B) Bicoordinate plot of cumulative intake of

protein and carbohydrate across the 6th stadium by replicate 1 6th instar cohort males ( ), across the 6th stadium by replicate 1 7th instar cohort males (◊) and across the 7th stadium by replicate 1 7th instar cohort males (Δ). For Figures 3A and 3B the numbers at the end of each rail indicate the ratio of percent dry weight protein to carbohydrate, respectively, for each diet.

Figure 4. Bicoordinate plot of cumulative intake across the 6th stadium by replicate 2 females (●) and males (O). The boxed insert within overall plot shows the protein- and carbohydrate-derived growth for 4 of the 7 diets over the 6th stadium for the replicate 2 insects females (●) and males (O). Numbers at the end of each rail indicate the ratio of percent dry weight protein to carbohydrate, respectively, for each diet.

Figure 5. Carbohydrate and protein intake by *E. acrea* larvae (sexes are combined) allowed to selectively feed on two different diets. Solid lines represent expected intake if equal quantities of foods were eaten with each treatment. Symbols external to plot, at the end of each line denote a treatment. ◊: 7P35C + 28P14C; O: 21P21C + 35P7C; : 7P35C + 35P7C; Δ: 21P21C + 28P14C.

Figure 6. Bicoordinate plot of growth derived from dietary protein and carbohydrate across the 6th stadium for replicate 1 6th instar cohort and replicate 2 females (● ■ ▲ ◆) and males (O Δ ◊) for diets 7P:35C (circles), 21P:21C (squares), 28P:14C (triangles) and 35P:7C (diamonds). Solid lines join symbols for males and females on each diet with numbers indicating replicate.

Figure 7. Bicoordinate plot of growth derived from dietary protein and carbohydrate across the 6th stadium for replicate 1 6th instar cohort females (●■▲◆) and males (○ △ ◊) and across the 7th stadium for replicate 1 7th instar cohort females (●■) and males (○) for diets 7P:35C (circles), 21P:21C (squares), 28P:14C (triangles) and 35P:7C (diamonds). Solid lines join symbols for males and females on each diet with numbers indicating cohort.

Figure 1A

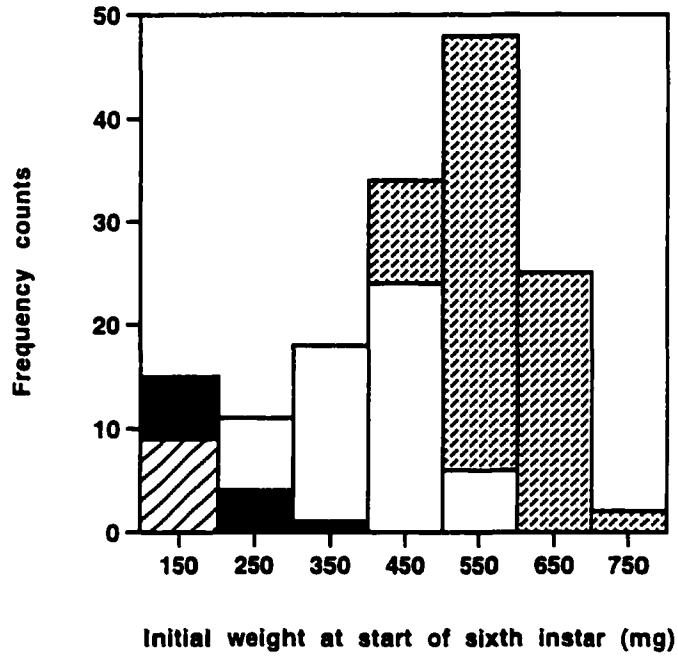
50
40
30
20
10
0
Frequency counts
150
250
350
450
550
650
750
Initial weight at start of sixth instar (mg)

Figure 1B

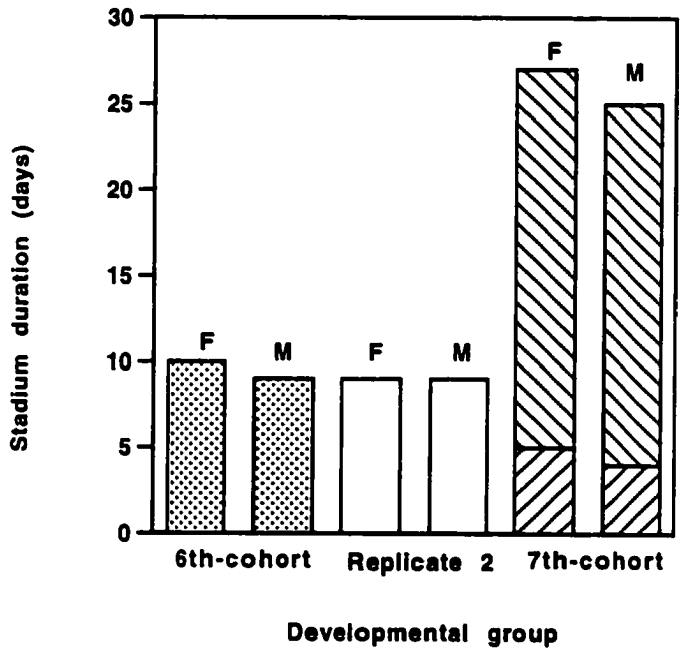
30
25
20
15
10
5
0
Stadium duration (days)
F
M
F
M
F
M
6th-cohort
Replicate 2
7th-cohort
Developmental group

Figure 2

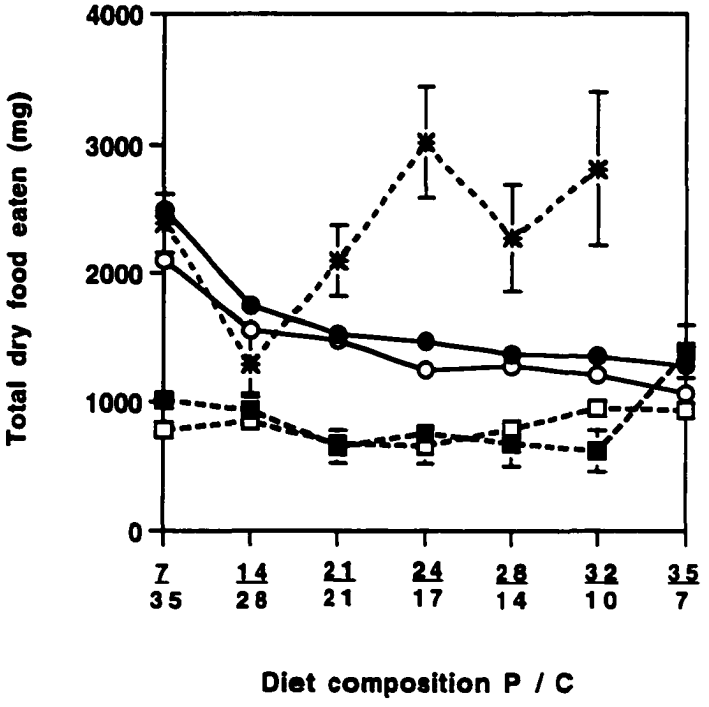
Total dry food eaten (mg)
4000
3000
2000
1000
0
7
35
14
28
21
21
24
17
28
14
32
10
35
7
Diet composition P / C

Figure 3A

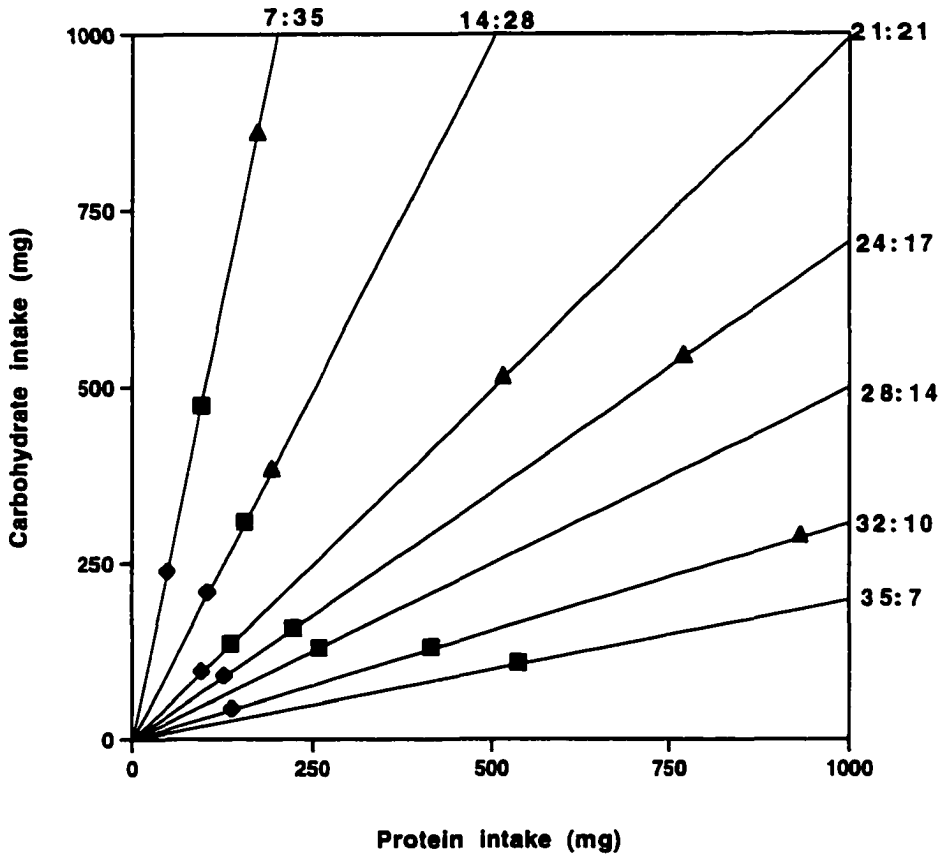
7:35
14:28
21:21
24:17
28:14
32:10
35:7
1000
750
500
250
0
0
250
500
750
1000
Carbohydrate intake (mg)
Protein intake (mg)

Figure 3B

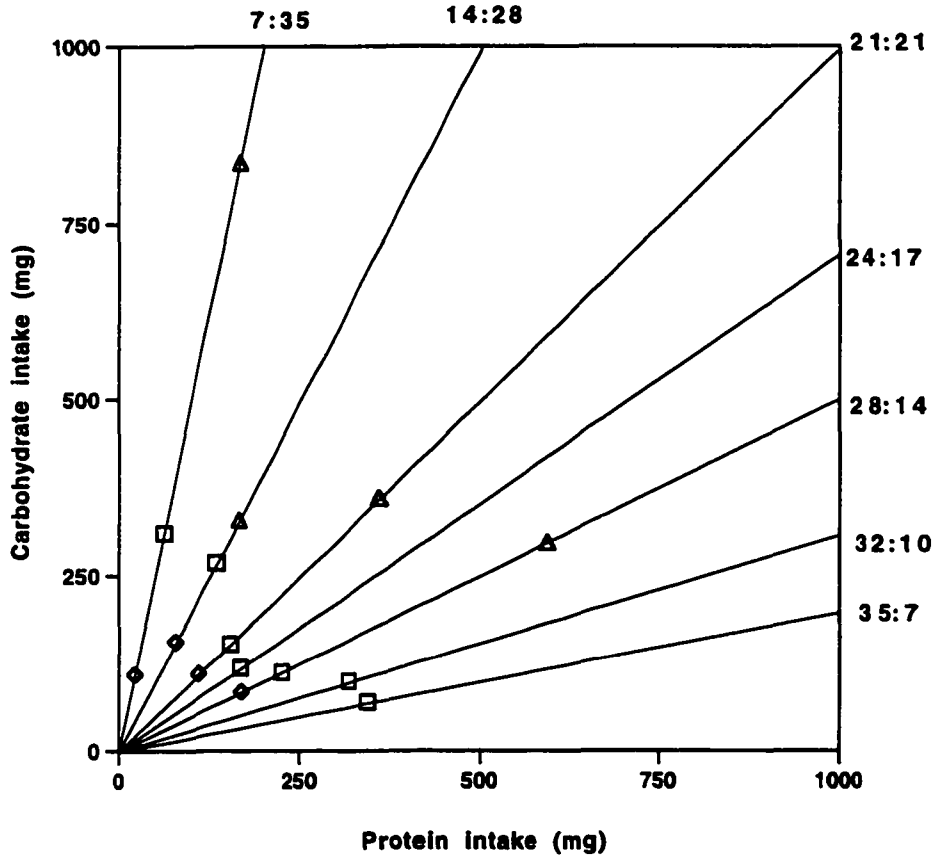
7:35
14:28
21:21
24:17
28:14
32:10
35:7
1000
750
500
250
0
Carbohydrate intake (mg)
0
250
500
750
1000
Protein intake (mg)

Figure 4

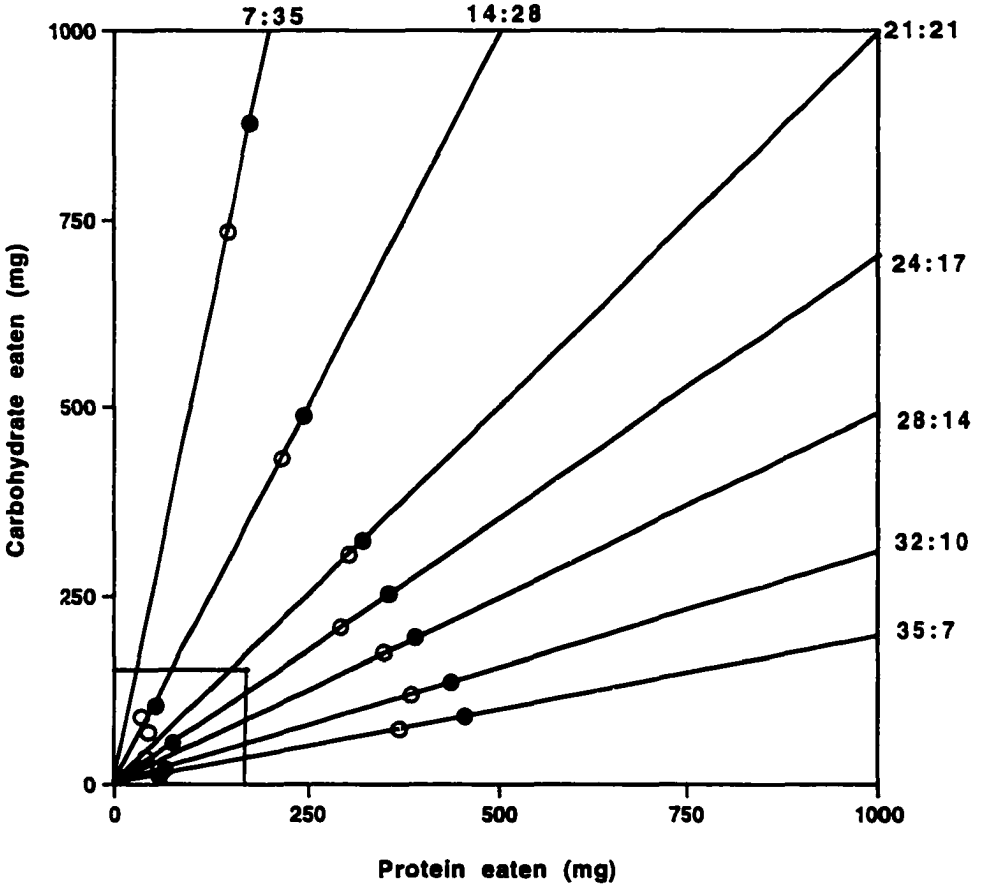
7:35
14:28
21:21
24:17
28:14
32:10
35:7
1000
750
500
250
0
Carbohydrate eaten (mg)
0
250
500
750
1000
Protein eaten (mg)

Figure 5

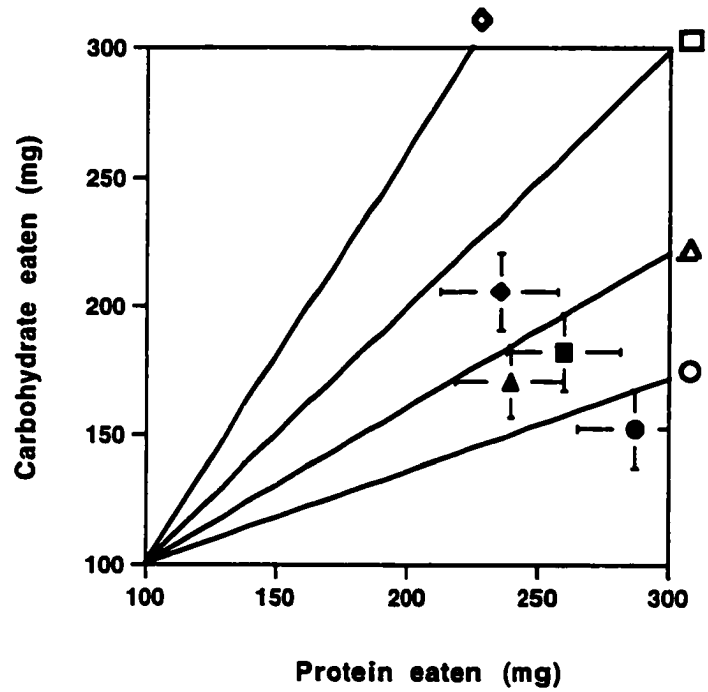
300
250
200
150
100
100
150
200
250
300
Carbohydrate eaten (mg)
Protein eaten (mg)

Figure 6

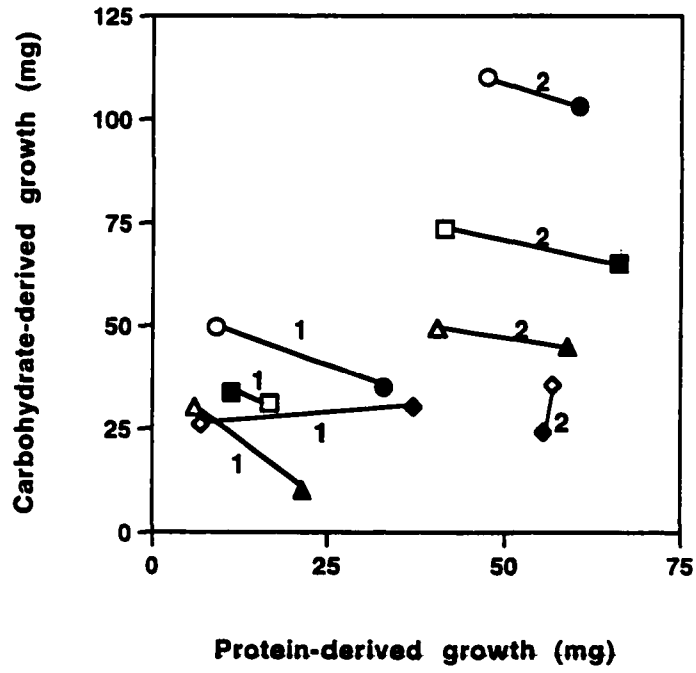
Carbohydrate-derived growth (mg)
125
100
75
50
25
0
0
25
50
75
Protein-derived growth (mg)
1
1
1
1
2
2
2
2

Figure 7

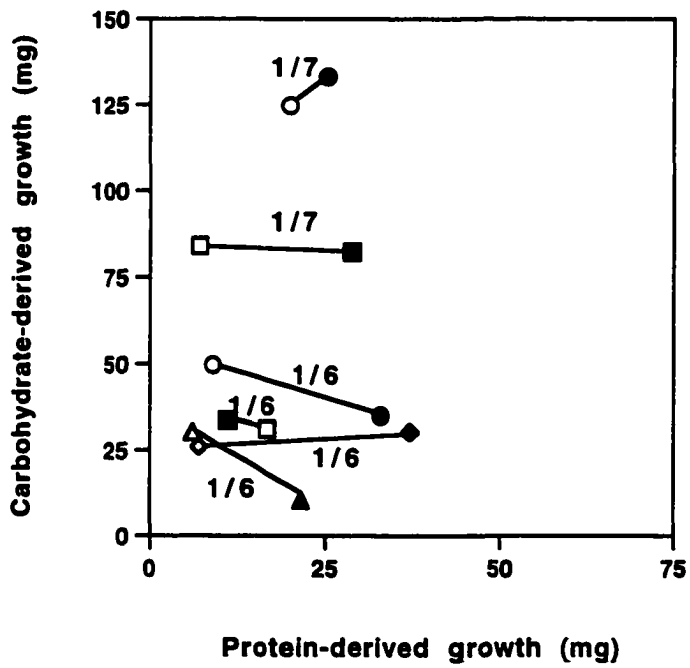
Carbohydrate-derived growth (mg)
150
125
100
75
50
25
0
0
25
50
75
Protein-derived growth (mg)
1/7
1/7
1/6
1/6
1/6
1/6

Table 1

F ratios and P values from ANCOVAs for consumption data in experiment 1.

Response Variables:	Total food eaten		Protein eaten		Carbohydrate eaten	
Effects (df)	F	P	F	P	F	P
Diet (6)	22.5	0.000	63.8	0.000	146.7	0.000
Sex (1)	9.9	0.002	13.2	0.000	4.2	0.041
Diet x Sex (6)	2.5	0.026	0.9	0.513	5.8	0.000
Replicate (1)	37.6	0.000	37.7	0.000	21.7	0.000
Covariate (1)	2.3	0.134	0.1	0.805	4.1	0.044
Error (178)						
Total (193)						

Table 2

F ratios and P values from ANCOVAs for performance data in experiment 1.

Response Variables:	Stadium duration		Initial fresh mass		MRGR		Protein growth (corrected) [c]	
Effects (df)	F	P	F	P	F	P	F	P
Diet (6)[a]	10.1	0.000	0.5	0.794	17.5	0.000	9.6	0.000
Sex (1)	13.7	0.000	30.5	0.000	3.6	0.060	51.9	0.000
Diet x Sex (6)[a]	0.5	0.831	1.4	0.199	0.6	0.721	1.4	0.237
Replicate (1)	5.8	0.017	211.7	0.000	144.2	0.000	82.5	0.000
Covariate (1) [b]	1.2	0.265			36.9	0.000	35.6	0.000
Error (178) [c]								
Total (193) [d]								

[a] For response variable protein growth, main effects Diet and Diet x Sex were associated with 3 degrees of freedom. [b] The covariate for protein growth (corrected) was protein eaten. For all other response variables, initial fresh mass served as the covariate. [c] For response variables initial fresh mass and protein growth (corrected), Error was associated with 179 and 94 degrees of freedom. [d] For response variable protein growth, Total degrees of freedom was 103. [c] Protein-derived growth is corrected for protein intake (covariate).

Table 3

F ratios and P values from ANCOVAs for consumption data in experiment 1 for replicate 1 insects only.

Response Variables:	Total food eaten (6/6)[a]		Protein eaten (6/6)[a]		Carbohydrate eaten (6/6)[a]		Total food eaten (6/7)[b]		Protein eaten (6/7)[b]		Carbohydrate eaten (6/7)[b]	
Effects (df)	F	P	F	P	F	P	F	P	F	P	F	P
Diet (6)	3.3	0.007	21.9	0.000	25.5	0.000	1.7	0.132	15.8	0.000	15.3	0.000
Sex (1)	2.9	0.089	2.0	0.158	2.7	0.105	7.4	0.008	8.4	0.005	2.6	0.113
Diet x Sex (6)	1.8	0.099	2.8	0.016	1.2	0.294	0.623	0.711	1.8	0.107	0.8	0.504
Cohort (1)	9.7	0.003	4.1	0.048	12.9	0.001	81.7	0.000	51.3	0.000	63.8	0.000
Cohort x Sex (1)	0.1	0.789	0.1	0.707	0.0	0.954	3.7	0.057	5.3	0.024	0.7	0.390
Covariate (1)	0.1	0.735	0.2	0.696	0.0	0.856	0.2	0.667	0.1	0.751	1.1	0.292
Error (58)												
Total (74)												

[a] 6/6 refers to analyses comparing the 6th instar stages of the 6th and 7th instar cohort and of insects that pupated at the end of their 7th instar stage.

[b] 6/7 refers to analyses comparing the 6th instar cohort with the 7th instar cohort over their final larval stages. Note: the 7th instar cohort data includes data only from their 7th larval stage and does not include data from their 6th larval stage.

Table 4

F ratios and P values from ANCOVAs for developmental data in experiment 1 for replicate 1 insects only.

Response Variables:	Stadium duration (6/6)[a]		Initial fresh mass (6/6)[a]		MRGR (6/6)[a]		Stadium duration (6/7)[b]		Initial fresh mass (6/7)[b]		MRGR (6/7)[b]	
Effects (df)	F	P	F	P	F	P	F	P	F	P	F	P
Diet (6)	1.5	0.178	1.6	0.155	1.4	0.215	1.7	0.138	1.1	0.343	1.5	0.177
Sex (1)	0.6	0.433	9.3	0.003	3.0	0.086	7.4	0.008	10.8	0.002	0.3	0.551
Diet x Sex (6)	0.5	0.748	2.5	0.031	1.3	0.254	0.8	0.543	1.7	0.134	1.3	0.261
Cohort (1)	19.8	0.000	120.2	0.000	53.6	0.000	118.7	0.000	1.9	0.162	12.2	0.001
Cohort x Sex (1)	2.1	0.155	0.0	0.967	1.1	0.296	0.8	0.360	0.4	0.515	0.0	0.935
Covariate (1)	1.8	0.185			4.1	0.048	5.1	0.026			2.4	0.124
Error (58)[c]												
Total (74)												

[a] 6/6 refers to analyses comparing the 6th instar stages of the 6th and 7th instar cohort and of insects that pupated at the end of their 7th instar stage. [b] 6/7 refers to analyses comparing the 6th instar cohort with the 7th instar cohort over their final larval stages. Note: the 7th instar cohort data includes data only from their 7th larval stage and does not include data from their 6th larval stage. [c] For response variable initial fresh mass Error was associated with 59 degrees of freedom.

Table 5

F ratios and P values from ANCOVAs for performance data in experiment 1 for replicate 1 insects only.

Response Variables:	Protein growth (corrected) [a]		Carbohydrate growth (corrected) [a]	
Effects (df)	F	P	F	P
Diet (3)	1.7	0.204	1.3	0.299
Sex (1)	3.8	0.063	0.0	0.863
Diet x Sex (3)	0.4	0.742	0.2	0.870
Cohort (1)	1.4	0.246	16.9	0.000
Cohort x Sex (1)	0.1	0.805	1.8	0.191
Covariate (1) [b]	5.4	0.030	6.1	0.021
Error (24)				
Total (34)				

[a] Protein-derived and carbohydrate-derived growth are corrected for protein and carbohydrate intake (covariates). [b] The covariates for protein and carbohydrate growth (corrected) was protein and carbohydrate intake, respectively.

APPENDIX C

DYNAMICS OF STORAGE PROTEIN LEVELS IN RESPONSE TO VARIATION IN DIETARY PROTEIN LEVELS

Dynamics of storage protein levels in response to variation in dietary protein levels

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Abstract

Storage proteins have been found to play a major role in insect metamorphosis and egg production and are accumulated during the actively feeding larval stage. Yet, few studies have focused on how nutrition affects storage protein levels. Three storage proteins were identified in male and female *Heliothis virescens* pupae, one arylphorin and two high-methionine hexamers. Storage proteins were quantified in early pupae and in pharate adults. Storage protein levels peaked in 48-hrs-old pupae and were more abundant in females across all stages. Both male and female pharate adults retained a portion of total storage protein levels and females retained greater levels overall. For females, post-eclosion protein reserves will undoubtedly be used toward egg manufacturing, while the role of protein reserves in males remains speculative. In our previous study of *H. virescens* larvae, we found that protein-derived growth in females progressively increased as dietary protein levels increased. Our present data show that levels of storage protein also increased progressively along with dietary protein levels. This suggests that females allocated protein, in excess of adult tissue formation needs,

toward storage protein. Our study is the first to demonstrate how dynamic storage protein levels can be in face of varying levels of dietary protein.

Keywords: Storage protein; Hexamerin; Female nutrition; Egg production; *Heliothis virescens*

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1. Introduction

Female reproductive success is nutrient-limited (Wheeler, 1996). In an earlier study we found that female *H. virescens* larvae increased consumption of high protein foods leading to greater protein intake, and, consequently, to greater protein-derived growth (Telang et al., 2001). In the present study we examine the dynamics of storage protein levels in response to variation in nutritional environment, especially with respect to dietary protein levels. Storage proteins are a family of insect proteins synthesized and secreted by the fat body during the active feeding stage of larvae. These proteins reach high concentrations in the hemolymph during this stage and are then sequestered by both the fat body and hemolymph during pupal development (Telfer and Kunkel, 1991).

The role of storage proteins in insect metamorphosis has been well-characterized (Telfer and Kunkel, 1991). Only recently have researchers focused attention on the role of hexamerins in female egg production (Pan and Telfer, 1996; Wheeler and Buck, 1996; Seo et al., 1998; Pan and Telfer, 1999; Wheeler et al., 2000; Pan and Telfer, 2001). Many of the studies regarding Lepidoptera have focused on species in which egg production and metamorphosis overlap making it difficult to assess the differential roles of hexamerins (Tojo et al., 1980; Ogawa and Tojo, 1981; Riddiford and Law, 1983; Pan and Telfer, 1996). The role of storage proteins in species in which females do not synthesize egg proteins until after eclosion has only recently been explored (Wheeler et al., 2000; Pan and Telfer, 2001). Both studies reported that females eclosed with some of their larval protein reserves remaining, presumably to be used in yolk protein synthesis. Like *Plutella* and *Danaus plexippus* (Wheeler et al., 2000; Pan and Telfer, 2001), *H. virescens* females begin vitellogenesis after eclosion (Zeng et al., 1997) and we predicted that the phenology of storage protein levels would be similar to that of *Plutella*. Few

studies have focused on how nutrition affects storage protein dynamics and these have done so by comparing fed versus starved insects (Tojo et al., 1981; Riddiford and Hice, 1985; Tojo et al., 1985; Kumaran et al., 1987; Webb and Riddiford, 1988). However, insects are more likely to face a situation in which their foods vary quantitatively and qualitatively. Our experimental design allowed us to manipulate the nutritional environment of larvae and measure protein reserves in response to varying levels of dietary protein.

2. Materials and methods

2.1. Animals

Egg masses of *H.virescens* were obtained from the Western Cotton Research Laboratories in Phoenix, Arizona and maintained in incubators at 25°C and a 12:12 h light:dark cycle. Upon hatching, neonates were placed individually in 30ml plastic rearing cups fitted with plastic lids to maintain moisture and were maintained at the same temperature and light regime as above. Larvae were reared on experimental diets fortified with wheatgerm to promote good growth and development since they were unable to develop properly when reared on strict chemically defined diets from hatching. Upon entry to their fifth, and last instar, insects were weighed, transferred to 150ml plastic cups fitted with plastic lids and randomly assigned to different wholly synthetic diets.

2.2. Synthetic diets

Experimental diets were the same as those used in our previous study (Telang et al., 2001). The protein source consisted of vitamin-free casein and the digestible carbohydrate was sucrose. All diets were agar based (3% solution w/v) and contained

54% cellulose (non-nutritive bulking agent) and 4% essential micronutrients (vitamins, cholesterol, salts and linoleic acid). The remaining 42% consisted of protein and carbohydrate at various levels. We chose 4 diets, based on nutrient-derived growth performance of *H. virescens* in our previous study, with the following dry weight percentages of protein (P) to carbohydrate (C): 7P:35C, 21P:21C, 28P:14C and 35P:7C. Our wheatgerm fortified diet, on which all larvae were reared to the fifth stage and used in the first experiment, utilized the same ingredients as in the experimental diets except that it consisted of 74% wheatgerm, 14% casein and 7.4% sucrose. Wheatgerm diets still contained 4% essential micronutrients but no cellulose was added. Each insect was given a fresh block of diet daily in all experiments and allowed to feed ad lib.

2.3 Experiments

Our first experiment was designed to gain phenological data on *H. virescens* storage protein levels. For this experiment, caterpillars remained on the wheatgerm enhanced diet throughout their larval period. We collected 12, 24, 48 hour old pupae, as well as pharate adults. The age of pupae was relative to that of a freshly pupated individual that had just shed its last larval cuticle and was considered "0" hours old. We determined from this experiment that storage protein level was constant during the pupal period but decreased substantially in pharate adults. Consequently, in our second experiment we sought to determine storage protein levels in response to variation in dietary protein levels in 48-hour-old pupae.

2.4 Sample preparation and electrophoresis

Upon pupation, insects were sexed, killed by freezing and later freeze dried until no further weight loss occurred. Freeze dried pupae were individually ground using a mortar and pestle cooled with liquid nitrogen. Pupae were subsequently dried again to

remove any water that may have condensed onto samples during grinding. Subsamples of 5-7 mg were homogenized in Tris-buffered saline (20 mM Tris, 150 mM NaCl, 5 mM EDTA, pH 7.5) containing the following protease inhibitors: leupeptin, antipain, chymostatin, aprotinin (all at 17 µg/ml), 1.7 µg/ml pepstatin A and 1 mM 4-(2-Aminoethyl) benzenesulfonyl Fluoride (AEBSF, an irreversible serine protease inhibitor). Samples were ground in 1.5 ml microfuge tubes with a pestle attached to a rotating shaft attached to a variable speed motor. Each sample was ground for 60 s at about 250 rpm. Samples were then centrifuged at 12,000g for 15 min at 4°C. For the phenology study, aliquots of the supernatant from all samples were diluted 1:10 in Tris-buffered saline solution, with the exception that supernatant from pharate female and 12 hrs old male pupae were diluted 1:5 and pharate male samples were undiluted. For the experimental diet study, aliquots of the supernatant from all samples were diluted 1:10. For all samples in both experiments, 10 µl aliquots of diluted supernatants were mixed with 20 µl sample loading buffer and applied to gels.

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out following the method of Laemmli (1970), adapted to 6-15% gradient slab gels. Staining and destaining procedures are given in Wheeler and Buck (1995). Molecular weights of proteins in SDS-PAGE were estimated using standards in the high molecular weight calibration kit (Biorad). To quantitate storage proteins, gels were scanned at 633 nm using a laser densitometer (LKB Ultrascan XL). Standard curves were generated for each experiment using bovine serum albumin (BSA) of known quantities from 0.2 – 4.0 µg. Internal standards of 1.0 and 3.0 µg BSA were included on each gel run for quantitative analysis to correct for gel to gel variation. Because storage proteins did not separate well on the 10x8 cm SDS gels (Hoefer Mighty Small II units), it

was only possible to measure densities of particular bands or combination of bands. However, we were able to estimate the amount of storage protein present at different stages of development (phenology study), at various dietary protein levels (experimental diet study) and in both sexes by totaling the densities of all bands in the region of interest.

2.5 Western blots

To better separate storage proteins, samples were run on a 20x16 cm gel slab unit (BRL. Model V16). We chose a representative sample of *H. virescens* female and male pupae from our phenology study and prepared them as described above except that a 1:12 dilution of supernatant with sample loading buffer was used and 30 µl was loaded onto each lane. Storage proteins were separated by SDS-PAGE and blotted onto nitrocellulose membrane. After blotting, the membrane was rinsed well, air-dried and stored at –70°C. Proteins from *H. virescens* were tested for cross-reactivity to several Lepidopteran based antibodies. A polyclonal antibody against arylphorin from *Manduca sexta* was obtained from J.H. Law (Ryan et al., 1984). M.L. Pan provided monospecific antibodies to moderately methionine-rich hexamerin and to arylphorin and also a polyclonal antibody against very methionine-rich hexamerin, all of which were isolated from *Hyalophora cecropia* (Pan and Telfer, 2001). The blot membrane was cut into strips with each strip containing both female and male sample lanes. Each blot strip was probed with a 1:1000 dilution of each primary antibody, incubated with a 1:3000 dilution of goat anti-rabbit secondary antibody and detected with alkaline phosphatase conjugate.

2.6 Amino acid analysis

A section of the SDS-PAGE gel used for Western blotting was used to transfer proteins onto a PVDF (polyvinylidene difluoride) membrane (Problott®). The PVDF membrane was rinsed, air-dried, kept in a container flushed with nitrogen gas and stored

at –70°C. The bands of interest were excised and analyzed at the University of Arizona (Laboratory for Protein Sequencing and Analyses) using an ABI 420A/H amino acid analyzer. Proteins were hydrolyzed by vapor-phase using 6N HCL at 155°C for 1hr 15min, then derivatized with phenylisothiocyanate (PITC) to form phenylthiocarbamyl amino acid derivatives (PTC-AA) which were extracted and transferred to an on-line HPLC for analysis at 254nm (W. Clark, pers. comm.).

3. Results

3.1 Characterization of storage proteins

We identified 3 storage proteins based on their positions in SDS-PAGE and their cross-reactivity to antibodies generated against known hexamerins in other Lepidopteran species. In SDS-PAGE (Figs. 1 and 2), storage protein subunits were similar in size and subsequently migrated to similar positions. Gels resolved three bands that showed relative mobility between 66-97 kDa. Both *H. cecropia* and *M. sexta* antibodies to arylphorin recognized the intermediate band. Amino acid analysis of this protein confirmed it was an arylphorin, with 14.6 Mol% (in males) and 15.8 Mol% (in females) of total amino acids made up of tyrosine and phenylalanine (Table 1). The uppermost and lowermost bands cross-reacted with antibodies to both moderately and very methionine-rich hexamerins, thus precluding any further identification. The molecular weights estimated for subunits by SDS-PAGE are consistent with a hexameric structure.

3.2 Phenology of storage proteins

Storage protein pattern was similar between males and females although all hexamerins were less abundant in males (Fig. 1). Total amount of storage protein per individual differed between the sexes (ANOVA $P = 0.010$) with females containing a

greater total amount of protein across all pupal stages (ANOVA $P = 0.464$ for interaction) (Fig. 3). Weight-specific level of storage protein also differed significantly between the sexes (ANOVA $P < 0.0001$), likewise females stored more weight-specific level of storage protein across all pupal stages (ANOVA $P = 0.971$ for interaction) (Fig. 3). There was strong evidence that both weight-specific level of storage protein and total amount of storage protein per individual differed among stages (ANOVA $P < 0.0001$ in both cases) (Fig. 3). Further analyses using *a posteriori* multiple comparison of means revealed that pharate adults contained less storage protein than did pupae in both absolute and weight-specific amounts in both sexes (rejection at $\alpha = 0.05$, Tukey-Kramer HSD).

3.3 Storage protein levels in response to variation in dietary protein

However, dietary protein levels affected the overall pattern of storage protein accumulation by both sexes (Fig. 2). Both total amount of storage protein per individual and weight-specific level of storage protein differed between the sexes (ANOVA $P < 0.0001$ in both cases) with levels of both being greater in females (Fig. 4). Females weighed the same or slightly more than males on all other diets, although this was not statistically significant (ANOVA $P = 0.227$). Regardless of the sexes being of similar size, females were compositionally more protein-rich (Fig. 4). Both weight-specific level of storage protein and total amount of storage protein per individual was influenced by diet (ANOVA $P < 0.0001$ in both cases) (Fig. 4). *A posteriori* multiple comparison of means revealed that individuals on the lowest protein diet (7P:35C) had less absolute and weight-specific amounts of storage protein than did individuals on the other diets (rejection at $\alpha = 0.05$, Tukey-Kramer HSD).

We were unable to quantitate the three storage proteins separately due to the size of small format gels. In many samples, the lower most and intermediate bands were

scanned and read by our densitometer as one band. Consequently, we analyzed resulting data as either bands 1+2 (lower most and intermediate) or band 3 (upper most). Weight-specific levels of both bands 1+2 and band 3 differed between the sexes (ANOVA $P < 0.0001$ and $P = 0.005$, respectively) and both responses were significantly affected by dietary composition (ANOVA $P < 0.0001$ in both cases). Insects made similar levels of band 3 across all diets but they made much less of bands 1+2 on the low protein diet compared to other diets (Fig. 5). Clearly, bands 1+2 showed a stronger response to dietary composition and larger differences between the sexes compared to band 3 (Fig. 5). On the higher protein diets, weight-specific levels of storage protein in females were more than 50% that of males and only around 25% on the 7P:35C diet. In addition, levels of bands 1+2 largely accounted for weight-specific levels of storage protein (compare Figs. 4 and 5). Levels of band 3 accounted for a greater proportion of weight-specific storage protein in males compared to females (compare Figs. 4 and 5).

4. Discussion

4.1 Characterization of Heliothis storage protein in relation to other Lepidopteran hexamers

In male and female *Heliothis virescens*, three putative storage proteins have been identified based on their positions in SDS-PAGE and their affinity to antibodies generated against known hexamerins in other Lepidopteran species. Based on SDS-PAGE, storage protein subunits were found to be similar in size as they migrated to similar positions (Figs. 1 and 2). We identified the intermediate protein band as an arylphorin, based on its immunoreactivity with two separate antibodies to arylphorin from both *Hyalophora cecropia* and *Manduca sexta*, as well as its amino acid

composition. The uppermost and lowermost bands were identified as high-methionine (HM) hexamerins based on immunoreactivity with two antibodies to either moderately (M-MtH) or very methionine-rich hexamerins (V-MtH) (Pan and Telfer, 2001). Cross-reactivity of both bands to both antibodies precluded any further identification. The HM hexamerins were found in both male and female *H. virescens* pupae.

4.2 Phenological profile of storage protein levels in Heliothis pupae

Storage proteins were present at an early pupal age in both male and female pupae but females contained more absolute amounts and weight-specific levels of storage protein compared to males across all pupal stages examined (Fig. 1). A significant amount of weight-specific level of storage protein, and possibly total amount of protein per individual, remained in female pharate adults (Fig. 3). Reduction of storage protein levels in females was the same as in males (i.e., they both used similar amounts in producing adult tissues) but females retained more because of higher initial levels. Of the total weight-specific levels of storage protein detected in 48 hrs old pupae, only 40% remained in female pharate adults. Storage protein retention was also observed in *Plutella xylostella* (Wheeler et al., 2000). Like *P. xylostella*, *H. virescens* initiates vitellogenesis and oocyte production soon after eclosion (Zeng et al., 1997) so that remaining protein presumably contributes to egg provisioning. We did not measure post-eclosion storage protein or vitellogenin levels in *H. virescens* but suspect that phenology would be similar to that of *Plutella* in which vitellogenin is not detected until the adult stage when egg-laying begins. Surprisingly, storage protein was found to be remaining in male pharate adults, although at lower levels compared to females (Fig. 3). Even the few

systems in which post-eclosion levels of storage protein has been measured report that males of most species eclose with their reserves depleted (Wheeler et al., 2000; Pan and Telfer, 2001). The only exception we are aware of is that of male Monarch butterfly (*Danaus plexippus*) which was found to eclose with low reserves of HM hexamers (Pan and Telfer, 2001). What role post-eclosion storage protein may play in males has not been looked at. An obvious role would be as a source of nutrients to donate to females during mating (Boggs and Gilbert, 1979; Boggs, 1981b; Boggs and Watt, 1981; Marshall, 1982; Zeh and Smith, 1985; Marshall and McNeil, 1989).

Phenological data also indicated that the three storage proteins were differentially utilized during metamorphosis (Fig. 1). Most of the uppermost protein, one of the putative HM hexamers, was depleted and so most of the remaining storage proteins in pharate adults consisted of the second HM hexamer and arylphorin. In addition to our findings, depletion of specific hexamers has also been reported in other systems in which egg development occurs in adults. For example, both *D. plexippus* and *Plutella* females preferentially exhausted arylphorin over HM hexamers during metamorphosis (Wheeler et al., 2000; Pan and Telfer, 2001). However, data on *D. plexippus* also showed that both males and females used a large proportion of pupal HM hexamers during metamorphosis, clearly toward somatic tissue development (Pan and Telfer, 2001). The high aromatic amino acid content of arylphorins suggests they play a role in cuticle formation (Kanost et al., 1990). Our data suggests that, unlike what is considered typical for most lepidopterans, *H. virescens* pupae utilized some of both HM hexamers and arylphorins during metamorphosis but preferentially depleted one of the HM hexamers. This result suggests that remaining levels of arylphorin and one of the HM hexamers would be used toward egg production in *H. virescens*. Evidence in support of arylphorin utilization

toward egg production comes from three very different systems. First, a stock of mutant *Drosophila melanogaster* were found to be deficient in larval serum protein (LSP1), an arylphorin-like storage protein in that it has a high content of aromatic amino acids. The LSP1-deficient mutants produced fewer eggs, many of which had defective egg chorion, but pupal survival was not affected (Roberts, 1987). Second, gypsy moth, *Lymantria dispar*, females synthesize arylphorins throughout larval development, with maximal levels reached during their last larval instar (Karpells et al., 1990), and synthesize vitellogenin from their last larval instar through pupal development (Lamison et al., 1991). Female gypsy moths lack HM hexamers (Lamison et al., 1991) and data suggest that arylphorin may furnish vitellogenesis during the pupal stage (Wheeler et al., 2000). Third, arylphorin was found to be as effective as a HM hexamer in providing for vitellogenesis and chorion formation in pharate adult *Actias luna* moths (Pan and Telfer, 1996). Lastly, amino acid analyses of *H. virescens* storage proteins revealed that both putative HM hexamers have relatively high aromatic amino acid content (11.7 Mol% for the lower-most band and 8.3 Mol% for the upper-most band) and perhaps such levels suffice for metamorphic needs of *H. virescens* pupae (Table 1).

4.3 Dietary levels of protein influences storage protein levels

Previously we found that female H. virescens larvae exhibited greater protein-derived growth on high protein diets (Telang et al., 2001). The present study was conducted to examine more closely the functional aspect of the total protein accumulation previously measured. Our data clearly show that females responded to increasing levels of dietary protein by storing more protein protein (Fig. 4). Few studies have looked at how nutrient supply affects storage protein levels and most have focused on how storage

protein synthesis responds under fed versus starved conditions (Tojo et al., 1981; Riddiford and Hice, 1985; Tojo et al., 1985; Kumaran et al., 1987; Webb and Riddiford, 1988). In *B. mori*, both HM hexamerin and arylphorin synthesis was negatively affected under conditions of nutrient deprivation (Tojo et al., 1981). This is not surprising since both storage proteins are utilized toward metamorphosis and egg development over a similar time range (Tojo et al., 1980; Ogawa and Tojo, 1981). Alternatively, in both *M. sexta* and *Spodoptera litura* only synthesis of arylphorin was dependent on nutrient supply (Riddiford and Hice, 1985; Tojo et al., 1985; Webb and Riddiford, 1988). Our results indicated that *H. virescens* increased production of the putative arylphorin and one of the HM hexamers (bands 1+2) in response to increasing levels of dietary protein while production of the other putative HM hexamer (band 3) responded to a lesser degree (Fig. 5). Weight-specific storage protein was composed largely of bands 1+2 so that these particular hexamers undoubtedly contributed significantly to storage protein levels in females but band 3 contributed more to storage protein levels in males (compare Figs. 4 and 5). In males, bands 1+2 contributed around 65% of weight-specific storage protein but these proteins contributed around 95% of weight-specific storage protein in females, indicating a large difference in synthesis (Fig. 5).

Phenology data show that female pharate adults, which are a few hours shy of eclosion, retained, on average, 40% of weight-specific levels of storage protein detected in 48 hrs old pupae. If we apply this figure to those insects reared on experimental diets, females feeding on the highest protein diet (35P:7C) should retain approximately 95 µg of storage protein / mg insect body weight (a total of 2580 µg storage protein). Females feeding on the lowest protein diet would retain only about 54 µg of storage protein / mg insect body weight (a total of 1207 µg storage protein). In our previous study of *H.*

virescens larvae, we found that protein-derived growth in females progressively increased as dietary protein levels increased. However, our present data show that levels of storage protein were similar among balanced and protein-biased diets. This suggests that females allocated protein, in excess of storage protein needs, toward adult tissue formation. Increased protein reserves can directly affect female adult reproductive output (Boggs, 1981a) and perhaps indirectly through increased longevity. Overall, a distinct fecundity advantage may go to female *H. virescens* feeding on high protein foods. However, for insects that feed during both their juvenile and adult stages, reproductive output potentially relies on both larval- and adult-derived nutrients (Boggs, 1981a). The majority of Lepidoptera feed on nectar as adults, a diet always considered to be deficient owing to the lack of abundant amino acids (Slansky and Scriber, 1985). However, a recent study on a hawkmoth (*Amphion floridensis*) reported that almost 42.3% of the amino acids in egg protein were essential and were derived exclusively from larval diet. In contrast, non-essential egg amino acids were synthesized by obtaining carbon skeletons from dietary sugars, obtained during adult feeding, and using an endogenous source of amine nitrogen (O'Brien et al., In Press). These researchers suggest that female egg production may ultimately be constrained by the availability of essential amino acids since these cannot be synthesized by animals and must be obtained from diet. Interestingly, amino acid analyses of *H. virescens* storage protein found that, on average, all three hexamers contained 50 Mol% essential amino acids (Table 1), a figure similar to that observed in many other lepidopteran storage proteins (Seo et al., 1998 see their Table 1). The dietary source of protein in our experiments was vitamin-free casein, analysis of that shows it to contain 49.3 Mol% essential amino acids (ICN technical support). In the field, late instar *H. virescens* feed on cotton anthers, which are protein-rich and have the

highest amino acid content relative to other cotton floral tissues. Amino acid analysis of cotton anthers showed that the same 12 essential amino acids indicated in our Table 1 comprise 52.1% of total amino acids detected in anthers. This indicates that the larval diet of *H. virescens* may not be limiting with respect to these important amino acids (Hedin and McCarty, 1990; Hedin et al., 1991).

In summary, our data show that both male and female pharate adults retain a portion of total storage protein levels although females retained greater levels overall. For females, post-eclosion protein reserves will undoubtedly be used toward egg manufacturing, while the role of protein reserves in males can only be speculated on. In our previous study of *H. virescens* larvae, we found that females increase consumption, and rely on efficient post-ingestive processing, on high protein diets leading to a greater protein intake compared to males. Our present data show that females also increase protein storage on high protein foods. Our study is the first to demonstrate how dynamic storage protein levels can be in face of varying levels of dietary protein.

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FIGURE CAPTIONS

Figure 1. Developmental changes in males and females, during the first 48h of pupal life to nearly eclosed pharate adults, in the level of storage protein (SDS-PAGE). Lanes containing female pupal samples represented 1.2-1.4% of single individuals and that of male pupal samples represented 1.3-1.8% of single individuals. Lanes are marked on top by pupal age (12, 24 or 48hrs old) or stage (PhA, pharate adult). The two dots on the right indicate molecular weight standards of 66 and 97 kDa.

Figure 2. Differences in storage protein levels (SDS-PAGE) in 48h old male and female pupae in response to varying levels of dietary protein. Lanes containing female pupal samples represented 1.5-1.8% of single individuals and that of male samples represented 1.7-1.9% of single individuals. Lanes are marked on top by %dietary protein found in the following diets (P:C ratio): 7P:35C, 21P:21C, 28P:14C and 35P:7C. The two dots on the right indicate molecular weight standards of 66 and 97 kDa.

Figure 3. Weight-specific and total storage protein levels in females (●) and males ( ) during the first 48h of pupal life and in eclosed pharate adults of *H. virescens*, based on quantitative SDS-PAGE of storage protein bands (totaled) produced by partial, homogenized body extracts. Error bars represent standard errors.

Figure 4. Weight-specific and total storage protein levels, in 48h old females (●) and males ( ) reared on diets varying in %dietary protein. Values are based on quantitative SDS-PAGE of storage protein bands (totaled) produced by partial, homogenized body

extracts. Error bars represent standard errors. Diets are labeled according to P:C ratio: 7P:35C, 21P:21C, 28P:14C and 35P:7C.

Figure 5. Weight-specific storage protein in 48h old females (●) and males ( ) reared on diets varying in %dietary protein. Values are based on quantitative SDS-PAGE of either storage protein bands 1+2 (solid line) or band 3 (dashed line) produced by partial, homogenized body extracts. Diets are labeled according to P:C ratio: 7P:35C, 21P:21C, 28P:14C and 35P:7C.

Figure 1

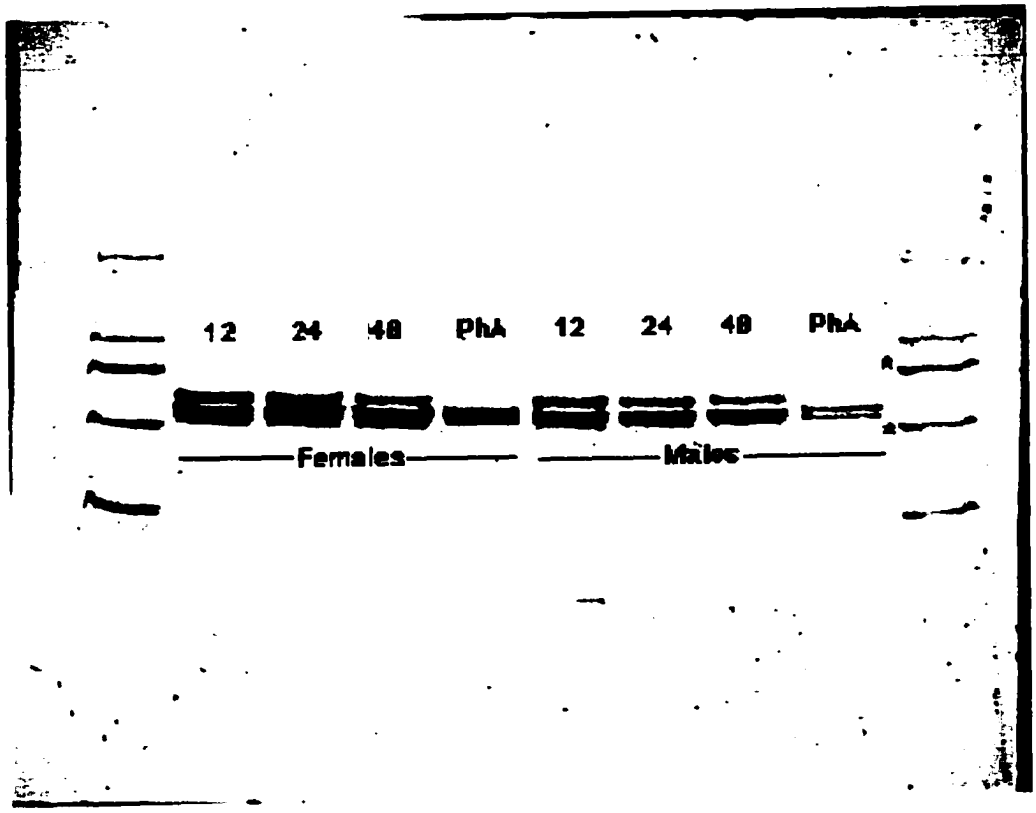

Figure 2

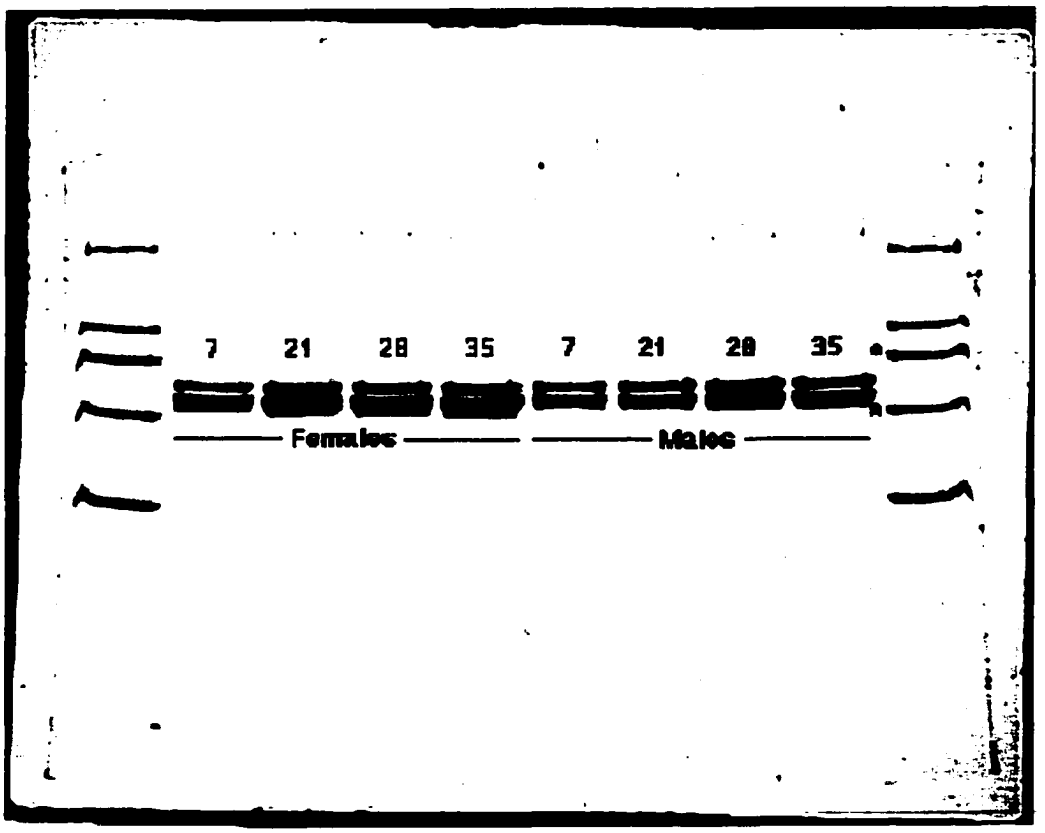

Figure 3

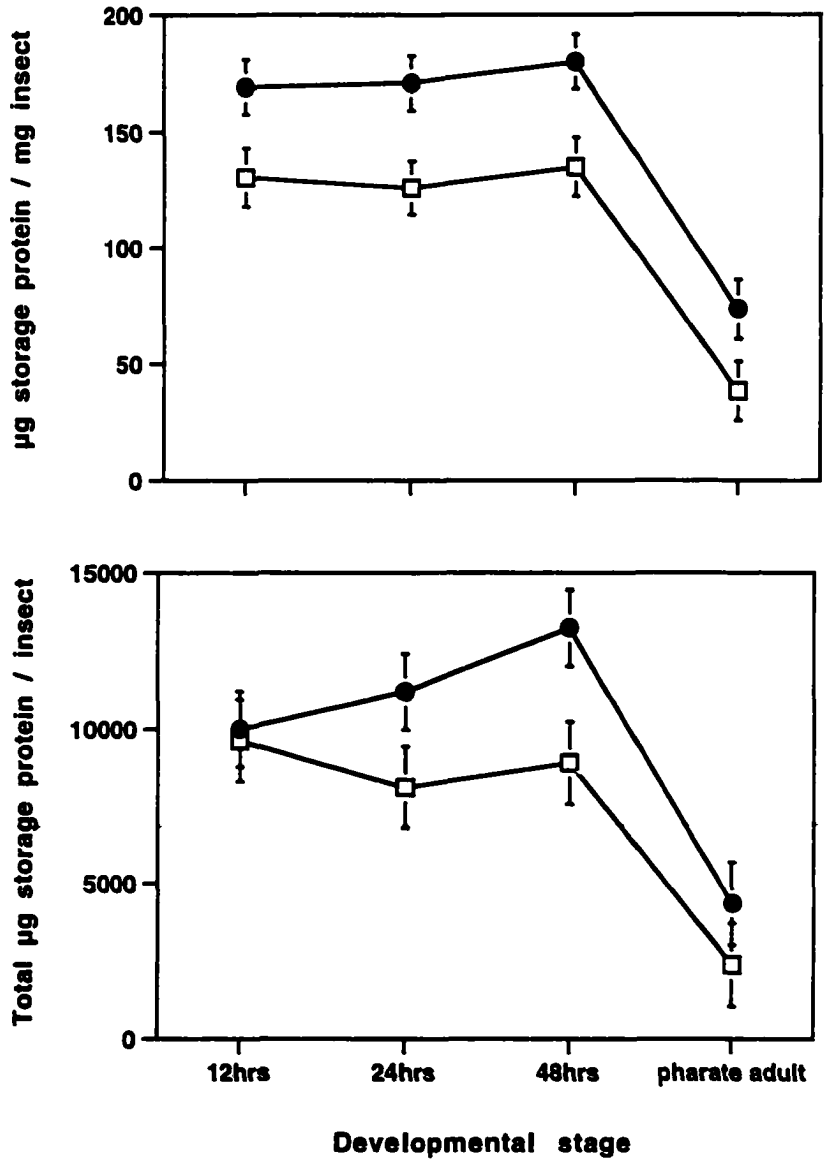
200
150
100
50
0
µg storage protein / mg insect
15000
10000
5000
0
Total µg storage protein / insect
12hrs
24hrs
48hrs
pharate adult
Developmental stage

Figure 4

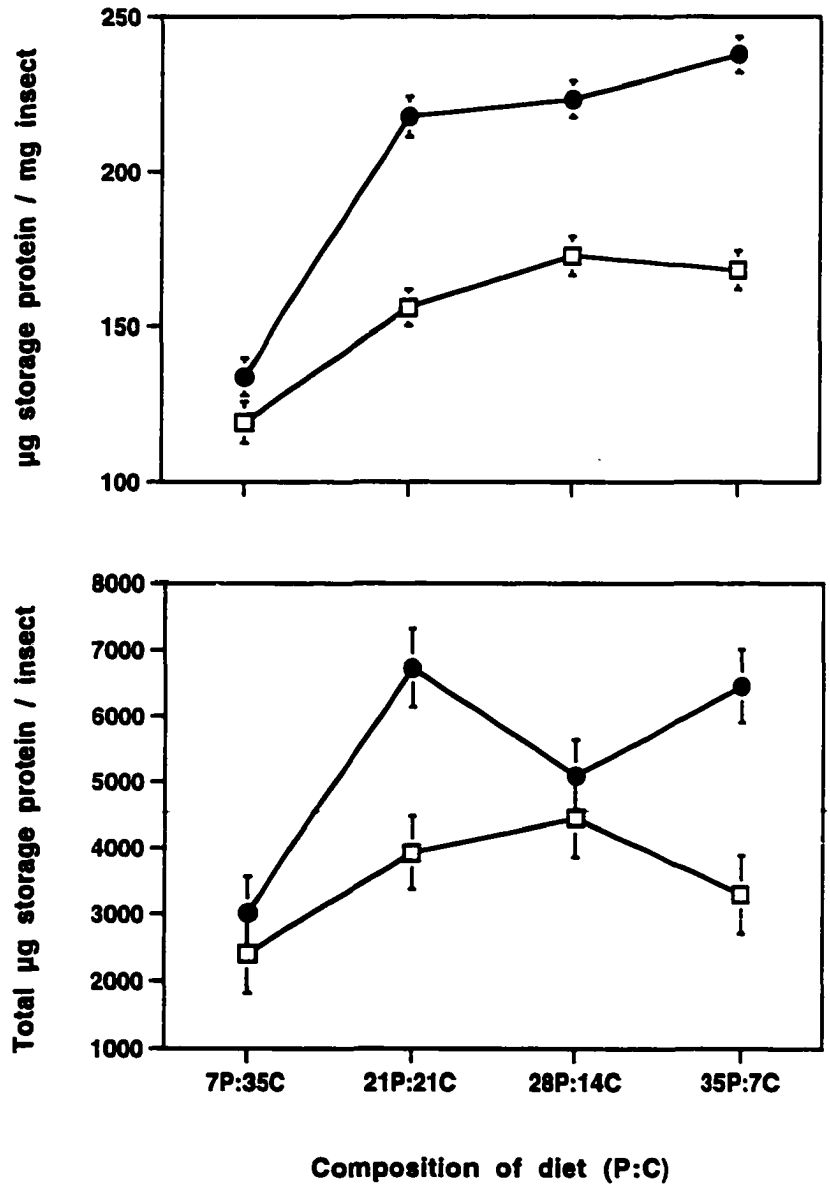
µg storage protein / mg insect
250
200
150
100
Total µg storage protein / insect
8000
7000
6000
5000
4000
3000
2000
1000
7P:35C
21P:21C
28P:14C
35P:7C
Composition of diet (P:C)

Figure 5

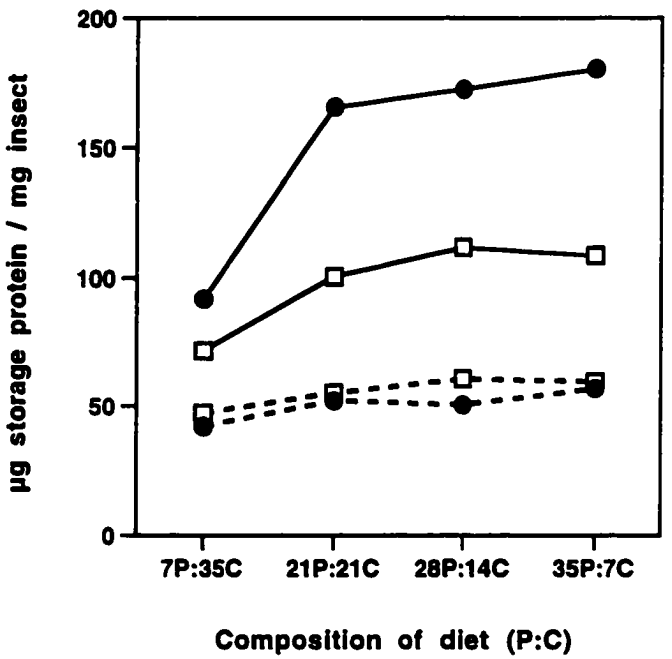
200
150
100
50
0
µg storage protein / mg insect
7P:35C
21P:21C
28P:14C
35P:7C
Composition of diet (P:C)

Table 1. Amino acid composition of *Heliothis virescens* storage proteins

Amino acid (Mol%)	HM1[a]	HM2[a]	Arylphorin
Histidine*	1.6	2.9	1.7
Arginine*	6.3	4.0	4.3
Threonine*	4.2	3.0	3.4
Tyrosine*	5.7	4.8	8.3
Valine*	6.9	7.6	6.0
Methionine*	0.9	0.7	0.5
Cysteine*	0.1	0.2	0.1
Isoleucine*	4.7	4.0	3.5
Leucine*	10.4	12.4	8.8
Phenylalanine*	6.0	3.5	7.7
Lysine*	8.5	4.9	8.0
Aspartic acid/Asparagine	15.0	11.2	12.5
Glutamic acid/Glutamine	7.5	11.4	9.9
Serine	4.1	6.6	5.5
Glycine	7.5	8.8	8.4
Alanine	5.2	6.9	5.2
Proline	5.5	7.1	6.3

[a] Putative high-methionine hexamer 1 and 2

* 11 of 12 amino acids are designated as essential; tryptophan was not determined in our analyses. Some classifications do not include tyrosine or cysteine as essential because they can be derived from the strictly essential amino acids phenylalanine and methionine, respectively. Other classifications include histidine and arginine as essentials.

APPENDIX D

THE REGULATION OF GROWTH BY CATERPILLARS THROUGH POST-INGESTIVE PROCESSING OF DIETARY PROTEIN AND CARBOHYDRATE

The regulation of growth by caterpillars through post-ingestive processing of dietary protein and carbohydrate.

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Running page head: Sexual differences in nutrient utilization

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ABSTRACT

Our previous studies indicated that female *Heliothis virescens* and *Estigmene acrea* caterpillars use both feeding and post-ingestive processing as mechanisms to meet nutrient demands. We present utilization and nutrient budget data on the post-ingestive partitioning of nutrients into pre- and post-absorptive components to determine the extent of their contributions toward regulating larval growth. Both species were found to efficiently utilize carbohydrate except at very high ingestion rates. Of the carbohydrate utilized, more was unaccounted for as intake increased and we assume the unaccounted material served as a respiratory substrate. In contrast, the pattern of nitrogen utilization

differed between the species. Larval *H. virescens* were highly efficient at retaining nitrogen except at very high ingestion rates whereas *E. acrea* showed a progressive increase in nitrogen excretion in response to increased nitrogen ingestion. Regardless, females of both species utilized nitrogen more efficiently than did males at all ingestion levels, thus contributing to their greater protein-derived growth. Results for *H. virescens* also showed that protein and amino acids accounted for a small proportion of fecal nitrogen and that most must be of post-absorptive nature. In contrast, nitrogen excreted by *E. acrea* could be partitioned into both pre- and post-absorptive components. The manner of post-ingestive processing by these two species reflects differences in their larval diet.

Introduction

Asymmetry in gamete size and associated investment in offspring is a fundamental difference between the sexes in many animal species. Female insects produce eggs richly provisioned with nutrients, including a high proportion of protein, so that oogenesis is a nutrient-limited process (Wheeler 1996). Few lepidopterans protein-feed as adults and so rely heavily on larval protein stores to provision eggs. Given that oogenesis is a nutrient-limited process for females, how the necessary protein and other nutrients are acquired from their environment and utilized is an important question. The implication is that female caterpillars accumulate greater stores of protein and other nutrients than do males. In some species, such as the gypsy moth, *Lymantria dispar*, this is achieved by the female undergoing an additional larval stage. However, in many species this is not the case, and the question arises "How do female larvae acquire greater nutrient stores? Do they feed at different rates or do differences between the sexes arise postingestively?

Previously, we reported that when insects were restricted to one of four completely artificial diets varying in protein:carbohydrate ratio, growth of both sexes of larval *Heliothis virescens* was highly clustered compared to ingestion values (Telang et al. 2001). A similar pattern was observed in the closely related arctiid species *Estigmene acrea* (manuscript in prep.) A pattern of tightly clustered growth in comparison to widely ranging ingestion values is taken to indicate that animals are relying on post-ingestive processing of nutrients to regulate their growth. Post-ingestive compensatory responses have been reported in a number of other insect species (Simpson and Simpson 1990; Slansky 1993). In addition, females achieved higher mean relative growth rates (MRGR) and protein-derived growth on all diets (Telang et al. 2001). In the present paper, we report on the post-ingestive effects of restricting insects to single diets

containing protein and carbohydrate at various concentrations. We further partitioned post-ingestive utilization of these nutrients into pre- and post-absorptive components to determine the extent of their contributions toward regulating larval growth.

Material and Methods

Animals

Egg masses containing *H.virescens* were obtained from the Western Cotton Research Laboratories in Phoenix, Arizona and maintained in incubators at 25°C and a 12:12 h light:dark cycle. Insects were reared individually in 30ml plastic rearing cups fitted with plastic lids to maintain moisture and were maintained at the same temperature and photoperiod as above. *Estigmene acrea* caterpillars were originally collected from the field in Arizona and were maintained in the laboratory on a synthetic, wheatgerm-enhanced diet. Eclosed adults were allowed to mate in large plastic boxes decorated with wax paper, which served as their egg laying substrate. Eggs were collected from several females since several individuals of both sexes were housed together in one box. Egg masses were maintained at LD 16:8 h, 25°C until hatching. Upon hatching, neonates were placed individually in 30 ml plastic cups fitted with plastic lids and were maintained in an environmental chamber at LD 16:8 h, 28:25°C. Larvae were reared on experimental diets fortified with wheatgerm to promote good growth and development (Telang et al. 2001). We determined from previous experiments that larvae were unable to develop properly when reared on strict chemically defined diets from hatching. However, from these same experiments we determined that later stages, upon being transferred to experimental chemically defined diets, develop normally.

Synthetic diets

Artificial diets were modified from diets previously described for *Heliothis virescens* (Telang et al. 2001). The protein source consisted of vitamin-free casein and the digestible carbohydrate was sucrose. Diets were agar based (3% solution w/v) and contained 54% cellulose (non-nutritive bulking agent) and 4% essential micronutrients (vitamins, cholesterol, salts and linoleic acid). The remaining 42% consisted of protein and carbohydrate at various levels.

Earlier experiments with *E.acrea* larvae on synthetic diets, designed for *H.virescens*, resulted in pupae with poorly developed cuticle, a condition subsequently remedied upon adding phenylalanine to experimental diets (E. A. Bernays, pers. comm.). These diets were similar to the *H. virescens* diets described above but contained 53.2% cellulose and 0.8% phenylalanine. All experimental diets were pre-weighed prior to offering and were replaced daily.

Experimental Protocol

Two replicates of each experiment were run for both species. Upon entry to their fifth, and last instar, *H. virescens* larvae were weighed, transferred to 150ml plastic cups fitted with plastic lids and randomly assigned to diet treatments. Caterpillars were restricted to one of seven diets with the following dry weight percentages of protein (P) to carbohydrate ©: 7P:35C, 14P:28C, 21P:21C, 24P:17C, 28P:14C, 32P:10C, 35P:7C. The experimental protocol for E. acrea was comparable to that applied to *H.virescens* except that sixth instar *E.acrea* larvae were experimental subjects.

Frass from each insect was collected daily, stored at –80°C and later oven dried at 50°C to constant weight, after which frass was prepared for chemical analyses. Frass

from insects from only four of the seven diet treatments were included for chemical analyses. The four diet treatments were chosen based on growth performance of insects.

Chemical analyses

Dried frass was ground using a Teflon pestle on a rotating shaft attached to a variable speed motor. Frass were analyzed for total nitrogen using a flash combustion method on a Model 440 CHN/O/S Elemental Analyzer. Carbohydrate content of frass was determined using a slightly modified anthrone method (Wheeler and Buck 1992; Telang et al. 2001). A standard curve ranging from 0 to 50 µg sucrose was run for each batch of samples and sample values were then read from this standard curve.

Frass protein content was measured using the Bradford Reagent (Sigma Chemicals, product number B6916). The standard curve, ranging from 0 to 12 µg, was constructed using Bovine Serum Albumin (BSA) because insolubility of casein precluded its use as a reliable standard. Samples of frass, 30-40 mg, were homogenized in 1.5 ml microcentrifuge tubes in 800 µl of 1.0 M NaOH, boiled for 3min and then centrifuged for 15 min at 12,000g at 4°C. According to the percent dry weight of protein in a particular diet, either 25, 40 or 50 µl aliquots of supernatant were neutralized with an equal volume of 1.0 M HCL, brought up to a volume of 0.5 ml with 0.01 M NaOH and absorbance was read at 595 nm. Previous trials indicated that 0.01 M NaOH did not adequately extract casein from frass samples but yields improved using 1.0 M NaOH. Subsequently, aliquots had to be neutralized to avoid interference with the Bradford Assay.

Total frass amino acids were measured using a Ninhydrin Reagent (Sigma Chemical Company, Product No. N1632). A standard curve was prepared using leucine in the range of 0 to 0.4 µM made up in 0.05% acetic acid. Again, according to the percent dry weight of protein in a particular diet, 30-60 mg of frass samples were placed

in 1.5 ml microfuge tubes. Amino acids were extracted and homogenized in 1000ul of 6% TCA (Trichloroacetic acid) to first precipitate protein in samples. Extracts were centrifuged for 15 min. and 20ul aliquots of supernatant were mixed with 800 µl of 0.05% acetic acid. To all samples and standards, 0.50 ml of ninhydrin reagent was added and vortexed. Samples and standards were boiled for 10 min., cooled to room temperature and quenched with 2.5 ml of 95% Ethyl alcohol. Absorbance of standards and samples were read at 570nm and values reported as % amino acids nitrogen as leucine.

Uric acid in the frass was measured using the Sigma enzymatic kit (Procedure No. 292-UV). A standard curve, ranging from 0 to 25 µg, was prepared using uric acid made up in 0.6% lithium carbonate. Uric acid was extracted from 15, 20 or 40 mg of frass samples using 0.6% LiCO3, following the procedure of Bhattacharya and Waldbauer (1969) and were centrifuged at 12,000g for 10 min. Uricase enzyme was added to either 25 or 50 µl supernatant aliqouts and all samples and standards were then incubated for one hour until absorbances had stabilized. Absorbances of standards and samples were then read at 292 nm and values were subsequently reported as %uric acid nitrogen.

Data analysis

Utilization data were interpreted according to the methodology of the geometrical framework (Simpson and Raubenheimer 1995). Budgets for each nutrient were constructed using values of total amount of nutrient eaten (Telang et al. 2001) to show how this is partitioned between growth and excretion. The amount of each nutrient found in frass was statistically analyzed using ANCOVA with amount of each nutrient eaten as a covariate. Data were statistically analyzed using JMP IN (version 3.2.1, SAS Institute Inc.). Adjusted mean values for nutrient budget components were obtained from

statistical models and used in their graphic illustrations, whereas bicoordinate utilization plots show raw data.

Results

Carbohydrate utilization by *H. virescens* and *E. acrea*

In our analyses of *H. virescens* frass samples, the covariate, amount carbohydrate eaten, strongly influenced the amount of carbohydrate found in frass (Table 1). Independent of this effect, the amount of carbohydrate excreted, by both sexes, was strongly influenced by dietary composition but no evidence was found that the sexes excreted different amounts. Figure 1A shows the relationship between amounts of carbohydrate eaten by the end of the fifth stadium and the amounts of carbohydrate in each insect's frass. When insects ingested roughly 40 to 150 mg of carbohydrate, a relatively small amount appeared in their frass, but at higher ingestion levels, a much larger amount was excreted. Similar results were obtained for carbohydrate levels measured in *E. acrea* frass samples (Table 2). As was observed for *H. virescens*, *E. acrea* larvae utilized carbohydrate efficiently at lower ingestion levels but increased carbohydrate excretion at the highest ingestion levels (Fig. 1B).

Nitrogen utilization by *H. virescens*

We found that the covariate, amount of nitrogen eaten, strongly influenced the amount of nitrogen excreted (Table 1). After accounting for this effect, we found strong evidence that dietary composition influenced the amount of nitrogen found in frass but no evidence for sexual differences, although the amounts excreted were increasingly variable at higher ingestion levels. A lower proportion of ingested nitrogen appeared in the frass of insects on low rather than high protein foods (Fig. 2A). Analyzing each diet treatment separately as a one-way ANOVA model, we found suggestive evidence for differences

between the sexes on diet 35P:7C only (two-sided ANOVA *P*-value = 0.043) with females excreting less nitrogen in frass than did males.

The amount of nitrogen eaten had no effect on amount of protein, amino acids or uric acid excreted by insects but the covariate did influence levels of calculated unaccounted nitrogen (i.e. the difference between total nitrogen and that accounted for by protein, amino acids and uric acid). All four responses were significantly affected by dietary composition but no differences between the sexes were found (Table 1). However, we obtained suggestive evidence for differences between the sexes with respect to unaccounted nitrogen on diet 35P:7C only (two-sided ANOVA $P = 0.046$) with males excreting more unaccounted nitrogen in frass compared to females. As observed for total nitrogen, the amounts of protein, amino acids, uric acid and unaccounted nitrogen excreted were increasingly variable at higher levels of nitrogen ingestion (Figs. 2B, 2C and 2D).

Nitrogen utilization by *E. acrea*

The covariate, nitrogen eaten, strongly influenced nitrogen excretion. Upon accounting for this effect, we found that dietary composition also significantly affected nitrogen excretion with strong evidence that the sexes excreted different amounts of nitrogen (Table 2). Fecal nitrogen levels were similar between the sexes at the lower ingestion levels associated with diet 7P:35C, but, at ingestion levels of 40 mg and greater, males excreted more total nitrogen than did females (points representing female data fall to the right of male data points) (Fig. 3A), thus explaining the significant interaction term between the main effects (Table 2).

Amounts of nitrogen eaten significantly affected levels of protein, amino acids, uric acid and unaccounted nitrogen in frass. Likewise, dietary composition was found to

influence all four responses but the sexes only differed in levels of uric acid and unaccounted nitrogen in frass with significant interactions between these main effects (Table 2). Males excreted similar levels of uric acid at low nitrogen ingestion but excreted greater amounts than did females across higher ingestion levels (male data points fall to the left of female data points starting at around 35 mg ingested nitrogen) (Fig. 3D). Female larvae excreted greater levels of unaccounted fecal nitrogen at low nitrogen ingestion but excreted far less compared to males at higher ingestion amounts (Fig. 3E). The significant interaction between main effects detected in fecal protein levels is due to females excreting more protein at lower ingestion levels compared to males. Upon reaching ingestion levels of 50 mg nitrogen or greater, protein excretion levels were more or less similar (Fig. 3B).

Nutrient partition (budgets) for *H. virescens* and *E. acrea*

Figure 4A summarizes the carbohydrate budget for both sexes of *H. virescens* larvae. Carbohydrate-derived growth was similar for both males and females on all four diets but a marked increase in amounts of unaccounted carbohydrate was observed on the 7P:35C diet, this amounted to 53% of that ingested. Figure 4B shows the overall nitrogen budget for both sexes. Nitrogen-derived growth was similar across all levels of % dietary protein but fecal nitrogen increased progressively with greater nitrogen ingestion associated with higher protein diets. A significant proportion of nitrogen was unaccounted for across all levels of dietary protein, with highest unaccounted levels associated with the 28% P diet.

Figures 5A and 5B summarize carbohydrate budgets for female and male *E. acrea* larvae, respectively. Carbohydrate-derived growth was similar for both males and females on the highest carbohydrate diet but was greater for males across the other

diets (Manu. in prep.). In contrast to the case for *H. virescens* frass, fecal carbohydrate levels for both sexes progressively decreased as levels of dietary carbohydrate decreased. Likewise, levels of unaccounted carbohydrate steadily decreased along with decreasing levels of dietary carbohydrate (Figs. 5A and 5B). Nitrogen budgets for female and male larvae are summarized in figures 5C and 5D, respectively. Nitrogen-derived growth was greater for females across all levels of % dietary protein but levels of unaccounted fecal nitrogen remained more or less similar. Levels of fecal nitrogen excreted by males increased sharply from the 7% to the 21%C diet whereas levels excreted by females progressively increase across all dietary levels.

Discussion

Previously we reported that caterpillars of *Heliothis virescens*, when restricted to one of seven diets varying in levels of protein and carbohydrate ingested different amounts of both nutrients. In contrast to widely varying ingestion values, growth derived from both nutrients was highly clustered (Telang et al. 2001) indicating that both sexes were relying on post-ingestive processing of nutrients to regulate growth (Raubenheimer and Simpson 1993; Zanotto et al. 1993). Such post-ingestive compensatory responses have also been reported in a number of other insect species, including caterpillars (Simpson and Simpson 1990; Slansky 1993).

Heliothis virescens larvae ingesting up to around 150mg carbohydrate utilized up to 90% but, at higher ingestion rates, relatively large quantities were excreted so that with an intake of about 250mg utilization fell to 70%. A similar utilization pattern was observed for *Estigmene acrea* larvae but at different ingestion levels. In addition, of the carbohydrate utilized, much greater quantities was unaccounted for as intake increased. Again, a similar profile was obtained for *E. acrea* larvae. We assume that

this material served as a respiratory substrate as suggested by studies on *Locusta migratoria* (Zanotto et al. 1993; Zanotto et al. 1997), although we have no evidence that caterpillars adjust their metabolic rate as a means of disposing of excess carbohydrate.

Bicoordinate plots between nitrogen eaten and excreted by *H. virescens* indicated a high efficiency of retention in the body up to an intake of about 20 mg of nitrogen, above this a greater amount of ingested nitrogen appears in frass. In contrast, results for nitrogen utilization by *E. acrea* larvae show a pattern of a progressive increase in nitrogen excretion in response to increased nitrogen ingestion. The nitrogen budget shows that caterpillars of both species tightly regulated growth derived from nitrogen and that they increased growth on higher protein diets. Our present results showed that females of both species utilized nitrogen more efficiently than did males on all diets, thus contributing to their greater protein-derived growth values (Telang et al. 2001 for *H. virescens* data).

With increasing nitrogen intake, relatively more was found in frass. In *Locusta migratoria*, Zanotto et al. (1993) observed a sharp increase in nitrogen excretion above 30 mg of intake (their Fig. 2A), suggesting a switch to a different method of processing at this point. In both *H. virescens* and *E. acrea* however, the change appears to be progressive rather than a switch. Such post-ingestive compensation for nitrogen has been reported in other insects (Slansky and Feeny 1977; Slansky and Wheeler 1989; Taylor 1989; Slansky and Wheeler 1991; Raubenheimer 1992; Zanotto et al. 1993). As was reported for *L. migratoria* (Zanotto et al. 1993), nitrogen excreted by both *H. virescens* and *E. acrea* in excess of growth requirements was mainly of post-absorptive origin. In the case of *H. virescens*, only a small proportion of fecal nitrogen was accounted for by protein and amino acids indicating that most of the ingested protein

was digested and absorbed across the midgut. Most of the fecal nitrogen was not accounted for but showed a similar relationship with amount of nitrogen eaten to total fecal nitrogen (as did fecal uric acid). It is unlikely that our measure of unaccounted nitrogen is of pre-absorptive nature although it is possible that our biochemical assay for protein determination underestimated fecal protein levels due to the insoluble nature of casein, our source of dietary protein (but see remaining Discussion for further explanation). In the case of *E. acrea*, fecal nitrogen could be partitioned into both pre- and post-absorptive components. Fecal protein levels was found to be low and showed a shallow relationship with amounts of nitrogen eaten. In contrast, levels of fecal amino acids showed a strong relationship with increased nitrogen ingestion indicating that protein was effectively digested but amino acids were not absorbed well across the midget as nitrogen intake increased. Levels of fecal uric acid were detected in very low quantities up to an intake of around 50 mg of nitrogen, at which point excretion increased, indicating that much of the excess nitrogen was being metabolized post-absorptively.

In the present results, only 45-50% of total fecal nitrogen excreted by *H. virescens* could be accounted for as fecal protein, amino acids or uric acid (although up to 70% was accounted for in *E. acrea* frass). One possible explanation is that the nitrogen missing was lost as volatile ammonia during drying of frass prior to chemical analysis (Mullins and Cochran 1972). Terrestrial insects may excrete a higher proportion of their nitrogenous waste as ammonia than was previously thought (Harrison and Phillips 1992; Chapman 1998). Ammonia excretion is not only important in maintaining acid-base balance (Harrison and Phillips 1992) but is energetically less expensive than further metabolism to uric acid. On diets 28P:14C

and 35P:7C, the caterpillars are essentially experiencing conditions of greater nitrogen load and deficiency of carbohydrate fuel which may favor the economics of ammonia excretion.

The above experiments indicate that *H. virescens* and *E. acrea* female larvae differ in the manner of post-ingestive processing of dietary protein. Female *H. virescens* larvae increased nitrogen excretion markedly at the highest nitrogen ingestion levels (levels associated with feeding on high protein foods). Much of the fecal nitrogen was of a post-absorptive nature indicating excess ingested nitrogen was being metabolized. Their response to high dietary protein levels may reflect their typical larval diet in the field in which late instar larvae feed and grow well on the anthers of developing cotton floral buds (Shaver et al. 1977; Hedin et al. 1991). Cotton anthers contain the highest total amino acid levels compared to other cotton floral tissues (Shaver et al. 1977; Hedin and McCarty 1990; Hedin et al. 1991) which may explain the ability of *H. virescens* to effectively digest high protein foods.
In contrast, *E. acrea* larvae are highly polyphagous and feed on the leaves of a number of hostplants (M.S. Singer, pers. comm.). Plant leaves are considered to be sources of high carbohydrate but low in nitrogen (Slansky and Scriber 1985) and this may explain why *E. acrea* wasted less nitrogen only at low nitrogen intake levels (associated with low protein foods).

In summary, a review of studies regarding insect nutritional physiology concludes that females are heavier than males due to greater nutrient accumulation as a result of greater food consumption (Slansky and Scriber 1985). Clearly, results of our study indicate that female *H.virescens* caterpillars regulate both nutrient consumption and post-ingestive physiology to achieve greater growth. Additionally, our study, in

using chemically defined artificial diets, explains the nutritional physiology of female caterpillars in terms of specific macronutrients important to them.

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Figure captions

Figure 1A. Relationship between the amounts of total carbohydrate eaten and total amount measured in frass over the fifth stadium of *H. virescens* for both females (●) and males ( ). Figure 1B. Relationship between the amounts of total carbohydrate eaten and total amount measured in frass over the sixth stadium of *E. acrea* for both females (●) and males ( ).

Figure 2. Relationship between the amounts of total nitrogen eaten and total amount measured in frass over the fifth stadium of *H. virescens* for both females (●) and males ( ). (A) nitrogen, (B) protein, (C) amino acids, (D) uric acid, and (E) unaccounted nitrogen.

Figure 3. Relationship between the amounts of total nitrogen eaten and total amount measured in frass over the sixth stadium of *E. acrea* for both females (●) and males ( ). (A) nitrogen, (B) protein, (C) amino acids, (D) uric acid, and (E) unaccounted nitrogen.

Figure 4. Summary plot of the overall carbohydrate budget for *H. virescens* (4A) showing partitioning of total ingested carbohydrate toward growth, excretion and unaccounted on all four diets. Summary plot of the overall carbohydrate budget for *E. acrea* females (4B) and males (4C) showing partitioning of total ingested carbohydrate toward growth, excretion and unaccounted on all four diets.

Figure 5. Summary plot of the overall nitrogen budget for *H. virescens* (5A) showing partitioning of total ingested nitrogen toward growth, excretion and amount unaccounted

for on all four diets. Summary plot of the overall nitrogen budget for *E. acrea* females (5B) and males (5C) showing partitioning of total ingested nitrogen toward growth, excretion and amount unaccounted for on all four diets.

Figure 1

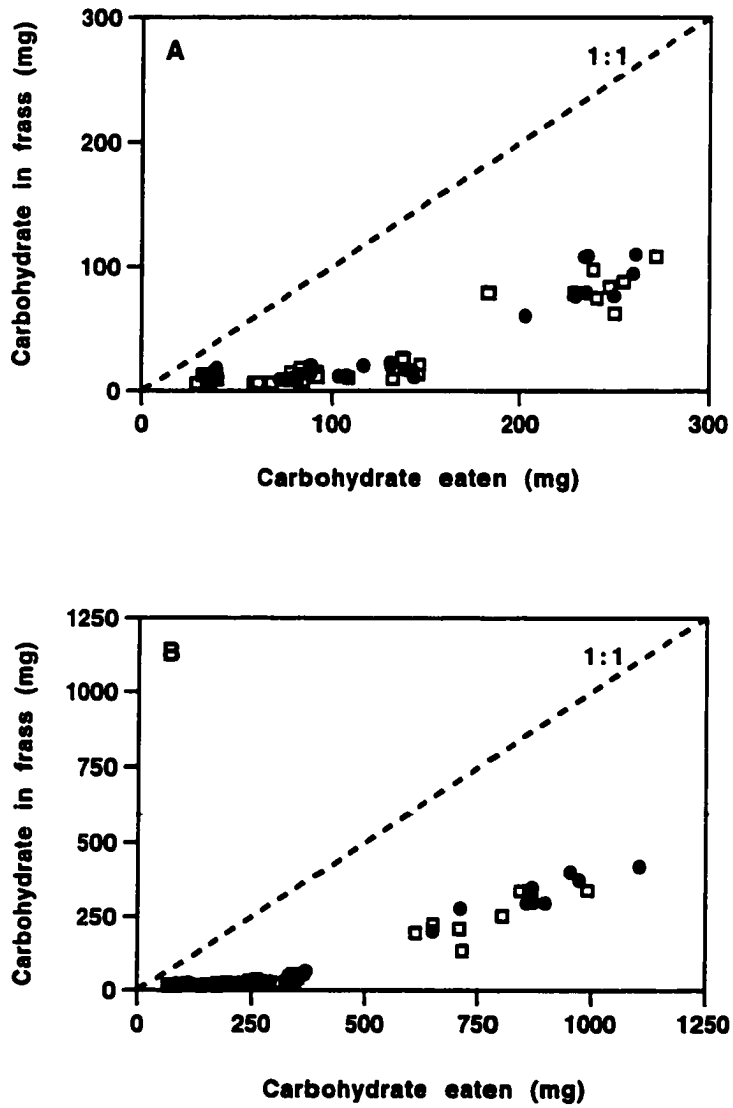
A
1:1
Carbohydrate in frass (mg)
Carbohydrate eaten (mg)
0
100
200
300
B
1:1
Carbohydrate in frass (mg)
Carbohydrate eaten (mg)
0
250
500
750
1000
1250

Figure 2

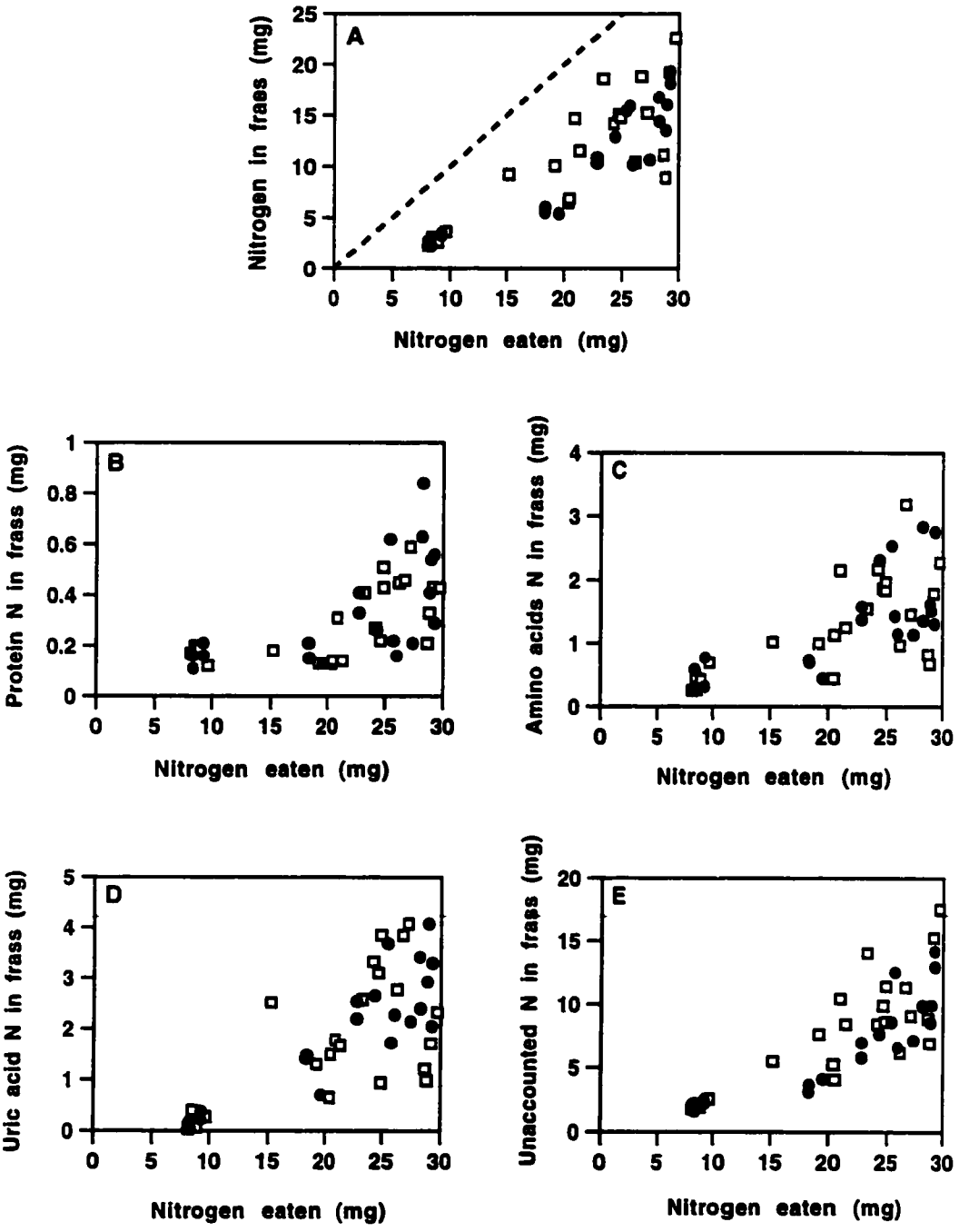
A
Nitrogen in frass (mg)
Nitrogen eaten (mg)
B
Protein N in frass (mg)
Nitrogen eaten (mg)
C
Amino acids N in frass (mg)
Nitrogen eaten (mg)
D
Uric acid N in frass (mg)
Nitrogen eaten (mg)
E
Unaccounted N in frass (mg)
Nitrogen eaten (mg)

Figure 3

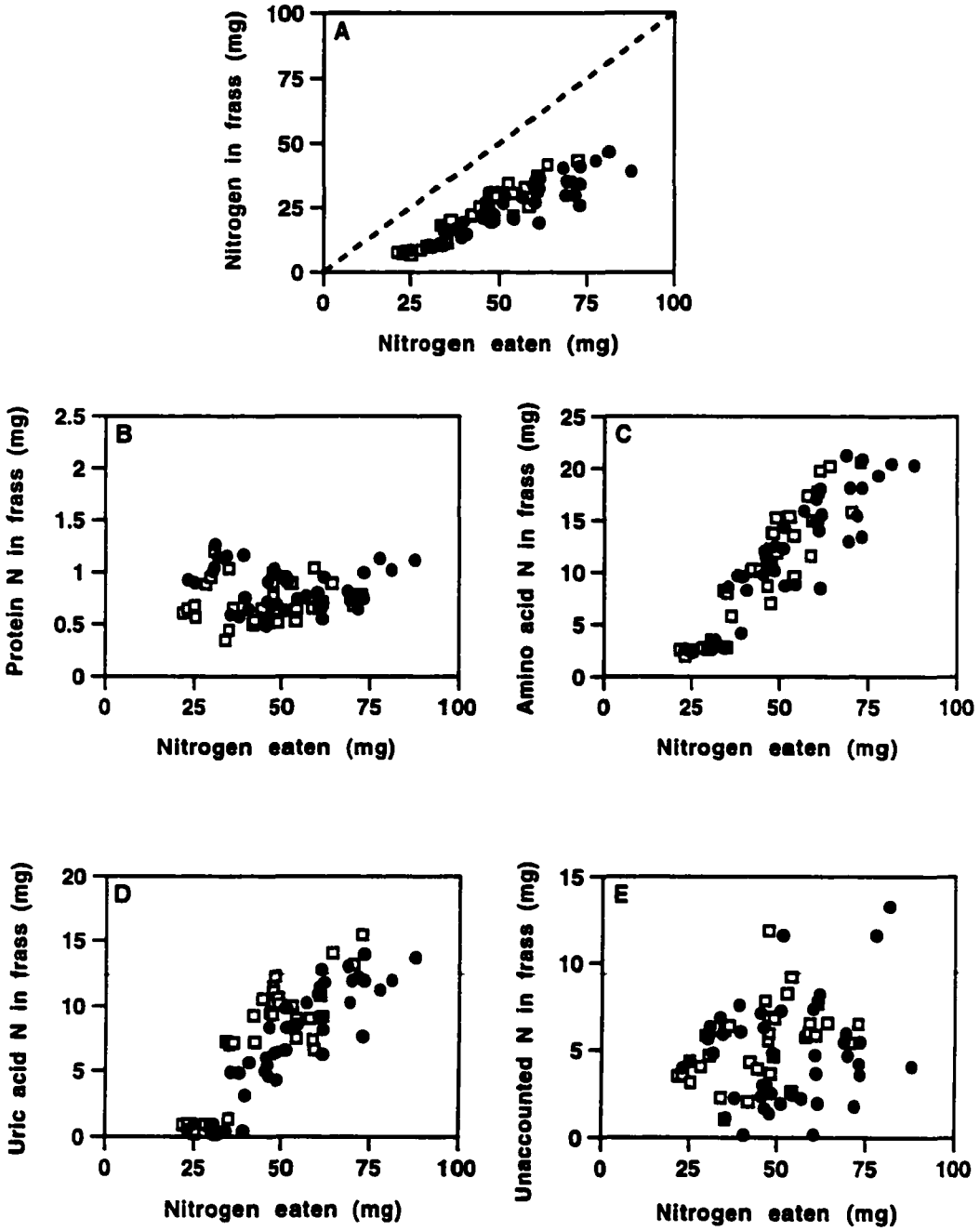
A
Nitrogen in frass (mg)
Nitrogen eaten (mg)
B
Protein N in frass (mg)
Nitrogen eaten (mg)
C
Amino acid N in frass (mg)
Nitrogen eaten (mg)
D
Uric acid N in frass (mg)
Nitrogen eaten (mg)
E
Unaccounted N in frass (mg)
Nitrogen eaten (mg)

Figure 4

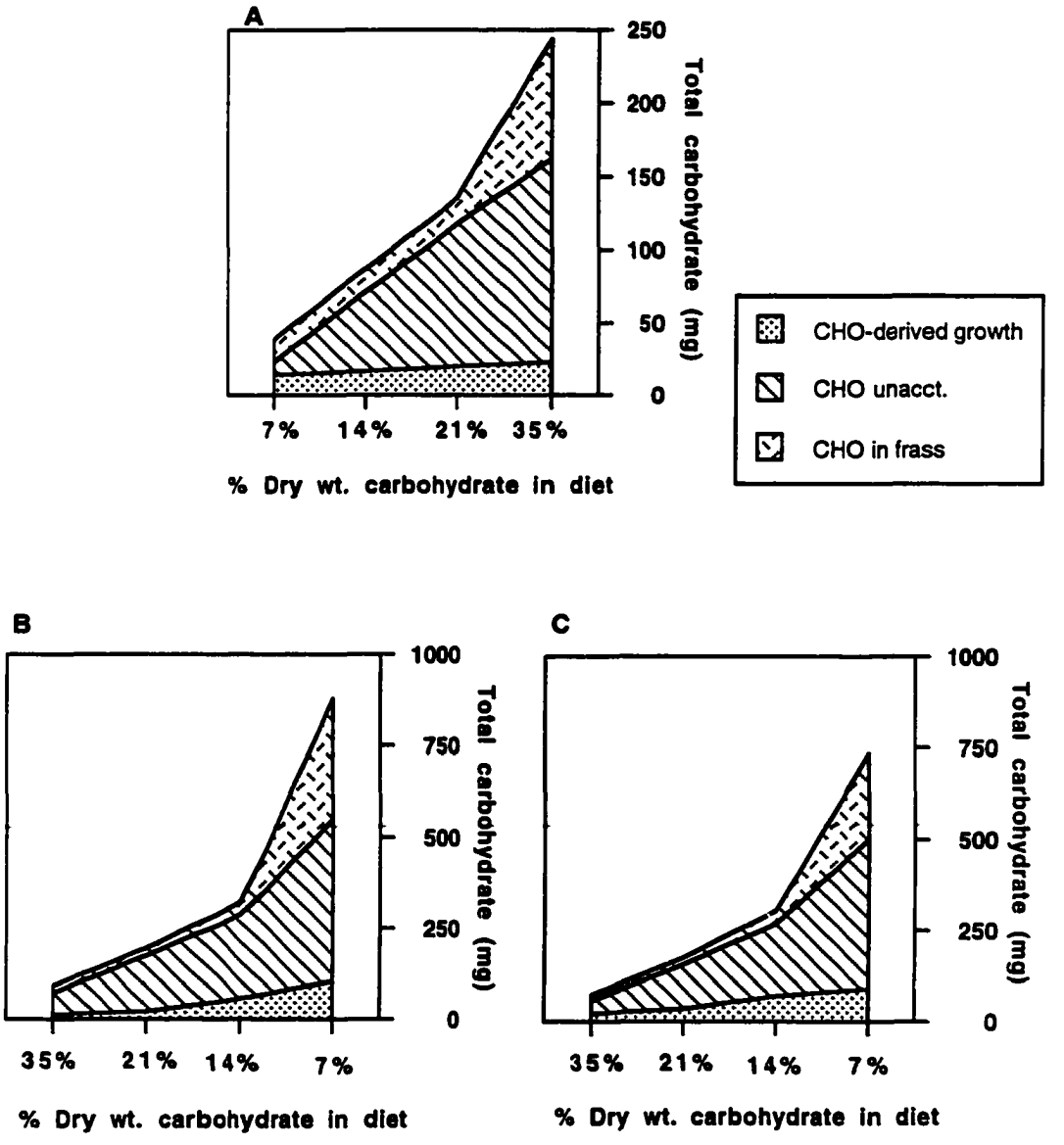
A
250
200
150
100
50
0
Total carbohydrate (mg)
7%
14%
21%
35%
% Dry wt. carbohydrate in diet
CHO-derived growth
CHO unacct.
CHO in frass
B
1000
750
500
250
0
Total carbohydrate (mg)
35%
21%
14%
7%
% Dry wt. carbohydrate in diet
C
1000
750
500
250
0
Total carbohydrate (mg)
35%
21%
14%
7%
% Dry wt. carbohydrate in diet

Figure 5

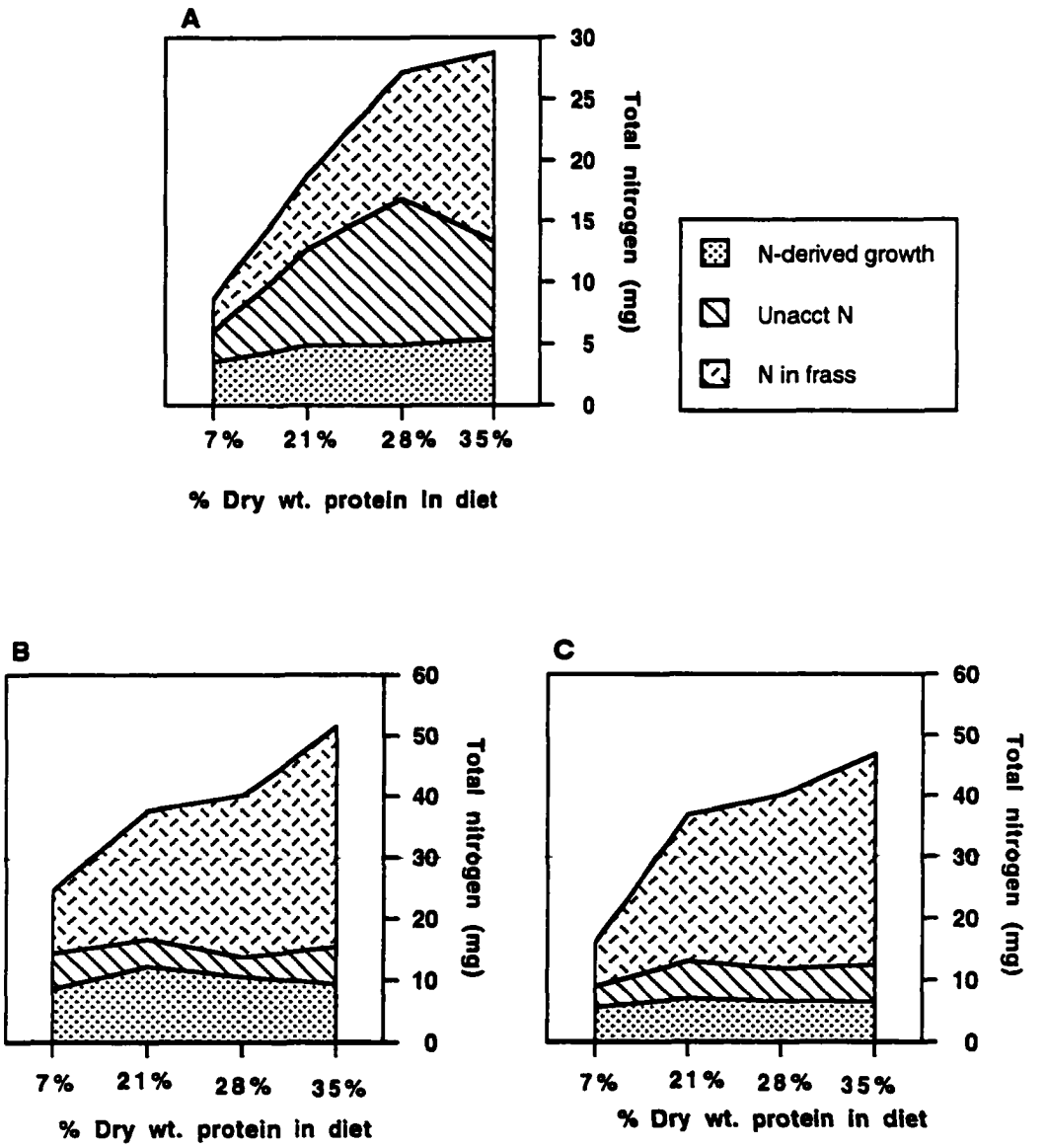
A
30
25
20
15
10
5
0
Total nitrogen (mg)
N-derived growth
Unacct N
N in frass
7%
21%
28%
35%
% Dry wt. protein in diet
B
60
50
40
30
20
10
0
Total nitrogen (mg)
7%
21%
28%
35%
% Dry wt. protein in diet
C
60
50
40
30
20
10
0
Total nitrogen (mg)
7%
21%
28%
35%
% Dry wt. protein in diet

Table 1
F ratios from ANCOVAs for excretion of nitrogen and carbohydrate in fifth instar *H. virescens*.

Response Variables:	Fecal Carbohydrate	Fecal Nitrogen	Protein N	Amino acids N	Uric acid N	Unacct. N
Effects (df)						
Diet (3)	45.1***	16.0***	14.5***	8.3***	2.9*	6.7***
Sex (1)	0.9	1.9	0.7	0.1	0.2	2.6
Diet x Sex (3)	0.4	0.8	0.8	0.3	0.5	1.1
Replicate (1)	0.0	12.5***	1.4	38.4***	0.2	4.4*
Covariate (1)[a]	12.9***	7.7**	0.0	1.9	0.1	6.6**
Error (34)[b]						
Total (43)[b]						

[a] The covariate for fecal nitrogen and fecal carbohydrate are nitrogen eaten and carbohydrate eaten, respectively. [b]For response variable fecal carbohydrate, Error and Total were associated with 57 and 66 degrees of freedom, respectively.
***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$.

Table 2
F ratios from ANCOVAs for excretion of nitrogen and carbohydrate by sixth instar *E. acrea*.

Response Variables:	Fecal Carbohydrate	Fecal Nitrogen	Protein N	Amino acids N	Uric acid N	. Unacct. N
Effects (df)						
Diet (3)	30.7***	21.3***	29.9***	16.6***	51.0***	4.8**
Sex (1)	0.2	16.8***	1.4	1.1	26.2***	3.8*
Diet x Sex (3)	1.5	3.3*	4.2**	1.2	4.6**	3.3*
Replicate (1)	11.7***					
Covariate (1) [a]	345.3***	101.1***	53.6***	54.4***	27.6***	17.2***
Error (71)[b]						
Total (79)[b]						

[a] The covariate for fecal nitrogen and fecal carbohydrate are nitrogen eaten and carbohydrate eaten, respectively. [b]For response variable fecal carbohydrate, Error and Total were associated with 94 and 103 degrees of freedom, respectively.
***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$.

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