DIETARY EFFECTS ON THE PERFORMANCE AND BODY COMPOSITION OF THE GENERALIST INSECT HERBIVORE, *Heliothis virescens* (LEPIDOPTERA: NOCTUIDAE)

A Thesis

by

KARL ADAM ROEDER

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2010

Major Subject: Entomology

Dietary Effects on the Performance and Body Composition

of the Generalist Insect Herbivore, *Heliothis virescens*

(Lepidoptera: Noctuidae)

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Approved by:

Chair of Committee,	Spencer Behmer
Committee Members,	Gil Rosenthal
	Robert Wharton
Head of Department,	Kevin Heinz

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ABSTRACT

Dietary Effects on the Performance and Body Composition
of the Generalist Insect Herbivore, *Heliothis virescens*
(Lepidoptera: Noctuidae). (August 2010)
Karl Adam Roeder, B.S., Texas A&M University
Chair of Advisory Committee: Dr. Spencer Behmer

All animals, including insect herbivores, eat to acquire nutrients that are essential for fueling physiological processes associated with growth, development, and reproduction. Protein and digestible carbohydrates are two nutrients required in large quantities by insect herbivores, but the amounts in which they occur in plants can be highly variable. In this thesis, I explore how the amounts and ratios of protein and digestible carbohydrate in an insect herbivore's food affect lifetime performance and body elemental composition. I do this by confining a generalist caterpillar, *Heliothis virescens*, to semi-synthetic foods with fixed protein-carbohydrate amounts and ratios.

I show that foods with protein-carbohydrate ratios that match the self-selected protein-carbohydrate intake of final instar caterpillars correlate strongly with best performance, and that small deviations away from this optimal protein-carbohydrate ratio can result in large drop-offs in overall performance, particularly for males.

I also show the importance of protein-carbohydrate balance over total macronutrient content. Finally, my results demonstrate that *H. virescens* caterpillars do not practice strict elemental homeostasis. My result, when contrasted with earlier work on

caterpillars, suggests that hemimetabolous and holometabolous insect herbivores practice different degrees of elemental homeostasis.

DEDICATION

This thesis is dedicated to my parents, Eric and Leslie Roeder; my brother, Stephen Roeder; and to my uncle, Brian Wiegmann. To my mom and dad, I cannot describe in words how much you mean to me on a daily basis. From unconditional love to all kinds of support, you have done everything and more that I could ever want. I do not think you realize how important both of you were to me in the last semester when I was on the verge of disappearing and leaving. Thank you so much for everything and hopefully one day I will be able to repay the same kindness, love, and, support to my children.

To Stephen, thank you for being the best brother and friend that anyone could ever want. You convinced me to stay and never give up even when I was on the verge of quitting.

Finally to my uncle, I have you to thank for turning me to a field I had no idea about. Without that summer job so many years ago, I probably would not be here writing the closing sentences of a master thesis. Without you, I am not sure where I would be.

ACKNOWLEDGEMENTS

I would like to give thanks to my committee chair, Dr. Spencer Behmer, who made all of this possible. Thank you for guidance and support for the five years I have known you. From the first pan of caterpillar eggs to my first day teaching to the signing of my defense, you have provided me with unbelievable opportunities and support that few get to experience in a lifetime. I would also like to thank Dr. Robert Wharton who gave me my first real job as an entomologist and continued that with his participation on my committee. A special thanks to Dr. Gil Rosenthal who made me come out of my entomological shell and think more about the overall biology of the animals, which is something that I truly did do until our talks.

I would also like to thank the entire Behmer lab. Everyone has been a pleasure to work with and provided unforgettable experiences. Without your help I doubt I would be finishing. Thank you for everything!

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CHAPTER I

INTRODUCTION

Acquisition and consumption of food are fundamental steps that any animal must travel through in order to obtain nutrients for growth and reproduction. Animals have been shown to require about 30 nutrients, including amino acids, carbohydrates, sterols, phospholipids, fatty acids, vitamins, minerals, trace elements, and water (Chapman 1998, Schoonhoven et al. 2005). Although many of these required elements are similar for different species (Sterner and Elser 2002), the blend of nutrients that they may need can be quite different (Behmer and Joern 2008). For animals that feed on plants, obtaining the correct blend can be challenging due to the variable nutrient profiles of the plants that they are consuming, and because plants often contain nutrients in less than optimal amounts and/or ratios (White 1978, 1984). For example, previous work has shown that the nitrogen, phosphorus, and sodium content is generally lower in plants than in animal tissue (Mattson 1980, Sterner and Elser 2002, Pennings and Simpson 2008) and that plants are highly variable with respect to their protein and digestible carbohydrate content (Slansky 1993, Behmer 2009, Bernays and Chapman 1994, Schoonhoven, et al. 2005), which supply nitrogen and energy, respectively (McNeil and Southwood 1978, Mattson 1980, Scriber and Slansky 1981).

The most abundant and arguably successful animal herbivores are insect herbivores – it is estimated that they contain a quarter of all known living organisms

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This thesis follows the style of Ecology.

(Wilson 1992, Chapman 2006). Throughout time, herbivorous insects have been quite successful at exploiting various nutritional landscapes due to unique morphological and physiological adaptations. Additional divergences among insect herbivores have also been demonstrated through the use of various feeding strategies – some are specialist, feeding on a very narrow range of plants, while others are generalists, and fed on a broad range of plants, often representing many different plant families. What is interesting about these two categories is that both groups of herbivores, specialists and generalists, have been shown to experience varying degrees of nutritional quality in nature (Raubenheimer and Simpson 1999). Plant nutritional variation is not uncommon, and differences can occur between species, populations, and even individuals, (Mattson 1980, McNeill and Southwood 1978, Scriber and Slansky 1981). However, because variation in nutritional quality is likely to be much greater between different plants species than within plants species, specialist insect herbivores are likely to occupy a narrow nutritional niche, while generalists, which feed on a broad range of different plants, will experience a much broader nutritional niche. The consequences associated with having evolved under different nutritional environments may be important in terms of how food nutrient content, especially food macronutrient content (proteins and carbohydrates), influences performance (including development time, survival, and even reproductive output).

The exploration of nutrient regulation in insect herbivores has been slowly pushed towards the measuring of nutrients in controlled choice or no choice artificial environments with fixed amounts or ratios of proteins, carbohydrates, and/or sterols on

various herbivorous insects like grasshoppers and caterpillars (reviewed by Behmer 2009). Models, like the Geometric Framework (GF), that explore these relationships in more detail have been developed to understand how nutrients interact and the mechanisms animals use to regulate different classes of nutrients (Behmer 2009). Specifically the GF is a state-space model that examines how organisms with changing nutritional needs are able to solve the ever-changing problem of a varied nutritional environment by simultaneously regulating the intake of multiple nutrients (usually limited to two types shown on 2 –axes) (Simpson and Raubenheimer 1993, 1995). The GF is particularly good at showing the priority that an insect places on particular nutrients and how these decisions affect an individual's performance by placing greater emphasis on the physiology and behavior of individuals (Behmer 2009). This is an important distinction to make since the GF can be used to explore how animals, e.g. herbivorous insects, change their behavior by feeding across a range of diets with varying protein: carbohydrate ratios (p:c) with possible lifetime implications that may have been inadvertently passed over.

The GF, in addition to being used to measure nutrient regulation, is also well suited to compare the effects of a food's nutrient content on performance. This has been primarily observed in grasshoppers (Simpson and Abisgold 1985, Raubenheimer and Simpson 2003, Behmer et al. 2001) and caterpillars (Despland and Noseworthy 2006, Telang et al. 2001, Lee et al. 2006) but in all current published instances, the studies have focused on the last or just a few of the late larval instars (reviewed in Behmer 2009). While these short duration studies provide insight on the physiological processes

occurring within those time periods, they fail to address a few key issues associated with lifetime consequences. Single stadium studies, for example Lee et al's (2006) work on *H. virescens,* fail to take in account early instar dietary affects. In most cases (reviewed in Behmer 2009) a culture diet is fed to the earliest instars and then they are transitioned to the experimental food only in the last stage. These procedures produce statistically similar survival rates for vary different diets. Additionally, at least in the terms of fixed p:c macronutrient foods, reproduction and adult performance have yet to be measured. And although various pupal performance measurements, e.g. mass, lipid, elemental composition, have been measured, the affects of consuming a particular food for an entire lifetime has not yet been associated with a true fitness number in part due to the inability of hemimetabolous insects to produce viable young.

A related question, in both lifetime and single stadium regards, is how does the macronutrient content of a food influence how an organism builds itself (specifically its elemental composition). Since foods are composed of various elemental combinations, reactions can occur that rearrange or modify existing compounds (Sterner and Elser 2002) and measuring this elemental flow is important not only for nutrient regulation within an organism, but also for overall elemental movement and flow in an ecosystem. Ecological Stoichiometry (ES) and its accompanying model were created to measure these movements (Sterner and Elser 2002). Specifically, ES is the balance of multiple chemical substances in ecological interactions and processes, or the study of this balance, which also sometimes refers to the balance of energy and materials (Sterner and Elser 2002). ES is quite useful in that regard as it helps explain the flow of elements, and

in turn how an organism uses certain elements and not others from the food it consumes. According to Sterner and Elser (2002), elements were chosen because they not only provide the framework for easy movement between biological levels but also because they are unchanging. They provide the structure for elemental body compositions across all living organisms. The focus of ES thus is elemental in nature, but broken down to exploring the relationship between three key macro elements: Carbon, Nitrogen, and Phosphorous. These three were primarily chosen due to their relevance in the building of an organism. Carbon, which makes up approximately 40-50% of the dry biomass of most living organisms (Sterner and Elser 2002), and nitrogen, which is an essential nutrient within proteins, nucleic acids, and amino acids, are of great importance since they make up a large percentage of the macronutrients that insects actively regulate (Joern and Behmer 1997, Simpson et al. 2004). Additionally phosphorous is a component of many of the building blocks found in DNA, RNA, ATP, and cell membranes. These three elements have currently been of great importance within aquatic invertebrate systems (Karimi and Folt 2006, Frost et al. 2004) as well as phosphorous levels within terrestrial food (Bertram et al. 2006), but measuring the physiological importance of the flow of carbon and nitrogen, within a terrestrial system has been slightly left behind.

ES, although relatively new, is a powerful tool that can be used to help answer critical questions concerning the flow of nutrients across a single stadium, multiple stadiums, and entire lifetimes. By incorporating both models, the GF and ES, proper mapping of elemental flow and the effects it has on the behavior and physiological

performance measurements of a holometabolous insect can finally be shown. Additionally, no completely controlled experiments have been performed on holometabolous insects (one study was done on a Hemimetabolous grasshopper – Boswell et al. 2008), nor have any been performed across an entire developmental stage. Thus it is our goal, and the first study to our knowledge, that aims to explore multi stadium performance measurements in the geometric framework by comparing the stoichiometric flow of elements through an individual holometabolous insect. This study will also follow a number of other potential important elements (S, Na, K, Ca, Mg, Fe, Cu, Mn, and B), and specifically ask how do bottlenecks in food macronutrient content influence the flow of non-related macronutrient elements into an organism.

Animals, including insect herbivores, all require nutrients in order to survive and grow. By manipulating the p:c ratios and/or amounts of macronutrient in an animal's food, questions about performance, both within developmental stages and across entire lifetimes, can be addressed. In the first part of my thesis, I will examine the lifetime consequences associated with eating different fixed amounts of proteins and carbohydrates. It will be the first study to measure the lifetime performance of an herbivorous holometabolous insect on artificially manipulated diets, which vary in their macronutrient ratios of protein to carbohydrate. In the second part of my thesis, I will measure and compare the flow of elements across the larval developmental stage in order to test whether the absolute amount of macronutrients or the ratio that they are present within a food is more important to not only improving performance but also for the better understanding of how an insect builds itself from the food it eats. Within both

of my thesis chapters, the same holometabolous lepidopteran species, *Heliothis virescens* or the tobacco budworm, will be used. This species is a generalist caterpillar, which feeds on a broad range of food plants (Neunzig 1969, Schneider et al. 1986), and thus potentially experiences a broad range of food macronutrient content.

CHAPTER II

LIFETIME CONSEQUENCES ASSOCIATED WITH FOOD MACRONUTRIENT CONTENT IN A GENERALIST INSECT HERBIVORE

Overview

Lifetime performance studies based on varied nutritional foods that mimic naturally occurring plants are vital to the overall understanding of the physiology of both hemi- and holometabolous insects. In this study seven unique artificial diets ranging from high protein: low carbohydrate (p31.5:c10.5) to low protein: high carbohydrate (p10.5:c31.5) were tested on *Heliothis virescens* Fabricus (Lepidoptera: Noctuidae) throughout an entire generation using a range of performance (survival, development, pupal mass, and lipid body percentage) and reproductive measurements (egg production and viability). Larval performance was highest on balanced (p21:c21) to slightly carbohydrate-biased diets (p17.5:c24.5). Pupal performance on the other hand was higher on balanced (p21:c21) to slightly protein rich diets (p24.5:c17.5), which has been shown previously to be the intake target of *H. virescens.* Males were more affected than females in regards to survival when any imbalance in macronutrients occurred. Highest eclosion and egg production rates were seen on the three middle range diets (p17.5:c24.5, (p21:c21, and p24.5:c17.5). Estimated population sizes for each diet treatment showed decreases in total size with each step down of diet variability away from p21:c21. Our findings suggest that single stadium studies are good indicators of

lifetime performance measurements, but fail to show the true effect that imbalanced foods have on individuals over an entire generation.

Introduction

The fundamental reason all animals eat is to acquire nutrients that are necessary for growth and reproduction. Animals generally require a collection of about 30 nutrients, consisting of amino acids, carbohydrates, sterols, phospholipids, fatty acids, vitamins, minerals, trace elements, and water (Chapman 1998, Schoonhoven et al. 2005), although the blend of nutrients that results in optimal performance is often species-specific. A key challenge, though, is that the foods an animal eats often contain nutrients in less than optimal amounts and or ratios (Bernays and Chapman 1994). This is particularly the case for herbivores (Slansky 1993, Behmer 2009, Schoonhoven, et al. 2005). For instance, the nitrogen, phosphorus, and sodium content of the plants is generally lower in plants than in animal tissue. It is also often the case that plants are highly variable with respect to their protein and digestible carbohydrate content, which supply nitrogen and energy, respectively (Mattson 1980, McNeil and Southwood 1978, Scriber and Slansky 1981).

Herbivores can overcome some of the variation in the nutritional content of their food by practicing selective feeding, either by eating from a range of different plants, or feeding on different vegetative tissues within a plant. Insect herbivores are particularly adept at regulating their nutrient intake, especially their macronutrient intake (Zanotto et al. 1993). A key question, though, is what are the consequences to an herbivore when it

cannot practice self-selection, and is instead restricted to foods with sub-optimal nutrient content. For example, in some situations food may be limiting, and insect herbivores will have no option but to eat food that may be nutritionally suboptimal (e.g. as a result of drought). Alternatively, where high quality food is available, it may not be eaten because of the threat of predation (Schmitz and Suttle 2001).

The effects of food macronutrient content on insect performance has received a great deal of attention, mostly in grasshoppers and caterpillars (reviewed by Behmer 2009), but the large majority of these studies have restricted their investigations to the final immature developmental stage. A serious limitation to this approach is that the foods given to the test insects prior to the start of the experiment were likely of high quality, and thus macronutrient related differences in performance (e.g., survival, growth rate) might be dampened because test insects likely would have had nutrient reserves to draw upon during the experimental phase. It is also the case that many of these experiments, particularly ones using caterpillars, did not measure eclosion success. Finally, to the best of our knowledge, none of these experiments explored the reproductive consequences associated with being restricted to foods with fixed macronutrient content.

Therefore, the aim of the current study was to explore, for the first time in an insect herbivore, the lifetime consequences (including reproductive output) associated with feeding on foods with a fixed macronutrient content. I explore this question using a generalist holometabolous caterpillar, *Heliothis virescens*. This caterpillar has a very broad diet, at both the individual and population level, so different individuals are likely

to experience a broad range of macronutrient ratios. I rear newly hatched neonate caterpillars, over their entire larval lifetime, on foods with different protein-carbohydrate (p:c) ratios, and measure the consequences of food p:c ratio on their larval, pupal and adult performance. Our results demonstrate that *Heliothis virescens*, despite being a generalist herbivore, performs best on a narrow range of p:c ratios, and that food p:c ratio affects males much more dramatically than females. We discuss our findings in relation to previous studies that have explored the short-term effects of food macronutrient content on insect herbivores, and their potential ecological implications.

Materials and Methods

Experimental Insects

Caterpillar eggs were obtained from a *Heliothis virescens* culture at North Carolina State University. These eggs came from adult female moths, which had been previously reared on a corn-soy-milk base diet (CSM) that had been modified from Burton (1970). All experimental neonates hatched at approximately the same time and within a few hours of hatching they were transferred, using a fine tipped paint brush, to 2 oz. Solo cups that contained a block of experimental food (see below). A lid was placed on each individual cup, and all cups containing caterpillars were transferred to an insect growth chamber (Percival Scientific Biological Incubator, Model I-66VL) set at 29ºC +/- 1ºC with a 12h: 12h L: D photoregime.

Experimental Diets

A total of seven CSM-based diets were used for this experiment. They all had similar combined total protein (p) and digestible carbohydrate (c) amounts (42% by dry mass) but differed in their p:c ratio: (1) p10.5:c31.5 (10.5% protein and 31.5% carbohydrate), (2) p14.0:c28.0, (3) p17.5:c24.5, (4) p21.0:c21.0, (5) p24.5:c17.5, (6) p28.0:c14.0, and (7) p 31.5:c10.5. The inclusion of a basal amount of CSM to the diet (20% of the total dry mass of the experimental food, which contributed 3.68% protein and 10.0% carbohydrate to each treatment) was necessary because initial pilot studies demonstrated that a pure synthetic diet (as used for grasshoppers (see Behmer et al. (2001)) did not support development of caterpillars from hatch to eclosion. The remaining 80% of the experimental diet was synthetic (originally based on a recipe for grasshoppers (Dadd 1961), modified later by Simpson and Abisgold (1985), and then modified further for caterpillars by Simpson et al. (1988)). The protein portion of the synthetic diet was a 3:1:1 mixture of casein, peptone, and albumen, while digestible carbohydrate was sucrose. Other nutrients in the synthetic diet included Wesson's salt (1.92%), cholesterol (0.4%), linoleic acid (0.4%), ascorbic acid (0.24%) and a vitamin mix (0.16%), with the remaining portion being non-nutritive cellulose. These dry ingredients were presented to the insects suspended in a 1:6 ratio in 1% agar solution. Mold inhibitors in the form of Aggie Microbial Inhibitor (Roeder et al. 2009) at 0.5ml per 200 ml, formaldehyde at 0.1ml per 200 ml, and methyl paraben at 0.4 grams per 200 ml were added to the wet diet mixtures of each treatment after the combination of dry and agar components had been completed.

Larval Protocol

Newly hatched neonates were randomly allocated to one of the nine diet treatments at hatch. There were sixty replicates per treatment and all treatments were run concurrently. Blocks of diet, each weighing approximately 1000 mg, were placed in arenas and replaced with fresh diet blocks of equivalent size every three days. Upon entrance into the 4^{th} instar, arena lids were perforated with small holes for ventilation to reduce high humidity (pilot studies revealed that high humidity levels negatively affected performance of late instar caterpillars (K.A.R. personal observation)). For each arena two measures of performance were recorded: (1) whether larvae pupated (survival success to the pupal stage) and (2) for those that pupated, the length of time it took to become a pupa (with this data I could also measure total development time, in days, from hatch to pupation).

Pupal Protocol

Five days after the larvae pupated, their mass and sex were recorded. I then split the pupae from each treatment into two groups. The first group, which was set-aside for mating experiments, contained 65% of the pupating individuals. The individuals for the mating experiment were randomly selected, but an equal number of males and females were selected (in order to maintain a 1:1 male-female sex ratio). These individuals were transferred to new arenas that contained a small square of damp paper towels, which increased eclosion success (K.A.R. pers. observation). For the individuals selected for mating, eclosion success was recorded as well as the number of days between pupation

and eclosion. The remaining 35% of the pupae were frozen and set aside for lipid extractions. Frozen pupae were dried to constant mass at 70° C, weighed to the nearest 0.1 mg and lipid extracted in three, 24-hour changes of chloroform before being re-dried and re-weighed (Loveridge 1973).

Adult Protocol

Overall survival success was calculated by subtracting 35% of the pupating individuals for each diet from the starting 60 individuals, creating a revised starting population size. The number of eclosing adults was then divided by this revised population number in order to determine an average total survival percentage for each diet. For individuals that successfully eclosed, development time was recorded in days. Upon successful eclosion, a single male and female from the same diet treatment were randomly paired and placed into breeding arenas for six days. These breeding arenas were composed of two key components. The first was a capped 50ml Corning plastic tube, standing upright, which held the mating pair. The second component was a 1.5ml VWR centrifuge tube filled with a 10% sucrose solution, pushed through a hole drilled in the cap on the corning plastic tube. A small hole had been drilled in the 1.5ml centrifuge tube, which allowed moths to access the sucrose solution. There was also a hole at the bottom of the Corning plastic tube that prevented leaked sucrose solution from building up in the bottom of the Corning plastic tube. Inside each large tube was a small sheet of paper towel for females to place their eggs. This sheet fit loosely inside the larger tube, and covered the entire tube in a single layer. The paper towel strip was

changed every two days. Adult moths were monitored daily, and when death occurred it was recorded and the dead adult was removed. Eggs were counted on each sheet and then placed into separate sealed deli cups in order to monitor offspring viability for each mating pair. Viability was also calculated, by dividing the total number of hatchlings by the number of eggs laid.

Statistical Analysis

Analyses were run using JMP 7.0.2. (SAS Institute Inc). Logistic regressions were used for survival success to the pupal stage, from the pupal to the adult stage, and for the total overall survival with odds ratios to make comparisons between treatments. Survival analyses were used for developmental time to pupation, from pupation to eclosion, and for the total time from neonate to eclosion with post hoc contrast comparisons. ANOVA was run to compare pupal wet and dry mass as well as the body lipid content (%) with Tukey post-hoc tests. Additionally, logistic regression was used to determine the significance of egg producing pairs across all treatments, and ANOVA was run to compare the average egg production and viability of egg producing pairs.

Results

Our results are divided into three sections based on the developmental stage (larval, pupal, and adult) of the experimental insects.

Larval Performance

Larval survival success, recorded as the percent of individuals pupating, was significantly different between treatments (Logistic regression: df = 6, $\chi^2 = 18.06$, $P = 0.006$). It was highest on the slightly carbohydrate rich p17.5:c24.5 diet, although survival success on the p14:c28 and p21:c21 diets did not differ statistically compared to the p17.5:c24.5 diet. Survivorship was lowest on the most protein-biased diet (p31.5:c10.5), but it did not differ statistically compared to the other protein-biased diets (p28:c14 and p24.5:c17.5), or the most carbohydrate-biased diet (p10.5:c31.5) (Figure 2.1a).

I also observed significant differences in development time (from hatch to pupation) between treatments (Survival analysis: df = 6, $\chi^2 = 72.52$, $P < 0.001$). Development was fastest on the p24.5:c17.5 diet, but there was no difference between treatments with at least 17.5% protein (Figure 2.1b). Development took the longest on the extremely carbohydrate-biased diet (p10.5:c31.5).

Pupal Performance

Upon pupation I recorded the sex of the pupae and then measured pupal wet mass (5 days post pupation), survival success (scoring whether or not they eclosed), and development time (days from pupation to eclosion). Additionally, for a subset of pupae on each treatment, I also recorded whole-body lipid content. With respect to pupal wet mass I observed a significant treatment effect (ANOVA: $F_{6,301} = 3.40$, $P = 0.003$), but no sex or treatment-by-sex interaction (ANOVA: $F_{1,301} = 3.48$, P = 0.063, and $F_{6,301} = 1.58$,

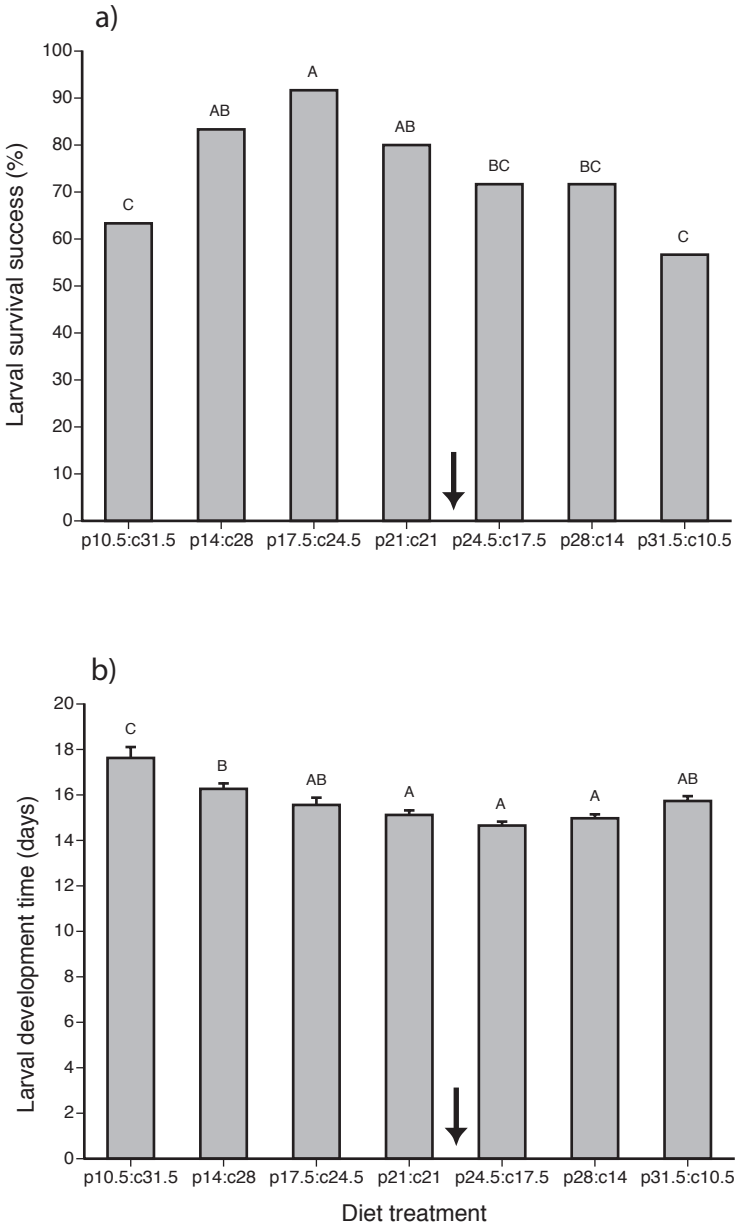

Fig. 2.1. Larval performance measures. Panel (A) shows survival success, measured as a percent. Panel (B) shows the mean (±SE) development time for larvae that successfully pupate. Panel (C) shows the mean (±SE) pupal wet mass.

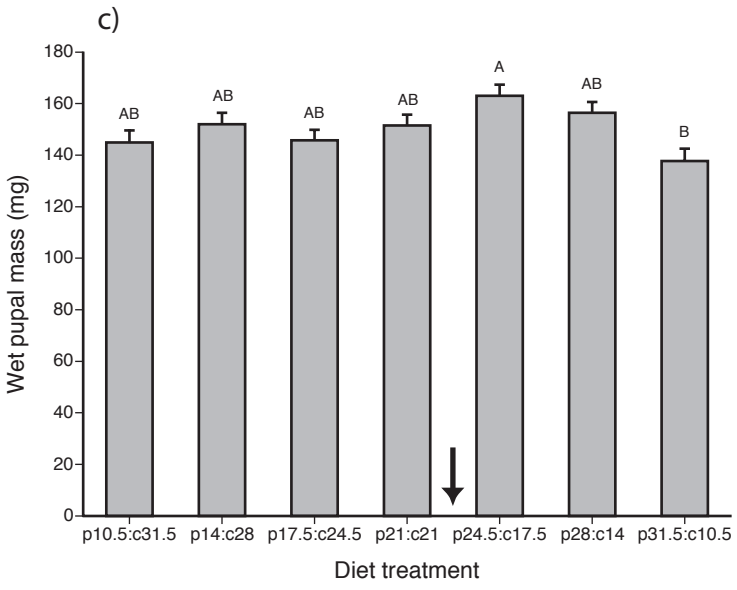

Fig. 2.1. continued

P = 0.152, respectively). Pupal wet mass was highest on the p24.5:c17.5 diet and lowest on the p31.5:c10.5 diet, but there was no statistical difference in wet mass between the other diets, and these diets did not differ compared to the former diets (Figure 2.1c).

Survival from the pupal to adult stages differed significantly between treatments (Logistic regression: df= 6, $\chi^2 = 68.17$, $P < 0.001$). Pupal survival was best on the balanced (p21:c21) and slight imbalanced diets (p17.5:c24.5 and p24.5:c17.5), and then dropped off in a symmetric fashion as the diets became more nutritionally imbalanced in both directions (Figure 2.2a). However, food macronutrient content had a much greater effect on survival success of male pupae compared to female pupae. Female survival was high on all but the most carbohydrate-biased diet (Figure 2.3a), while male survival was best on the p24.5:c17.5 diet, intermediate on the balanced (p21:c21) and slight carbohydrate-biased (p17.5:c24.5) diets, and then dropped-off greatly on diets with more extreme p:c imbalances (Figure 2.2a).

Pupal development time also differed significantly between the diets (Survival analysis: df = 6, $\chi^2 = 97.61$, $P < 0.001$), although this was mostly the result of longer development on the most carbohydrate-biased diet (Figure 2.2b). Additionally, females tended to develop faster than males. The only exception was on the most carbohydrate-biased diet, where male and female development time was similar (Figure 2.2b).

Finally, the lipid content of pupae (calculated on a dry mass basis) differed significantly between treatments (ANOVA: $F_{6,90} = 31.93$, $P < 0.001$). It was highest on the two most carbohydrate-biased diets, intermediate on diets with equal or slightly imbalanced p:c ratios, and lowest on the two most protein-biased diets (Figure 2.3). I

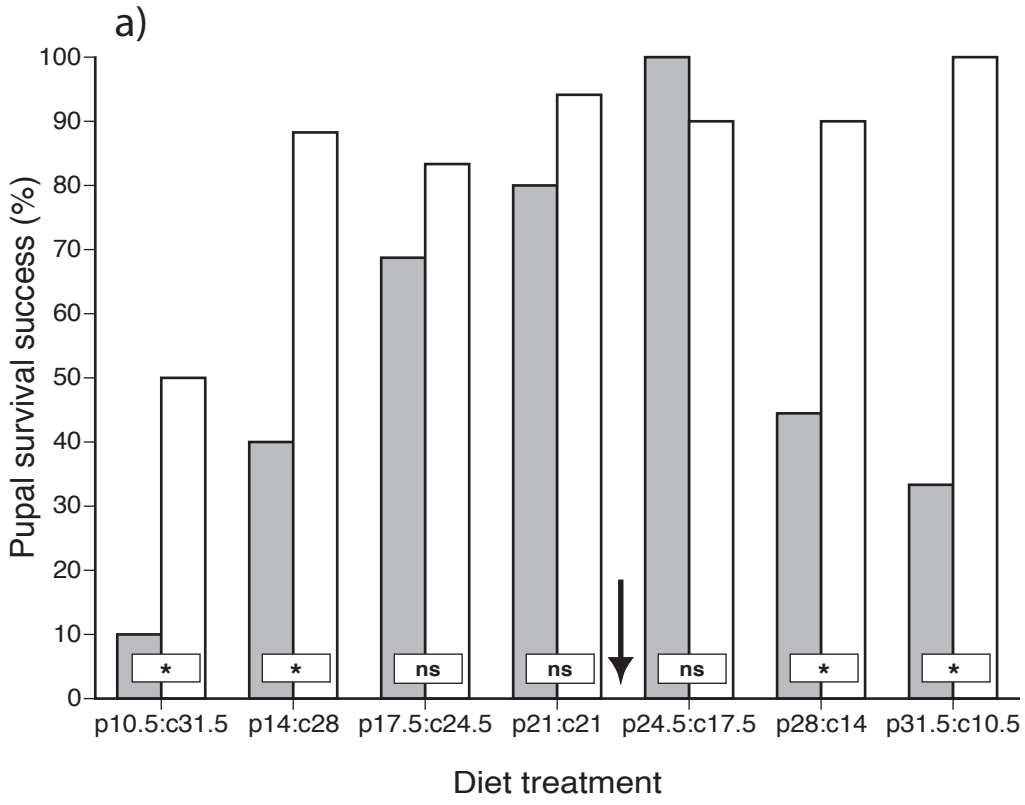

Fig. 2.2. Pupal performance measures. Panel (A) shows survival success, measured as a percent. Panel (B) shows the mean (±SE) development time for pupae that successfully eclose. Males (grey bars) and females (white bars) are shown seperately for each treatment. Small white boxes between bars identify significant differecnes between the sexes (ns= not significant and *= significant).

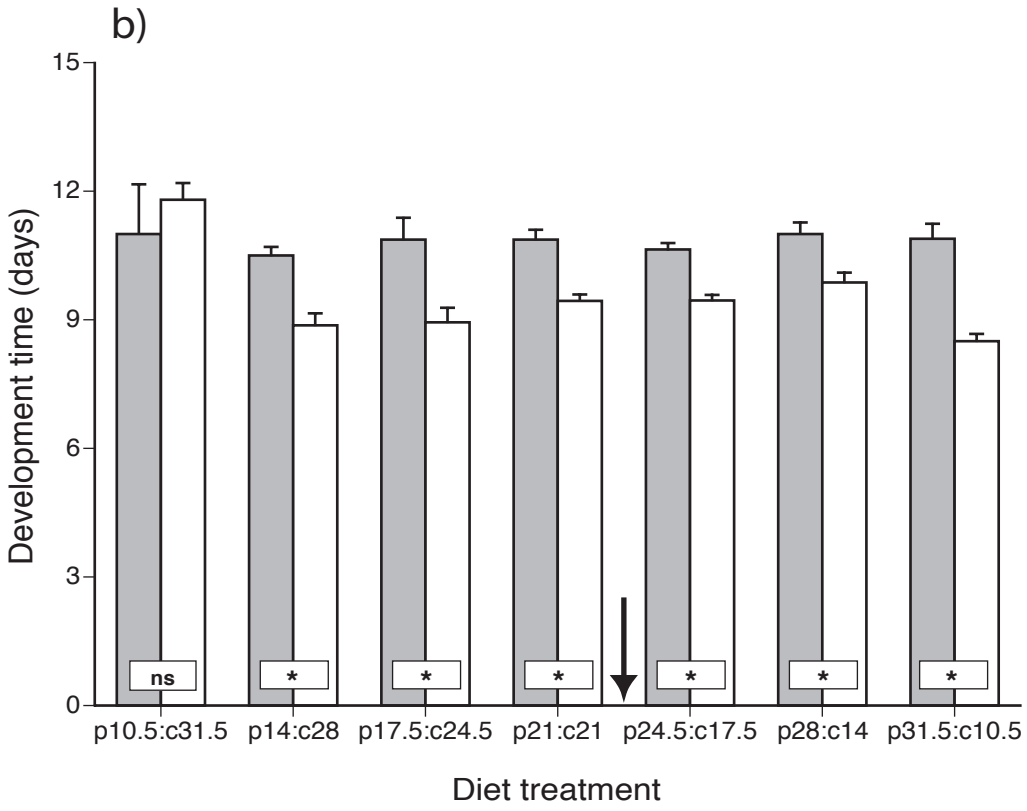

Fig. 2.2. continued

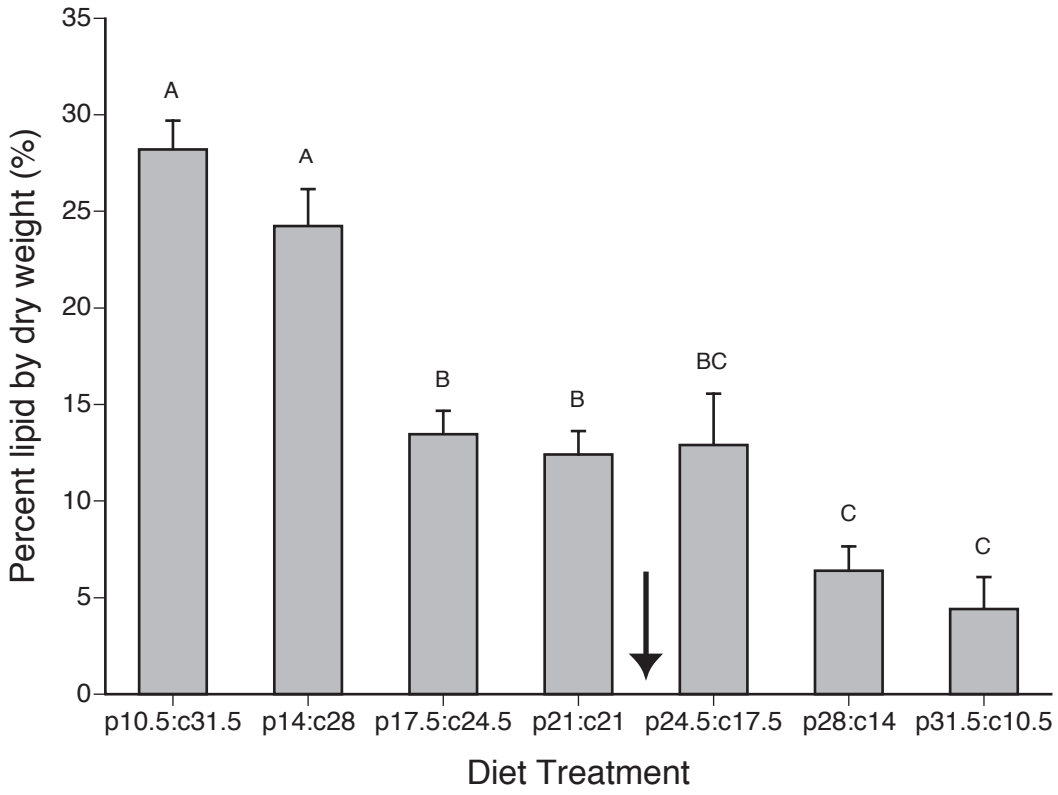

Fig. 2.3. Pupal performance measure. Panel shows the mean (±SE) percent body lipid content.

also observed a significant sex effect (ANOVA: $F_{6,90} = 6.86$, $P = 0.010$), with females having a higher average lipid content, but did not observe a significant treatment-by-sex interaction (ANOVA: $F_{6,90} = 0.31$, $P = 0.933$),

Cumulative Performance and Adult Reproduction

I analyzed total survival success (did individuals survive from hatch through to eclosion) and development time (days from hatch until eclosion). Survival from hatch to eclosion was significantly different across the seven treatments (Logistic regression: df = 6, χ^2 = 41.53, $P < 0.001$). It was highest on the diets with balanced (p21;c21), and slightly imbalanced p:c ratios (p17.5:c24.5 and p24.5:c17.5), but steadily declined as the p:c ratios of the diets became more imbalanced (Figure 2.4a). Development time from hatch to eclosion was also significantly different across the treatments (Survival analysis: df = 6, χ^2 =37.69, $P < 0.001$). It was equally fast on diets with balanced or protein-biased p:c ratios (Figure 2.4b), and slowest on the extremely carbohydrate-biased diet.

I also recorded the total number of mating pairs generated on each treatment, the number of egg producing pairs, the number of eggs for successful mating pairs, plus egg viability (Table 2.1). The number of mating pairs and egg producing pairs was highly variable due to the different eclosion and survival rates displayed across the treatments. When comparing the number of pairs to a hypothetical population that had 100% survival and the same 35% of individuals removed for lipid analyses, both the number of mating pairs (Logistic regression: df= 6, $\chi^2 = 31.38$, $P < 0.001$) and the number of egg

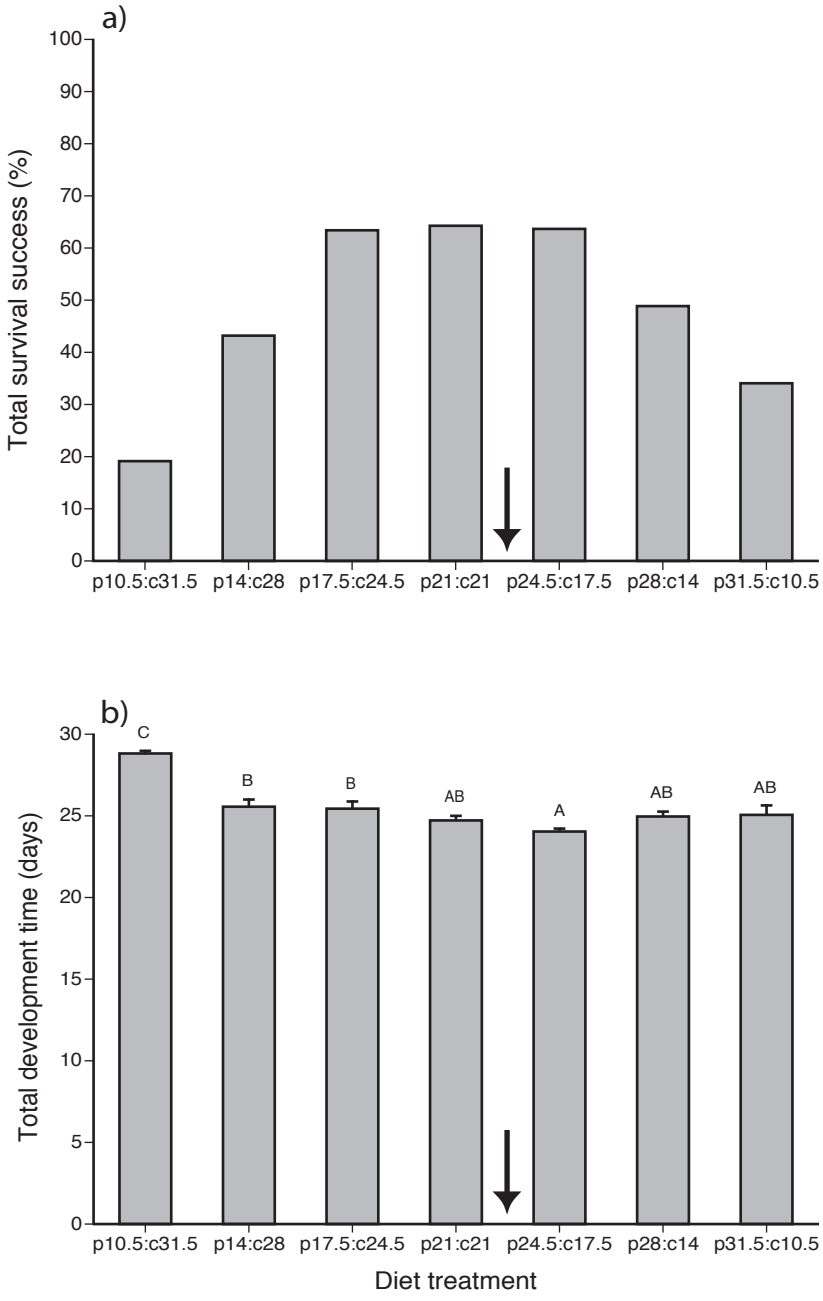

Fig. 2.4. Cumulative performance and adult reproduction. Panel (A) shows survival success, from hatch to successful eclosion, measured as a percent. Panel (B) shows the mean (±SE) development time for individuals from hatch to successful eclosion.

Table 2.1 Total values for number of mating pairs, number of egg producing pairs, the mean (±SE) values for average egg production per egg producing pair, and the average egg viability for each treatment.

Treatment	Mating pairs	Egg producing pairs	Mean eggs per egg producing pair	Average egg viability (%)
p10.5:c31.5	0	0	0	0
p14:c28	6	5	166 ± 33	65.3
p17.5:c24.5	11	5	173 ± 53	75.1
p21:c21	12	9	230 ± 32	82.5
p24.5:c17.5	11	9	162 ± 25	81.4
p28:c14	8	6	199 ± 52	81.7
p31.5:c10.5	4	2	358 ± 60	65.1

producing pairs (Logistic regression: df= 6, $\chi^2 = 22.14$, $P = 0.001$) were statistically different across treatments. However, the average egg production per mating pair that could produce eggs was not significantly different between treatments (ANOVA: $F_{5,30} = 1.70$, $P = 0.165$). The highest average egg production per mating couple was found on high protein, low carbohydrate diets while the lowest egg producing couples were found on low protein, high carbohydrate treatments and the slightly imbalanced treatments were not found to be significant from one another (Table 2.1). All treatments that produced mating pairs were capable of producing viable young, however the range of viability was significantly affected by the p:c ratio of a diet (ANOVA: $F_{5,30} = 5.48$, $P = 0.001$).

Discussion

The general trend among generalist caterpillars with respect to protein and carbohydrate regulation is that they self-select foods in such a way as to ingest more protein than carbohydrate, or at the minimum maintain a balanced protein-carbohydrate intake (reviewed by Behmer 2009). Often, though, caterpillars may be unable to regulate their macronutrient intake, but currently the full consequences of caterpillars eating a nutritionally suboptimal diet are poorly understood. In large part this is because most nutritional studies have only explored performance in the final stadium (e.g., Despland and Noseworthy 2006, Telang et al. 2001, Lee et al. 2006). In this current paper I build a comprehensive picture of how a food's nutritional qualities, particularly its macronutrient content, can affect an herbivorous insect over its entire lifetime,

including its reproductive output. My results demonstrate three key findings. First, self-selected protein-carbohydrate intake targets obtained during the final instar likely represent the optimal diet for the entire larval development period. Second, males are much more sensitive to nutritional imbalances than are females, but this effect is only revealed at the time of eclosion. Third, when larval and pupal performance are combined with reproductive output, it becomes clear that there is specific protein-carbohydrate ratio that is optimal, and that small deviations away from this intake target has strong negative consequences at the population level.

H. virescens and other lepidopteran species have been used in many previous studies that measured larval performance on fixed macronutrient foods (Lee 2007, Raubenheimer and Simpson 2003, Despland and Noseworthy 2006, Telang et al. 2001, Lee et al. 2002, 2003, 2004a, 2004b, 2006), but most of these studies only examined performance values for the final larval instar (with the single exception of Despland and Noseworthy 2006, who started their study with 2nd instar caterpillars) . Lee et al. (2006) studied last instar *H. virescens*, and identified a self-selected intake target between p21:c21 and p28:c14. In the current study survival values were highest (~90%) on a slightly carbohydrate enriched diet (p17.5:c24.5), and then dropped off gradually as the diets became both more carbohydrate-biased (e.g. survival on the most carbohydrate-biased diet was ~60%), and more protein-biased (survival was also ~60% on the most protein-biased diet). These results demonstrate the importance of exploring the effect of diet macronutrient profile on survival over the entire larval development period, as most other lepidopteran studies typically fail to show differences in survival when only a

single developmental stage is examined (e.g. Lee et al. 2006). In fact, in most of these studies survival across all diets is often 100%.

The best protein-carbohydrate combination, in terms of rapid development (from hatch to pupation), size (wet pupal mass), and growth rate (a performance measure that combines the two former variables combined), was the p24.5:c17.5 diet. This protein-carbohydrate ratio closely matches the self-selected protein-carbohydrate intake target seen in final stadium *H. virescens* caterpillars (Lee et al. 2006). Interestingly, though, protein-carbohydrate ratios do not seem to have large effects on development and final body size. The lack of large differences in larval performance across the p:c ratios examined in the current study might be the result of compensatory feeding. Although amounts of food eaten were not measured in the current study, Lee et al. (2006) using a similar range of diets, showed that *H. virescens* caterpillars on carbohydrate-biased diets ingested more food relative to caterpillars on high-protein diets. The outcome of increased food consumption was that caterpillars across all diet treatments ingested similar total amounts of protein. Rapid development and high protein consumption are often considered evolved traits in generalist caterpillars since both are thought to reduce the risk of predation and parasitism under natural conditions (Lee et al. 2006). However, a consequence associated with protein-driven compensatory feeding is that carbohydrates were eaten in excess of their requirements, and as a result caterpillars on these diets showed greatly elevated lipid content (on the two most carbohydrate-biased diets fat body content was near 25%). In contrast, caterpillars on the two most protein-biased diets ate less carbohydrate, and as a result showed incredibly low body fat levels

(~5-6%). For *H. virescens*, the ideal body fat content, based on the treatments that correlate with the best performance, seems to be about ~13%.

The current study is the first, to my knowledge, to explore the effects of food protein-carbohydrate content on eclosion success in a holometabolous insect. When eclosion success was measured, across both sexes, the best protein-carbohydrate combinations for eclosion success were the diets with equal or near-equal p:c ratios (p17.5:c24.5, p21:c21, and p24.5:c17.5). However, when males and females eclosion success was compared within treatments, male eclosion success, as compared to female eclosion success, was shown to be much more sensitive to diet p:c ratio. In particular, males on the two most carbohydrate-biased and two most protein-biased diets suffered significantly higher mortality than did females. These results suggest that males are much less well equipped to deal with extreme nutrient imbalances than are females. It is not clear why males would suffer more on high-carbohydrate diets compared to females. Males and females showed similar fat levels on the different diets, so perhaps females are better suited for handling high body lipid levels than are males. In contrast, fat levels were low for both males and females on the high-protein diets. Here males might suffer from nitrogen toxicity, as they attempt to increase their carbohydrate intake. This might occur because female caterpillars, but not males, have the ability, via storage proteins, to deal with excess nitrogen (Telang et al. 2001). The collection and storage of nitrogen is important during larval development as female moths and butterflies rarely consume nitrogen in the adult stage.

A second measure of pupal performance was pupal development time. Here, as was the case for larval development time, the time from pupation to eclosion was relatively similar across most treatments, except for the biased carbohydrate diet (p10.5:c31.5), although this difference was not large. However, even small differences in development might be important in the field, as extended development can increase the risk of predation and parasitism (Moran and Hamilton 1980, Benrey and Denno 1997, Lee et al. 2006).

This is also the first study, to my knowledge, to quantify the effects of diet p:c ratio on reproductive output in an adult insect herbivore. The reason for this lack of data relates to complications between the ingredients and the reproductive ability of individuals (Dadd 1960, Cavanagh 1963). When the reproductive data was compared, the highest egg production was seen on the highest protein diet; however this treatment only produced two pairs capable of laying eggs from a potential four out of a starting sixty caterpillars, while other treatments had over ten couples laying eggs at various amounts. This increase in egg production for lepidopterans on higher protein diets may be similar to the way diet quality, and especially the N content, has been shown to not only affect the rate of egg production but also the size of the laid eggs in grasshoppers (Joern and Behmer 1997). Egg size was unfortunately not measured in this study so a complete comparison between hemi and holometabolous insects cannot be made, but our results do offer similar findings to those that indicate increasing protein levels are generally associated with a higher offspring production rate.

The effects of a diets p:c ratio are even more pronounced when they are projected at the population level. I did this by using our data for survival success, egg production, and offspring viability to create an estimate of how large a population of *H. virescens* could grow if it was maintained on foods with different p:c ratios (sensu Behmer and Grebenok 1998). Each treatment was designated a starting population of 100 individuals in a 1:1 sex ratio, with no assumed mortality from biotic or abiotic factors. These individuals were run through multiple generations using our previously determined performance values in order to create estimated populations. Interestingly the slightly protein-biased food (p24.5:c17.5) which had previously been shown here, and by others (Telang et al. 2001, Lee et al. 2006) to produce better performing late instar *H. virescens* larvae, did not produce the largest population. Instead, the largest population growth was observed on the balanced diet (Figure 2.5). After only the third generation, the balanced diet (p21:c21) produced an estimated population size that was almost double that of two closest diets (p17.5:c24.5 and p24.5:c17.5), three times greater than the two most protein-biased diets, and 6x better than the p14:c28 diet.

In conclusion, my results indicate that for *H. virescens* a balanced to slightly protein enriched diets are optimal in terms of lifetime performance. Key questions concerning study length importance were addressed through the use of multiple developmental stages, and this study shows that in general the protein-carbohydrate intake target selected by caterpillars in single stadium studies (usually the final instar) correlate well with the performance of multiple stadium studies. The combined results, including the effects at the population level, indicate that the diet macronutrient content

has important effects on insect herbivores, and that even small departures from an optimal p:c ratio can have dramatic effects. Perhaps subtle changes in nutrient quality of available host plants has a much greater impact on population levels, and by extension community level patterns, than has been previously recognized.

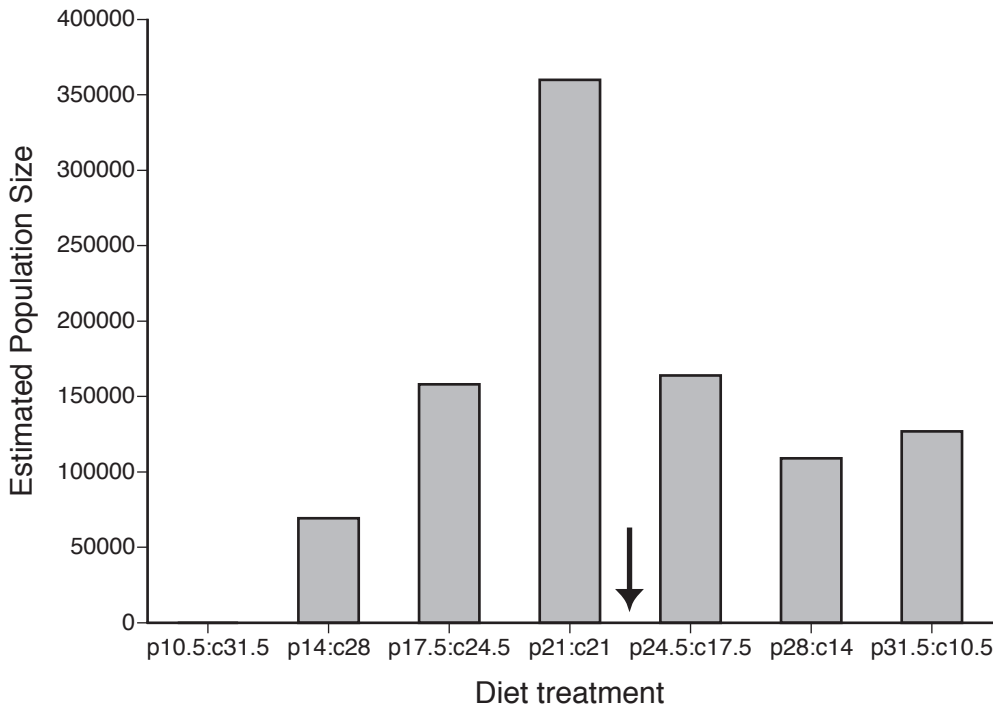

Fig. 2.5. Estimated population size at the end of the third generation of *H. virescens* caterpillars. Population sizes were based on the survival success, egg production, and offspring viability for each treatment.

CHAPTER III

THE EFFECT OF FOOD MACRONUTRIENT BALANCE AND AMOUNTS ON PERFORMANCE AND ELEMENTAL BODY COMPOSITION IN A GENERALIST INSECT HERBIVORE

Overview

Ecological stoichiometry, or the study of the balance of energy and materials in living systems, has previously focused on aquatic invertebrate systems due to the relative amount of control needed to measure the flow of elements. Little attention has thus been given to terrestrial systems that concentrate on the elemental flow in herbivorous insects. In particular, the studies that have previously looked at stoichiometry in insects have done so in a limiting manner that primarily focused on three key elements: carbon, nitrogen, and phosphorous. Currently, there is only one study on hemimetabolous grasshoppers that has investigated how macronutrient content influences the elemental body composition of an insect. Here I manipulated both the protein to carbohydrate ratio (p:c) and the total amount of macronutrient content in order to explore the performance and elemental consequences associated with each using *Heliothis virescens* Fabricus (Lepidoptera: Noctuidae) throughout their entire larval development. Many of the highest values for performance were seen on a slightly protein-biased ratio (p1.4:c1) when protein levels were highest (28%). Elemental results indicate that the amount and concentration of elements varied according to macronutrient ratio and/or amount that were being consumed. I discuss these results in the context of ecological stoichiometry

using a geometric framework to explain the relationships between macronutrient amounts and ratios in order to better understand how an insect performs and builds itself.

Introduction

All animals eat to obtain key nutrients that are required to fuel the processes of growth and reproduction (Chapman 1998, Schoonhoven et al. 2005). However, different foods can often vary with respect to the types and amounts of nutrients they contain. This is particularly true for animals that feed on plants, which are known to vary with respect to a broad range of important nutrients (Bernays and Chapman 1994). Two key nutrients that can be highly variable in plants, and which are known to influence insect performance, are protein and digestible carbohydrates (hereafter "carbohydrates"). The manner in which insect herbivores respond to variation in their food protein-carbohydrate level can best be understood using the experimental approach of the geometric framework (reviewed in Raubenheimer and Simpson 1999, Behmer 2009). The geometric framework (hereafter "GF") is a state-space modeling approach designed to study how an animal balances the intake of multiple nutrients in response to changing nutritional needs in multi-dimensional and variable nutritional environments. The GF was originally designed to study, in locusts and caterpillars, the multiple interactions among mechanisms regulating the intake of different classes of nutrients (Raubenheimer and Simpson 1993, 1997, 1999, Simpson and Raubenheimer 1993, 1995, 2001). Over recent years, though, it has also been used to explore nutrient regulation and interactions in a broad range of organisms, including chickens (Raubenheimer and Simpson 1997),

rats (Simpson and Raubenheimer 1997), mice (Sorensem et al. 2008), fish (Ruohonen et al. 2007), and even humans (Simpson et al. 2003).

The GF is also a useful tool for exploring nutrient utilization in insects, including the rate and efficiency of conversion of ingested carbohydrates and proteins into body lipids and body nitrogen (N), respectively. Recently, though, a stoichiometric approach, where the emphasis is on the flow of important biological elements from resources (e.g. plants) to consumers (e.g. insect herbivores), has also been used. This approach, called ecological stoichiometry (henceforth "ES"), is the study of the balance of energy and multiple chemical elements in ecological interactions (Sterner and Elser 2002), and it has become popular due to its recognition of species-specific regulatory physiology as the basic unit in ecological processes (Raubenheimer and Simpson 2004). Additionally, ES has broadened past emphasis on single variable studies (e.g. ones that focus solely on energy) to include several dimensions that examine multiple nutrients and energy (Reiners 1986, Sterner and Hessen 1994, Raubenheimer and Simpson 2004). ES has been successfully used to measure the energy and elemental flow in snails (Stelzer and Lamberti 2002), zooplankten (Boersma and Kreutzer 2002), insects (Perkins et al. 2004), fish (Borlongan and Satoh 2001), and birds (Grone et al. 1995) across terrestrial, marine, and freshwater ecosystems. However, since most ES studies have focused primarily on aquatic invertebrate systems (Karimi and Folt 2006, Frost et al. 2004), due to the relative amount of control needed to measure the flow of elements, few terrestrial systems have truly explored ES beyond measuring the effect of phosphorous levels in food (Fagan et al. 2002, Schade et al. 2003, Bertram et al. 2006, 2008). This discrepancy has prevented

a number of different physiological questions that specifically compare elemental imbalances in nature and how these imbalances directly affect a consumer's physiology and life history from being answered.

Despite being developed independently of one another, the GF and ES have many similarities. An important one is that both recognize that animals are often faced with potential imbalanced mixtures of energy, nutrients and important elements (Sterner and Elser 2002), and where this occurs it can place strong constraints on growth and reproduction (Brunning 1991, Sterner and Schulz 1998, Aerts and Chapin 2000). But there are also key differences, most notable being that the GF places a greater emphasis on absolute amounts of biomolecules (e.g. protein and digestible carbohydrates) consumed, retained and excreted, while ES primarily focuses on the concentration of elements (namely C, N, and P) in food and consumers (Raubenheimer and Simpson 2004). These differences are significant for a number of reasons. First, it is important to recognize that insects have evolved regulatory mechanisms for nutrient biomolecules, in particular protein (which contains amino acids, and thus N) and digestible carbohydrates (the key energy source), not for elements. Second, regulation of protein and digestible carbohydrates often takes precedence over other classes of nutrients (reviewed in Behmer 2009). Third, focusing on the elemental composition of foods can be problematic. For example, much of the carbon found in an insect herbivore's food is unavailable, because insects cannot digest cellulose (which often makes up more than 50% of a plants biomass (Martin et al 1991)). However, ES is a very useful approach because elements are a useful way to measure how an animal builds itself from the foods

it consumes, and linking important nutrient biomolecules, like protein and carbohydrates, with elemental body composition can provide novel insights into how tightly insect herbivores practice elemental homeostasis, and how food macronutrient content influences this physiological process (Frost et al. 2005). Measuring elemental flow is also important understanding ecosystem processes and functioning (Sterner and Elser 2002).

In the current paper I borrow experimental approaches both from the GF and ES to explore how food macronutrient content influences performance of an insect herbivore. I do this by rearing the generalist caterpillar, *Heliothis virescens*, from hatchling to pupa on a range of synthetic diets that differ in their protein-carbohydrate ratios and/or absolute amounts. This caterpillar has a very broad diet, at both the individual and population level (Neunzig 1969, Schneider et al. 1986), so different individuals are likely to encounter a broad range of macronutrient ratios in their food. For each insect I measure three key performance variables (survival rate from hatch to pupation, development time from hatch to pupation, and mass gain during the larval stadium), and then construct a composite variable that integrates these three different performance variables. I also explore how the protein-carbohydrate profile of a caterpillar's food affects its elemental composition (C, N, P, S, Na, K, Ca, Mg, Mn, Fe, Zn, Cu), as well as total lipid body levels. A key aim of this paper is to understand whether food macronutrient ratio/balance, or total macronutrient content is more critical for insect herbivores. I discuss these findings in relation to previous studies that have

explored the effects of food macronutrient content on insect herbivores, and their potential ecological implications.

Materials and Methods

Experimental Insects

Caterpillar eggs were obtained from a *Heliothis virescens* culture at North Carolina State University. These eggs came from adult female moths, which had been previously reared on a corn-soy-milk base diet (CSM) that had been modified from Burton (1970). All experimental neonates hatched at approximately the same time and within a few hours of hatching they were transferred, using a fine tipped paint brush, to 2 oz. Solo cups that contained a block of experimental food (see below). A lid was placed on each individual cup, and all cups containing caterpillars were transferred to an insect growth chamber (Percival Scientific Biological Incubator, Model I-66VL) set at 29ºC +/- 1ºC with a 12h: 12h L: D photoregime.

Experimental Diets

A total of twelve CSM-based diets that differed in their protein (p) and carbohydrate (c) content were used for this experiment. In total there were 3 protein concentrations (14, 20, and 28%) and 4 digestible carbohydrate concentrations (10, 14, 20, and 28%), and the total macronutrient content of these twelve diets ranged from 24-56%: (1) p14:c10 (14% protein and 10% carbohydrate; combined macronutrient content = 24%), (2) p14:c14, (3) p14:c20, (4) p14:c28, (5) p20:c10, (6) p20:c14, (7) p 20:c20,

(8) p20:c28 ,(9) p28:c10, (10) p28:c14, (11) p28:c24, and (12) p28:c28. A two dimensional plot of the various treatments graphically depicts the various locations of each protein-carbohydrate combination within nutritional space (*sensu* Raubenheimer & Simpson 1999).

The inclusion of a basal amount of CSM to each diet (20% of the total dry mass of the experimental food, which contributed 3.68% protein and 10.0% carbohydrate to each treatment) was necessary because initial pilot studies demonstrated that a pure synthetic diet (as used for grasshoppers (see Behmer et al. 2001)) did not support full development of caterpillars from hatch to pupation. The remaining 80% of the experimental diet was synthetic (originally based on a recipe for grasshoppers (Dadd 1961), modified later by Simpson and Abisgold (1985), and then modified further for caterpillars by Simpson et al. (1988)). The protein portion of the synthetic diet was a 3:1:1 mixture of casein, peptone, and albumen, while digestible carbohydrate was sucrose. Other nutrients in the synthetic diet included Wesson's salt (1.92%), cholesterol (0.4%), linoleic acid (0.4%), ascorbic acid (0.24%) and a vitamin mix (0.16%), with the remaining portion being non-nutritive cellulose. These dry ingredients were presented to the insects suspended in a 1:6 ratio in 1% agar solution. Mold inhibitors in the form of Aggie Microbial Inhibitor (Roeder et al. 2009) at 0.5ml per 200 ml, formaldehyde at 0.1ml per 200 ml, and methyl paraben at 0.4 grams per 200 ml were added to the wet diet mixtures of each treatment after the combination of dry and agar components had been completed This table also lists concentrations for 10 additional elements.

Experimental Protocol

Newly hatched neonates were randomly allocated to one of the twelve diet treatments at hatch. There were sixty replicates per treatment and all treatments were run concurrently. Blocks of diet, each weighing approximately 1000 mg, were placed in arenas and replaced with fresh diet blocks of equivalent size every three days. Upon entrance into the 4th instar, arena lids were perforated with small holes for ventilation to reduce high humidity (pilot studies reveled that high humidity levels negatively affected performance of late instar caterpillars (K.A.R. personal observation)). For each arena two measures of performance were recorded: (1) whether larvae pupated (survival success to the pupal stage), and (2) for those that pupated, the length of time it took to pupate (in days, from hatch to pupation).

Five days after the larvae pupated, individuals were removed from their arenas, weighed for wet mass on an excellence plus XP analytical balance (Mettler Toledo), and then sexed. Pupae were then frozen till each individual had been weighed, after which they were dried to constant mass at 70° C and then reweighed to the nearest 0.1 mg for dry mass. Three sets of ten individuals (or evenly distributed groups depending on survival rates) with an equal male: female sex ratio were dried to constant mass at 70° C and set aside for lipid extractions, carbon and nitrogen analyses, and elemental body composition analyses.

The first set of pupae used for lipid extractions were washed three times over three days in vials with chloroform, and redried to constant mass at 70° C (Loveridge 1973). They were then reweighed in order to measure the change in body mass that

occurred during the extraction and an overall lipid percent was determined. The second group of ten insects was prepared for nitrogen (N) and carbon (C) analyses by vortexing individual dried pupae over a thirty second time period followed by a reweighing to the nearest 0.1mg (modification of a technique demonstrated in Boswell et al. 2008). The samples were then wrapped in small sheets of tin foil, placed in steel crucibles, and burned in an Elementar vario MAX CN high temperature carbon-nitrogen analyzer that was set at 950º C. Results were analyzed using methods demonstrated by McGeehan & Naylor (1988). The third set of pupae was vortexed into a fine powder, weighed to the nearest 0.1 mg, and then transferred to polypropylene digestion tubes. The samples were then digested using trace metal grade nitric acid on a 105º C graphite block and analyzed using a Spectro axial CIROS inductively coupled plasma – Atomic Emission Spectrometry (Havlin and Soltanpour 1980) in order to measure elemental body composition. Each diet treatment was also tested for C and N analyses as well as elemental composition so later comparisons between an insect's body and the composition of the food it ate could be made (Values listed in Table 3.1). Finally, an estimated overall performance measurement was calculated using the total wet mass of pupating individuals, their associated larval development time, and the average survival percentage for that each of the selected diets

Statistical Analysis

Analyses were run using JMP 7.0.2. (SAS Institute Inc) to examine how the balance and ratio of proteins to carbohydrates affected an insect’s performance and body

Table 3.1. Elemental concentrations (expressed as a % or ppm) present in 12 artificial diets with different protein-carbohydrate ratios (p = protein, c = carbohydrate; the numbers in each treatment represent the amount of protein and carbohydrate, respectively (expressed as a percent dry mass of the diet)).

Diet	C (%)	N (%)	P (%)	S (ppm)	K (ppm)	Na (ppm)	Ca (ppm)	Mg (ppm)	Zn (ppm)	Fe (ppm)	Mn (ppm)	Cu (ppm)
p14:c10	43.61	2.57	6.72	21058	83154	27533	53053	4720	117	414	17	152
p14:c14	43.77	2.64	4.55	20732	69831	24027	44463	3511	116	337	22	61
p14:c20	43.67	2.38	5.26	18173	68207	21649	40561	3370	104	287	18	49
p14:c28	43.41	2.19	3.66	16902	57289	20886	39499	2670	101	417	22	60
p20:c10	44.13	3.37	5.89	25332	68340	26795	41147	3767	141	456	18	48
p20:c14	43.97	3.24	5.19	24498	69528	26930	43727	3601	150	483	20	69
p20:c20	43.96	3.19	4.41	22709	62523	25315	40426	2997	194	469	22	60
p20:c28	43.79	2.92	4.24	21035	56520	22131	36132	2706	113	397	20	55
p28:c10	44.52	4.19	5.69	33068	69587	30609	39287	3823	164	1396	20	53
p28:c14	44.52	4.19	5.82	30541	66025	29672	38743	3488	158	461	19	51
p28:c20	44.38	3.86	4.78	28312	61253	27674	38024	2839	160	435	21	58
p28:c28	44.01	3.66	4.89	26744	57485	25487	36013	2892	149	388	20	68

composition throughout the larval stage. A response surface approach (Lande and Arnold 1983, Blows and Brooks 2003, and Chenoweth and Blows 2005) was utilized for all statistical analyses in order to estimate how certain amounts and ratios of proteins and carbohydrates affected larval performance in terms of survival, development, mass, and a composite estimate of these three variables. A response surface approach was also used to measure body composition for 12 elements (including C and N), plus total lipid content. These variables were analyzed in terms of absolute amounts and as a percent.

Results

Performance

Survival success (recorded as the percent of pupating individuals), development time (recorded as the number of days from hatch to pupation), and pupal mass were used as measurements of *H. virescens* caterpillar performance.

Survival success was affected in a linear fashion by protein and carbohydrate, and in a quadratic fashion by carbohydrate (Table 3.2). It was best on the p20:c14, p28:c20, and p28:c28 diets, and then dropped off as protein concentrations decreased (Figure 3.1a). With respect to carbohydrates, survival was optimal at intermediate carbohydrate concentrations, and then decreased as carbohydrate concentrations became both lower and higher (Figure 3.1a). Our second measure of performance, development time (recorded as the number of days from hatch to pupation), was only affected by protein (Table 3.2). Development was fastest on diets with the highest protein content, and decreased in a linear fashion as protein concentration dropped (Figure 3.1b). Our

Table 3.2. Response surface significance (shown as a p value) for the full model, main effects (P=protein and C=carbohydrate) and the interactions (PxP=protein by protein, CxC=carbohydrate by carbohydrate PxC=protein by carbohydrate) as well as sex over 4 performance measurments (Survival, Development time, Wet pupal mass, Larval Performance).

Source	Survival	Development	Mass	Performance
FULL MODEL	**<0.001**	**<0.001**	**0.008**	**<0.001**
P	**0.003**	**<0.001**	**0.040**	**<0.001**
C	**<0.001**	0.066	0.524	**<0.001**
P X P	0.059	0.963	0.077	0.427
C X C	**<0.001**	0.984	**0.002**	**<0.001**
P X C	0.069	0.859	0.978	**<0.001**
SEX	-	0.363	0.290	-

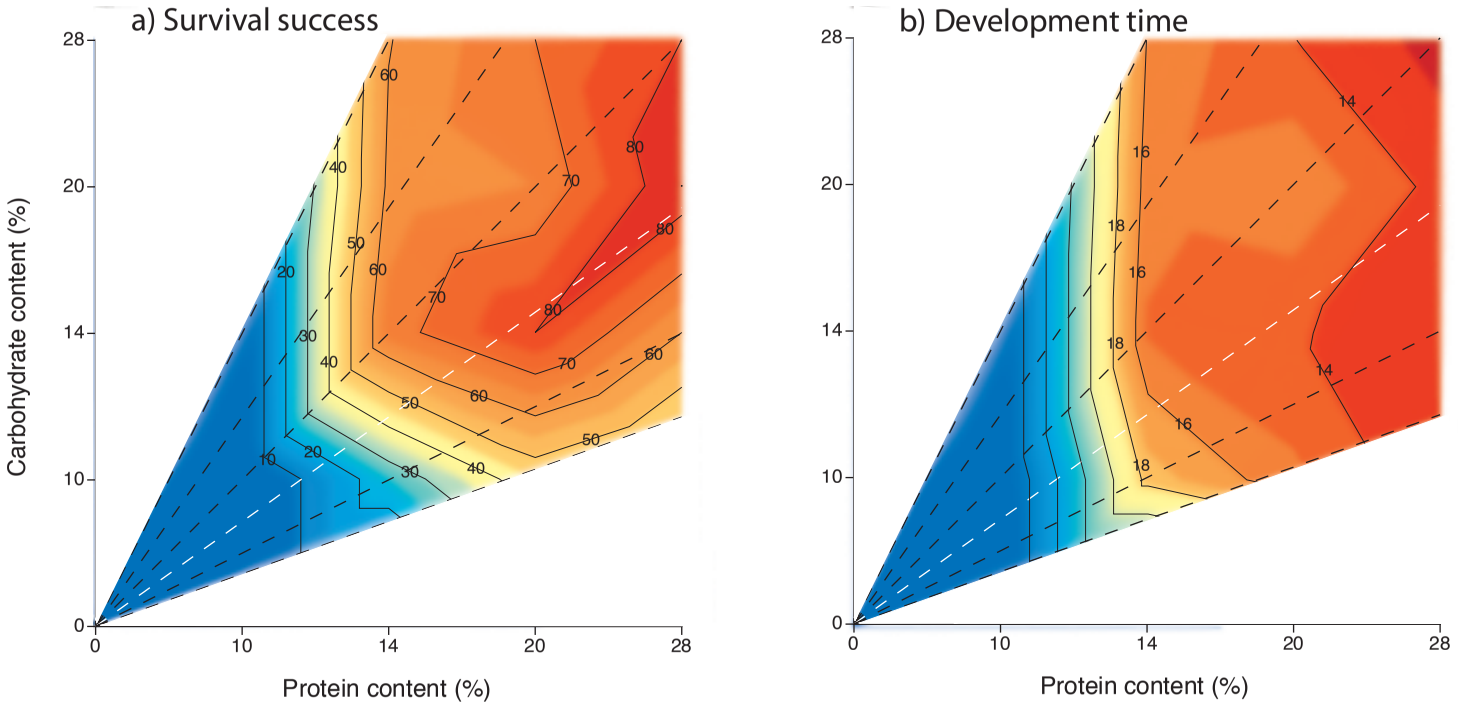

Fig. 3.1. Performance measures for the larval stage. Panel (A) shows survival success, measured as a percent. Panel (B) shows the mean development time for larvae that successfully pupated in days. Panel (C) shows the mean pupal wet mass as milligrams. Panel (D) shows overall "larval performance" as a composite variable that integrated the probability of survival on each diet, with the growth rate of individuals on each diet.

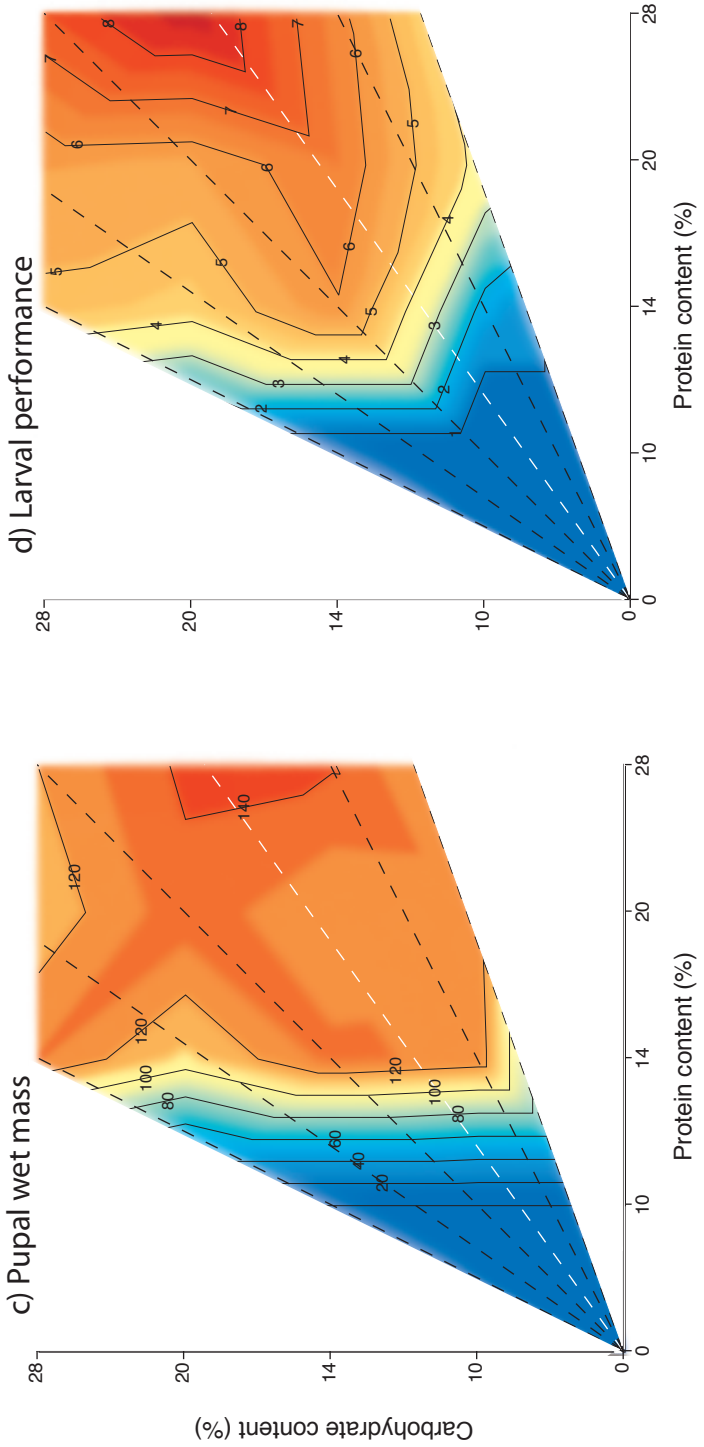

Fig. 3.1. continued

third measure of performance, pupal wet mass, was affected in a linear fashion by protein, and in a quadratic fashion by carbohydrate (Table 3.2). Pupal mass was highest on the p28:c14 and p28:c20 diets, and then decreased in a linear fashion as the dietary protein concentrations decreased (Figure 3.1c). With respect to carbohydrates, pupal mass was optimal at intermediate carbohydrate concentrations, and then decreased as carbohydrate concentrations became both lower and higher (Figure 3.1c).

A composite variable that that integrated the probability of survival on each diet, with the growth rate of individuals on each diet, was also generated (*sensu* Simpson et al. 2004). This variable, henceforth called 'larval performance', was significantly affected in a linear fashion by protein, and in a linear and quadratic fashion by carbohydrate (Table 3.2). There was also a significant protein-by-carbohydrate interaction (Table 3.2). Larval performance was clearly best on the p28:c20 diet, and then tended to fall away from this optimal peak in a horseshoe-like pattern (Figure 3.1d). Most notable was that larval performance on the p14:c14 diet was comparable to that of caterpillars on the p28:c10 and p20:c28 diets.

Body Elemental and Lipid Composition

Total amounts and concentrations of 12 biologically important elements (C, N, P, S, K, Na, Ca, Mg, Zn, Fe, Mn, and Cu), plus amounts and body fat composition (as a %), were measured for caterpillars on each diet, and analyzed using Response Surface methods.

The statistical results for total amounts of elements for caterpillars from the different diets are shown in Table 3.3a. Body amounts of K, Fe, and Mn were only affected significantly by protein. Both K and Fe increased linearly as the protein content of the food increased (Figure 3.2e and 3.2j, respectively), while Mn levels were highest at protein levels of about 14% (Figure 3.2k). In contrast, the amount of Na and Ca in caterpillars was only affected significantly by food carbohydrate content. With respect to Na levels, a significant quadratic trend was observed, with levels generally highest at intermediate carbohydrate levels (Figure 3.2f), while Ca levels followed a linear trend, increasing as carbohydrate levels in the diet decreased (Figure 3.2g). Body levels of C and Zn were significantly affected by both protein and carbohydrate. The total amount of C in the body of caterpillars increased in a linear fashion as the digestible carbohydrate content of the diet increased, but followed a quadratic trend with respect to food protein content (Figure 3.2a). In contrast, Zn body content decreased in a linear as dietary carbohydrate levels increased, but increased in a linear fashion as dietary protein levels increased. For the five remaining elements (N, P, S, Mg, and Cu) I observed significant protein-by-carbohydrate interactions with respect to the total amounts. In the case of P and S two identifiable peaks were observed, and for both elements the two peaks were intersected by the rail corresponding to the optimal p:c ratio for *H. virescens* caterpillars (Lee et al. 2006). The response surfaces generated for Mg and Cu also showed two peaks, but here the two peaks for these two elements were not, relatively speaking, much higher compared to amounts from caterpillars on the other diets. Finally, body N, in addition to showing a significant protein-by-carbohydrate interaction,

Table 3.3. Response surface significance (shown as a p value) for main effects (P=protein and C=carbohydrate) and interactions (PxP=protein by protein, CxC=carbohydrate by carbohydrate PxC=protein by carbohydrate) across 12 elements for both absolute amounts and concentrations.

		Structural				Electrochemical				Catalytic			
Source	**df**	**C**	**N**	**P**	**S**	**K**	**Na**	**Ca**	**Mg**	**Zn**	**Fe**	**Mn**	**Cu**
a) Amount													
P	1	0.133	**0.009**	0.101	0.084	**0.047**	0.053	0.757	**0.005**	**<0.001**	**0.044**	**<0.001**	0.189
C	1	**<0.001**	0.241	**<0.001**	**<0.001**	0.657	0.070	**0.026**	**0.036**	**0.010**	0.295	0.711	0.631
P X P	1	**0.023**	0.052	**0.037**	0.719	0.637	0.674	0.160	**0.017**	0.320	0.091	**0.001**	**0.023**
C X C	1	0.071	**0.036**	0.197	0.989	0.722	**0.043**	0.775	0.474	0.277	0.163	0.776	0.378
P X C	1	0.216	**0.007**	**0.030**	**<0.001**	0.062	0.161	0.483	**0.013**	0.151	0.657	0.842	**0.017**
b) Concentration													
P	1	0.830	0.307	**0.025**	**0.019**	0.364	**0.013**	0.877	**<0.001**	**<0.001**	**0.021**	**<0.001**	**0.025**
C	1	**<0.001**	**<0.001**	0.780	0.523	**<0.001**	0.431	**0.002**	**<0.001**	**<0.001**	0.070	**0.008**	0.346
P X P	1	0.084	0.292	0.632	0.055	**0.003**	**0.043**	**0.011**	0.148	0.590	**0.006**	0.816	0.307
C X C	1	0.446	0.311	0.961	0.457	0.480	0.399	0.499	0.561	**0.012**	0.148	0.660	0.423
P X C	1	0.468	**0.007**	**0.041**	**0.005**	**0.030**	0.808	0.972	**<0.001**	0.349	0.939	0.388	0.086

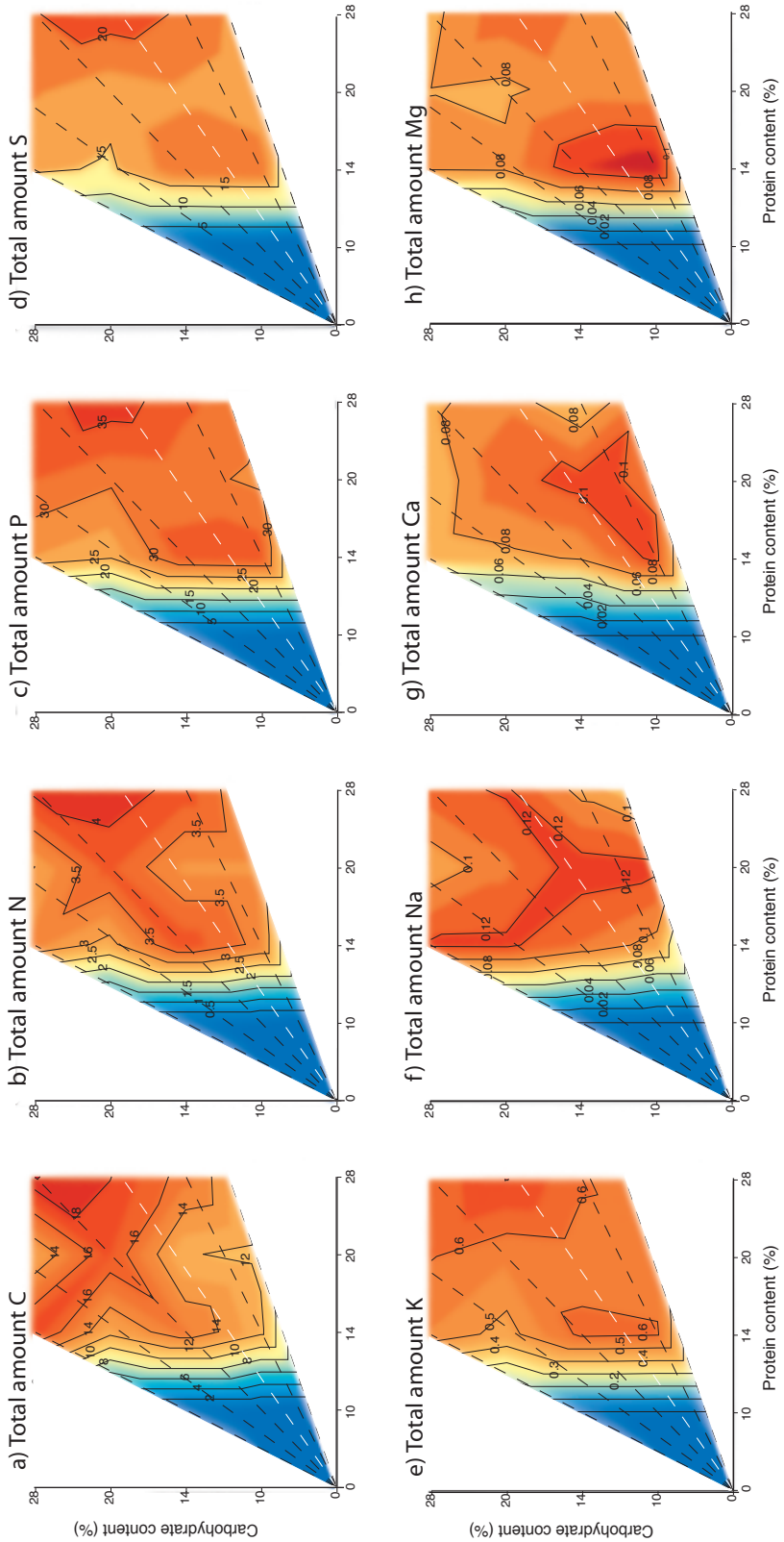

Fig. 3.2. Absolute amounts of elements. Panels (A=carbon), (B=nitrogen), (C=phosphorous), and (D=sulfur) show the amount as milligrams, of structural elements found in pupated *H. virescens*. Panels (E=potassium), (F=sodium), (G=calcium), and (H=magnesium) show the amount of electrochemical elements and panels (I=zinc), (J=Iron), (K=manganese), and (L=copper) show the amount of catalytic elements.

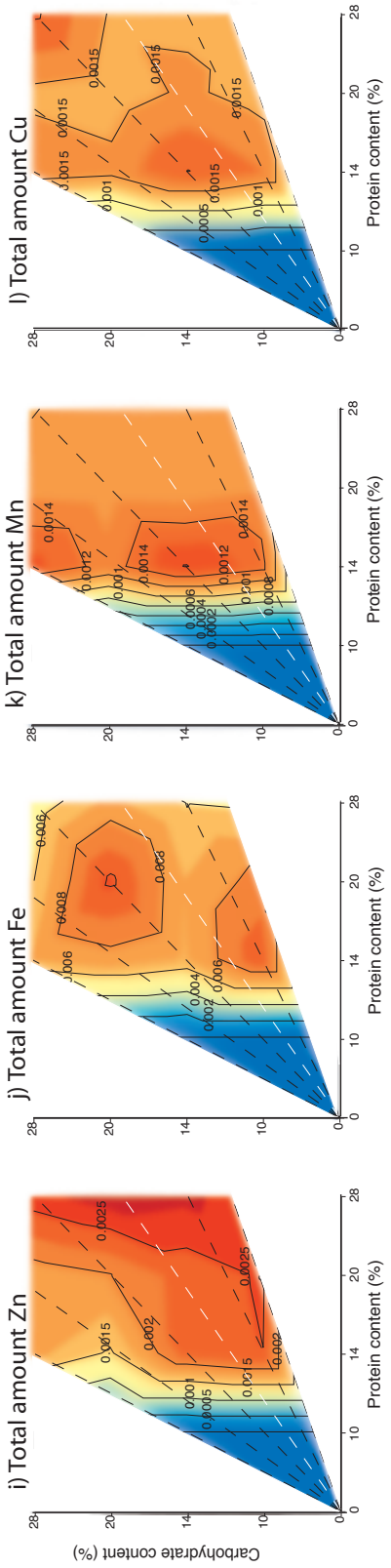

Fig. 3.2. continued

was significantly affected, in a quadratic fashion, by food carbohydrate content. Body N amounts peaked on diets with high protein, and moderate to high carbohydrate content, but were also relatively high on diets that had 1:1 protein-carbohydrate ratios (Figure 3.2b).

The statistical results for the concentration of elements for caterpillars from the different diets are shown in Table 3.3b. The concentration of Na, Fe, and Cu were only affected significantly by protein. Both Na and Fe concentrations were significantly affected by protein in a quadratic fashion, and were generally highest on moderate protein levels (Figure 3.3f and 3.3j, respectively). The C concentration of caterpillars was only affected by dietary carbohydrate – it increased in a linear fashion as digestible carbohydrate levels in the diet increased (Figure 3.3a). The concentration of Ca, Mn, and Zn were each affected by food protein and digestible carbohydrate content, but each in a different manner. Body Ca concentrations increased linearly as digestible carbohydrates decreased, but were affected in a quadratic fashion by protein, being highest on diets with 20% protein (Figure 3.3g). In contrast, Zn concentrations increased in a linear fashion as food protein content increased, and increased in a quadratic fashion as food carbohydrate content decreased (Figure 3.3i), while Mn concentrations increased linearly as food protein and carbohydrate content decreased (Figure 3.3k). For the five remaining elements (N, P, S, K, and Mg) I observed significant protein-by-carbohydrate interactions with respect to body elemental concentrations, and generally speaking the highest concentration of these elements occurred at low to moderate protein levels and

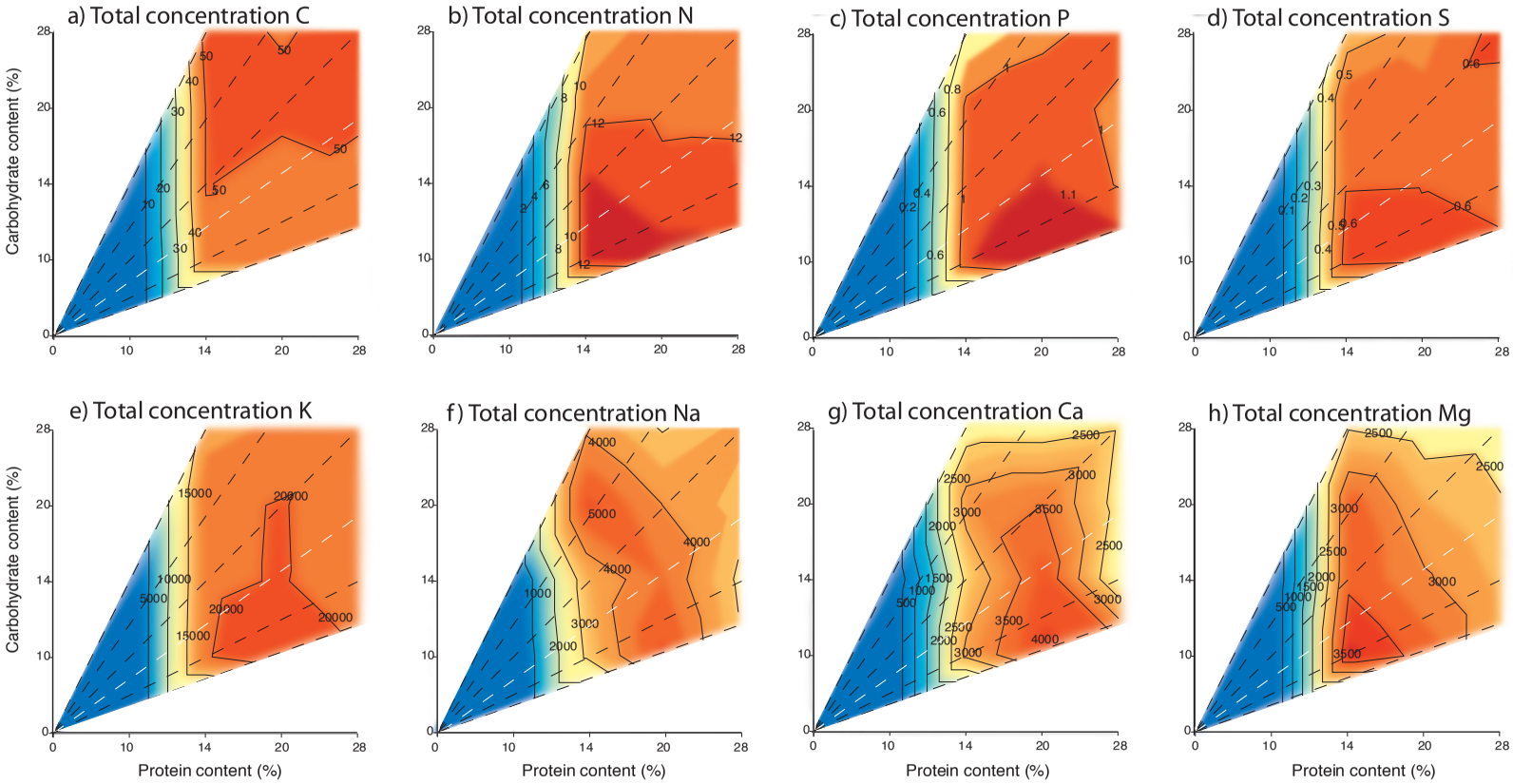

Fig. 3.3. Concentration of elements. Panels (A=carbon), (B=nitrogen), (C=phosphorous), and (D=sulfur) show the concentration, as parts per million, of structural elements found in pupated *H. virescens*. Panels (E=potassium), (F=sodium), (G=calcium), and (H=magnesium) show the concentration of electrochemical elements and panels (I=zinc), (J=iron), (K=manganese), and (L=copper) show the concentration of catalytic elements.

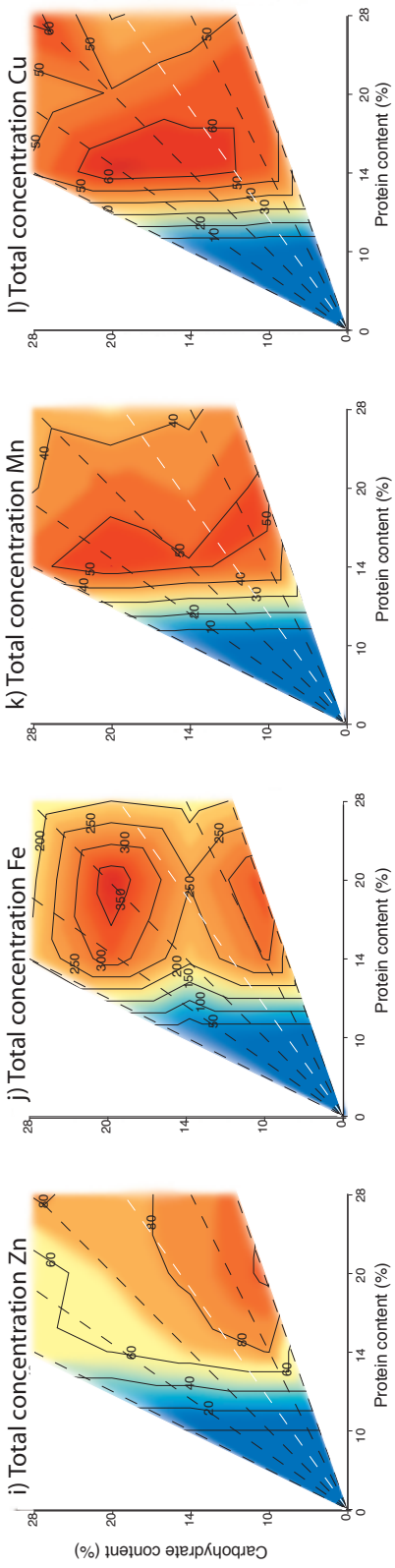

Fig. 3.3. continued

low to moderate digestible carbohydrate levels (Figures 3.3b, 3.3c, 3.3d, 3.3e, and 3.3h, respectively).

Lastly, the effect of food protein and carbohydrate content on body lipid levels was analyzed. Total lipid amounts increased linearly as the carbohydrate percent in a diet increased (Table 3.4a), and were highest on diets containing 28% carbohydrate (Figure 3.4a). Body lipid content, measured as a percent, was also affected by both food carbohydrate and protein content (Table 3.4b), increasing in a linear fashion as food carbohydrate content increases and as protein content decreases (Figure 3.4b).

Discussion

Protein and digestible carbohydrates are two important macronutrients for insect herbivores, although traditionally protein is considered to be the more limiting of the two (Joern and Behmer 1997). Protein certainly was the limiting factor with respect to development, but carbohydrates can also limit performance. For example, survival and mass gain were consistently low on diets with only 10% carbohydrate, even though protein content was relatively high (greater than 20%). The key message from the current study, though, is there is a particular blend of protein and carbohydrate that optimizes insect performance, as demonstrated by the response surface analysis for survival success, pupal wet mass, and larval performance.

The combined effect of protein and carbohydrate on larval performance has been explored in a range of generalist caterpillar species (Lee 2007, Raubenheimer and Simpson 2003, Despland and Noseworthy 2006, Telang et al. 2001, Lee et al. 2002,

Table 3.4. Response surface significance (shown as a p value) for the full model, main effects (P=protein and C=carbohydrate) and the interactions (PxP=protein by protein, CxC=carbohydrate by carbohydrate PxC=protein by carbohydrate) as well as sex for the total lipid amount and concentration.

Source	Lipid Amount	Lipid Concentration
FULL MODEL	**<0.001***	**<0.001***
P	0.255	**0.004***
C	**<0.001***	**<0.001***
P X P	0.960	0.689
C X C	0.377	0.195
P X C	0.304	0.582
SEX	0.650	0.625

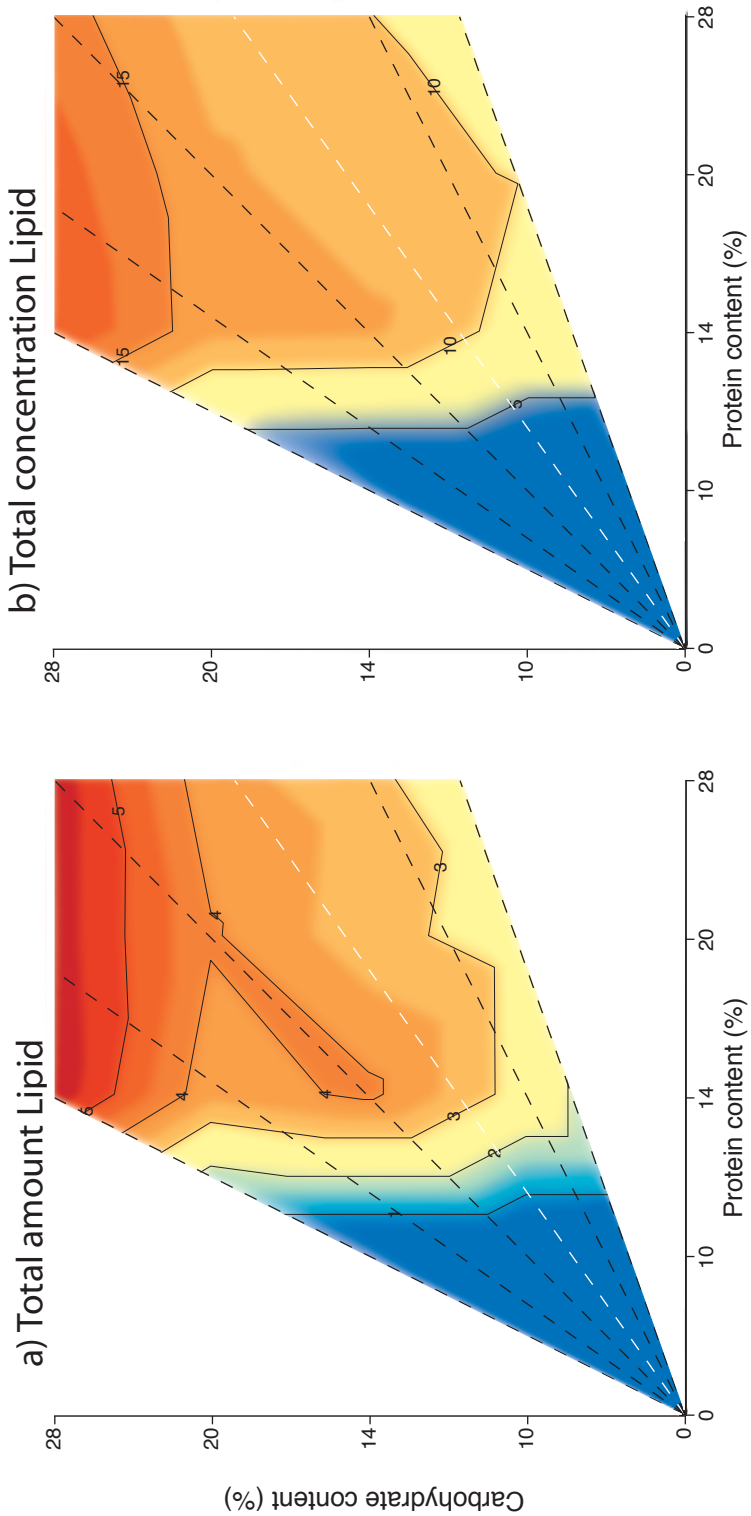

Fig. 3.4. Lipid content. Panel (A) shows the mean amount of bodily lipid. Panel (B) shows the mean percent body lipid content.

2003, 2004a, 2004b, 2006), but the great majority of these studies looked at only a single developmental stage and typically kept the total macronutrient density (protein + digestible carbohydrates) constant. In the current study I've simultaneously explored the effects of both food p:c ratio and nutrient density, across all of larval development, and my results strongly suggest that obtaining a balanced protein-carbohydrate intake (one that is optimal for that given species) is more important than maximizing total macronutrient intake. Previous work with *H. virescens* has shown that final instar caterpillars self-select a diet with a p:c ratio of 1.4:1 (Lee et al. 2006), and in the current study larval performance (a composite of survival, development time, and pupal wet mass; see Fig. 1d) was best on the food that had this p:c ratio at a high density (p28:c20). The significance of balance rather than total macronutrient content is further demonstrated by the finding that larval performance on the p20:c14 food was comparable to the p28:c28 and p28:c14 diets, even though these latter two diets had greater total macronutrient content (56% and 42%, compared to 34%). An even stronger case for the importance of balance is seen when performance on the p20:c14 diet is compared with performance on two other diets – p:20:c20 and p20:c28. These two diets have the same protein content, but greater carbohydrate content relative to the p20:c14 diet, yet larval performance on these two diets was reduced compared to that on the p20:c14. Performance was also reduced on three other diets with elevated total macronutrient content relative to the p20:c14 diet (e.g. p14:c28, p28:c14 and p28:c10). Reduced performance on imbalanced diets is likely due to physiological costs associated

with having to process nutrients ingested in excess of requirements (Simpson et al. 2004).

Identifying performance trends associated with varying amounts and ratios of dietary proteins and carbohydrates helps explain only a piece of an insect's physiology. Biomolecules such as proteins, carbohydrates, and lipids and the elements that comprise them (e.g., carbon, nitrogen, phosphorous, etc.) are not always the same between food and consumer (e.g., demonstrated by varying phosphorous level between plants and insects in Fagan et al. (2002)), so a key question is how insect herbivores redress this incongruence. One could postulate that larger animals might always have higher total amounts of elements due to their increased size, and my study has shown this to be the case for structural elements like carbon and nitrogen. However, this is not always the case, as many of the electrochemical and catalytic elements were recorded in amounts that did not correlate with body size. The true test of elemental regulation, and the degree of nutritional homeostasis being practiced, is to examine elemental profiles as a percentage of body mass. My data shows that caterpillars, when restricted to a broad range of foods that differ in their protein-carbohydrate ratios, and absolute amounts, do not practice strict homeostasis. This result is in strong contrast to studies conducted on a generalist grasshopper (Boswell et al. 2008).

Protein and digestible carbohydrates are the dominant macronutrients in plants and our diets, and they provide a large pool of key elements, particularly N and C (Sterner and Elser 2002). Carbon is a particularly important element to measure as it makes up a large percent of the total elemental profile, and is the dominant element

found in digestible carbohydrates, including sugar and starch. Carbon is also found in cellulose, a structural carbohydrate, but only C from sugar and starch (digestible carbohydrates) is available to insect herbivores. Importantly, I found that the concentration of C within individual caterpillars was positively correlated to a diet's digestible carbohydrate level. That body C concentrations increased on high carbohydrate diets is likely tied to the physiological fate of ingested digestible carbohydrates, which when ingested in excess of requirements can be either respired (e.g. Zanotto et al. 1993) or converted to fat (triglyceride (TAG)) and stored. Insects fed diets with high carbohydrate content (28%) would have been greatly overeating carbohydrates to meet their protein requirements (Lee et al. 2004a). In doing so, they would have only been able to respire a fraction of their ingested carbohydrates, with the remainder being converted to fat (mostly in the form of triglyceride (TAG). Lipid content on high carbohydrate diets was elevated (see Figure 3.4), and because TAG is mostly C (around 80% of its total molecular weight), an elevated body lipid content likely explains the inability to strictly regulate C.

Nitrogen, on the other hand, is found mostly in amino acids, which are the building blocks of protein. For insect herbivores, N is often recognized as one of the most limiting elements and in terms of its contribution to an organism's biomass, it ranks second behind C (Sterner and Elser 2002). Interestingly there was a clear N peak, in terms of concentration, and this peak occurred on foods with low nutrient densities (e.g., p14:c10, p14:c14, and p20:c10). This observation was likely a combination of small bodies that were lower in fat, and compensatory feeding (shown by Lee et al. 2004a) as a

means to acquire the correct amount of macronutrients needed for growth and development. In contrast, N body concentrations were lowest on high carbohydrate diets, which was likely a result of a dilution effect as a result of high body lipid.

The remaining two structural elements that were measured were phosphorous, which is an important element found in DNA, RNA, ATP, and cell membranes, and sulfur, which is an essential element found in the two amino acids cysteine and methionine. Although total amounts of these elements were affected by an insect's size, the concentration for both appeared to be negatively linked to the amount of carbohydrate in a given food. One possible reason for this is that when foods contained at least 14% carbohydrate, carbon concentrations were shown to increase and in turn reduce the available room for other structural elements (as shown for nitrogen, phosphorous, and sulfur). However when performance measurements were linked to the structural element's concentrations, I found that having a higher concentration of certain elements does not always lead to an increase in performance.

In order to understand how an insect truly builds itself I next looked at the elemental composition for electrochemical elements, which are important for message transmissions across nerves, cellular signaling, and energy metabolism (Sterner and Elser 2002), and catalytic elements, which are important for digestion, hydrolysis of urea, nitrogen fixation, and various reactions with O_2 (Sterner and Elser 2002). When these eight elements were compared, I found most to have a unique concentration peak that was not necessarily associated with any one particular dietary treatment. Therefore I propose that most of the electrochemical and catalytic elements were not directly

regulated to a certain concentration and reaffirm our hypothesis that caterpillars regulate their elemental concentrations at a lower level than grasshoppers.

In conclusion, my results show that for *H. virescens* a balanced to slightly protein-enriched diet was optimal in terms of performance and because of this I argue that the ratio of protein to carbohydrate was more important than the absolute amount as long nutrients are not too diluted. An insect's elemental balance was also found to be directly affected by the p:c ratio of a food indicating that subtle changes in nutrient quality of available host plants may have a much greater impact on how an insect builds itself.

CHAPTER IV

CONCLUSION

These experiments have helped provide information towards the better understanding of insect physiology, specifically in terms of what affects the performance and body composition of a generalist caterpillar like *Heliothis virescens*. I used the geometric framework to show how different protein and carbohydrate ratios in artificial diets, which simulated a wide range of plants that an insect may encounter in the wild, affected an insect's performance through multiple developmental stages. This type of study has generally limited the time frame to the last larval instar. However by comparing multiple developmental stages, a comprehensive picture of what an insect is doing and how it is being affected was created. While some of my results did not directly confirm all of my hypotheses due to varied performance across some variables in different developmental stages, caterpillars were generally shown to perform best around the diet ratio that they had previously self-selected in single instar studies. Additionally population sizes for different generations were built from multiple performance measurements and clearly indicate that imbalances in p:c ratio directly affect the potential future fitness of any number of individuals. Sex differences, which are generally quite hard to measure in the larval immature stages, were shown to have significant affect for males in terms of survival during the pupal stage. The combined results, including the effects at the population level, indicate that diet macronutrient content is very important

to insect herbivores, and that even small departures from an optimal p:c ratio can have dramatic physiological effects.

These experiments also allowed me to use both the geometric framework and ecological stoichiometry in order to fully explore what is happening to an insect throughout its larval development when placed on diets that were composed of different amounts and concentrations of protein and carbohydrate. Specifically this experiment focused on exploring performance (survival, development, mass, and "larval performance") and body composition (C, N, P, S, K, Na, Ca, Mg, Zn, Fe, Mn, Cu, and lipid) over twelve diets that were mapped to a rectangular area within nutrient space. Essentially the main questions revolved around what was more important to an insect in terms of nutrients, the absolute amount or the ratio that they were presented in, and how an insect built itself from these different foods. While performance clearly confirmed what had been shown in the first experiment, body composition was affected by a number of different variables ranging from a diets macronutrient content to its elemental balance. The key message from the this study, though, is that there is a particular blend of protein and carbohydrate that optimizes insect performance. Future research should focus more on explaining why the elements were found in different amounts and concentrations and why reduced regulation of elemental concentrations was occurring in our holometabolous insect compared to the tighter elemental regulation seen on some hemimetabolous insects.

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