DIET-MIXING IN A GENERALIST HERBIVORE: TRADE-OFFS BETWEEN NUTRIENT AND ALLELOCHEMICAL REGULATION

A Dissertation

by

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ABSTRACT

Despite decades of research, many key aspects related to the physiological processes and mechanisms insect herbivores use to build themselves remain poorly understood, and we especially know very little about how interactions among nutrients and allelochemicals drive insect herbivore growth processes. Understanding the physiological effects of these interactions on generalist herbivores is a critical step to a better understanding and evaluation of the different hypothesis that have been emitted regarding the benefits of polyphagy. I used both lab and field experiments to disentangle the respective effect of protein, carbohydrates and allelochemicals on a generalist herbivore, the grasshopper *Melanoplus differentialis*.

The effect of protein and carbohydrates alone were examined using artificial diets in choice and no-choice experiments. Results were plotted using a fitness landscape approach to evaluate how protein-carbohydrate ratio and/or concentration affected performance and consumption. Growth was best near the self-selected ratio obtained from the choice experiment, most likely due to the fact that the amount of food digested was also higher on that ratio. By contrast, development time was not best near the preferred ratio most likely due to the trade-off existing between size and development time. These results illustrate how nutrient availability can shape an ecological trade-off: growing big or growing fast.

When an allelochemical (gramine, an alkaloid commonly found in grass) was introduced to the artificial diets, it had an interactive effect with protein and carbohydrates on performance and consumption and performance were generally improved on diets that contained higher amount of protein.

Host plants of two wild populations were determined by gut content analysis and fed in choice and no-choice experiment. First the plant material was dried and ground and its protein, carbohydrate, terpenoid and phenolic content analyzed. Both populations regulated for the same protein-carbohydrate intake. However performance was different, due to variation in plant allelochemical content. This demonstrated for the first time that nutrient regulation, not toxin dilution, is directing food selection behavior in a generalist herbivore.

Finally the role of macronutrients was analyzed in a context of cost of detoxification by measuring microsomal p450 production in the presence/absence of gramine. In the presence of choice, nutrient regulation was altered when gramine was present in the protein-biased diet. In the absence of choice, insects performed better on carbohydrate biased diet. I found that gramine elevated the level of microsomal protein in the fat body.

DEDICATION

To my dad for whom grasshoppers are fish bait.

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NOMENCLATURE

C	Carbohydrates
GF	Geometric Framework
P	Protein
P:C	Protein: Carbohydrates ratio
GST	Gluthatione S-transferase

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Plants and insects have coexisted for as long as 350 million years, if the earliest forms of land plants and insects are included, and have developed a series of relationships which affect the organisms at all levels, from basic biochemistry to population genetics. Although some of these relationships, such as pollination, are mutually beneficial, the most common interaction involves insect feeding on plants, and plant defenses against herbivorous insects. So common is this consumer–host relationship that virtually every plant species is eaten by at least one insect species. All major crop plants are vulnerable to insect herbivores and, despite heavy use of insecticides, pre-harvest crop loss to insect herbivores is estimated at about 15% (Maxmen 2013).

As for any other animal, insect herbivores eat to acquire a mix of nutrients to fuel growth process, maintenance and reproduction. They have requirements for upwards of 30 different nutrients, including protein (amino acids), digestible carbohydrates, fatty acids, sterols, vitamins, minerals and water (Chapman 1998, Schoonhoven et al. 2005). Plants contain all the nutrients insect herbivores need, but securing these nutrients in appropriate amounts and ratios is often challenging because nutrient are diluted in a matrix of indigestible structural compounds like cellulose and lignin. Furthermore, in plants the absolute amounts and ratios of nutrients is highly variable. Variation is most notable between different species (e.g., forbs usually contains higher concentrations of protein than grasses), but also occurs within species as a result of genotypic differences

(Osier and Lindroth 2001, von Fircks et al. 2001, Gusewell and Koerselman 2002, Lindroth et al. 2002, Oleksyn et al. 2002) and insects are susceptible to such variation (Scriber and Slansky 1981, Slansky and Rodriguez 1987, Bernays and Chapman 1994, Raubenheimer and Simpson 1997, Schoonhoven et al. 1998).

Among plant nutrients, proteins and digestible carbohydrates are particularly important for herbivorous insects and both are highly variable (Chambers et al. 1995, Simpson et al. 2004). Protein is necessary to provide the amino acids needed for the production of new proteins, such as enzymes (e.g., P450), transport and storage proteins (e.g., lipoproteins), and receptor molecules (e.g., nicotinic acetylcholine receptor). Digestible carbohydrates are used mostly as fuels, but they can also be converted to fat, and contribute to the production of amino acids. Because the maintenance of a specific intake of protein and carbohydrate is crucial to insect fitness, they have evolved the ability to regulate their intake. This was first demonstrated in *Helicoverpa zea* using artificial diets (Waldbauer et al. 1984) and has since been repeatedly shown in various species (see Behmer 2009 for a review on insect herbivore nutrient regulation).

To explore how an animal attempts to solve the problem of balancing multiple and changing nutrient needs in a multidimensional and variable nutritional environment in 1993 Raubenheimer and Simpson imagined the Geometric Framework (GF) (Fig. 1.1) (Raubenheimer and Simpson 1993, Simpson and Raubenheimer 1993). The GF treats an organism within a multidimensional nutrient space where the axes are defined by functionally relevant (fitness-affecting) nutrients, for instance protein and carbohydrates for an herbivore. There is a mixture and blend of these nutrients that is optimal, the nutritional target (Raubenheimer and Simpson 1993, Simpson and Raubenheimer 1993, 1995). As mentioned previously, many animals have evolved a suite of behavioral and physiological mechanisms that enable them to approach this point (Raubenheimer and Simpson 1999). The position of this target can change over time depending on an organism's stage of development, and the environmental circumstances. The *intake target* represents the amount of nutrients that an animal needs to ingest in order to reach its nutritional target since not all ingested nutrients are absorbed.

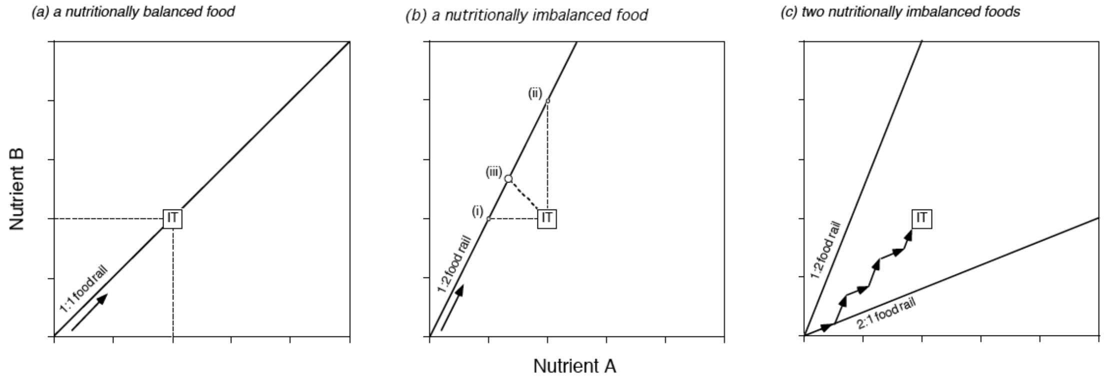

Figure 1.1 Nutritional space for two functionally relevant nutrients, A and B. The intake target (IT; shown in each panel) lies on the rail with the two nutrients in a 1:1 ratio. Panel (a) shows that the intake target can be reached by moving along an optimal food rail. In (b) the animal cannot reach its intake target. It can: (i) feed until its requirement for B is met, but suffer a deficit in A; (ii) feed until it meets its requirement for A but suffer an excess of B; (iii) move to some intermediate point between the first two extremes. Panel (c) shows our animal reaching its intake target by switching between the two nutritionally sub-optimal, but complementary foods. The nutritional space occupied by the hypothetical animal is defined by the space between the two food rails.

Using the GF, we have learned much over the past two decades about the importance of nutrient balance for insect herbivores, and have identified some of the physiological mechanisms insect herbivores use to build themselves (Raubenheimer and

Simpson 1999, Simpson and Raubenheimer 2000, Jonas et al. 2007, Behmer 2009). Still missing from the picture is the role of other food components (plant defenses for instance) play in regard to diet mixing and nutrient balance. Plant defenses are extremely diverse ranging from mechanical defenses such as trichomes, to the production of complex chemicals: the secondary compounds. The term secondary compound derived from the fact that they do not participate in plant primary metabolism. Allelochemicals are part of plant secondary metabolism and are very diverse, more than 100 000 substances described (Schoonhoven et al. 2005), and abundantly produced in plants. Allelochemicals can be defined as substances that negatively impact the physiology of another organisms (Behmer 2009). The range of mechanisms of toxicity shown by different plant defensive compounds is very wide, and includes membrane disruption, inhibition of transport or signal transduction, inhibition of metabolism, and even disruption of hormonal control of developmental processes (Bennett and Wallsgrove 1994, Harborne 2007). By acting as feeding deterrents and/or toxins, they can interfere with an insect herbivore's ability to secure an optimal nutrient mix, including negatively affecting post-ingestive metabolic processes (Broadway and Duffey 1986, Slansky and Wheeler 1992a, b, Simpson and Raubenheimer 2001).

Secondary compounds that exert repellent, antinutritive, or toxic effects on herbivores are commonly referred to as direct defenses by opposition to indirect defenses that protect the plant by enhancing the probability of attracting natural enemies of herbivores (Howe and Jander 2008). Research in the past decade has largely focused on induced defense (Paul et al. 2000, Walling 2000, Kessler and Baldwin 2002). In contrast, studies on the roles and mechanisms of constitutive chemical defense are rather rare because of the difficulty of manipulating constitutive compounds in experimental settings (Wittstock and Gershenzon 2002). Only two studies (Simpson and Raubenheimer 2001, Behmer et al. 2002) have looked into the interactive effects of allelochemicals and nutrients on herbivorous insects. Interestingly, Simpson and Raubenheimer found that the negative effects of tannic acid on performance (developmental time, mass gain, survival) varied with tannic acid concentration but also, and more importantly, with food nutrient profile. When insects were fed nutritionally unbalanced food, the effects of tannic acid were more severe (Simpson and Raubenheimer 2001).

These results are of great significance for many reasons. First, they highlight the need to further our understanding of the interactions occurring among different food components and the gaps existing in our current knowledge of herbivore nutrition. We know very little about the basis of diet mixing and its function for organisms; the investigation of nutrient-allelochemical interaction could provide a novel mechanism by which the advantage of polyphagy could be explained. Two hypotheses are usually advanced to explain why generalist herbivore present enhanced performance when given a mixed diet. The first states that polyphagy offers a greater availability of food in the environment and the possibility of optimizing dietary quality through the intake of foods complementary in terms of nutrients. The second suggests that polyphagous insects can maintain the intake of a particular allelochemical low by switching from plant to plant (Bernays and Raubenheimer 1991, Singer et al. 2002). Both hypotheses have receive some support (Bernays et al. 1994, Unsicker et al. 2008, Karban et al. 2010) but few studies have actually addressed the issue of diet mixing through rigorous tests of multiple hypotheses (Singer and Bernays 2003) and the role of nutrient-allelochemical interactions is largely unknown. Finally, plant allelochemicals are heavily used in herbivorous pest control and furthering our understanding of the factors that increase their effectiveness is obviously of great interest for agriculture, particularly in the actual context where human society faces a global-scale issue regarding food production.

In summary, a number of factors make insect herbivore nutrient acquisition a difficult proposition. These include: 1) variation in amounts of nutrients, both within and between plant species (Mattson Jr 1980, Scriber and Slansky Jr 1981, Bernays and Chapman 1994), and 2) the use of defensive compounds by plants to deter and/or make insect herbivores sick (Bernays and Chapman 1994, Simpson and Raubenheimer 2001, Behmer et al. 2002). These factors are not independent and we currently know very little regarding their interacting effects. In this dissertation I used a generalist grasshopper, *Melanoplus differentialis*, as my model insect herbivore and conducted a series of laboratory and field experiments, within an explicit physiological framework (the GF), to explore how a food's nutrient profile drives the growth process in insect herbivores. I explored how different amounts of allelochemical interfere with growth process, and how the effectiveness of an allelochemical is a product of a food's nutritional background.

The effect of food components on the insect performance were assessed and plotted using a fitness landscape approach. The fitness landscape approach was developed by Simpson et al. (2004) and combines state-space models of nutrition (Geometric Framework) with Tilman's models of resource exploitation (Tilman 1982). The idea is to construct a smooth fitness landscape over nutrient space, centered on the intake target. The nutritional strategies of animals can then be mapped on to the performance surface, including the position of regulated points of intake and points of nutrient balance when fed suboptimal foods. In my first chapter I used this approach with artificial diets to understand how varying ratio and concentration of protein and carbohydrates affected *M. differentialis.* In my second chapter, I added an alkaloid to artificial diets varying in their protein-carbohydrate profiles to investigate the existence of nutrient-allelochemical interactions. Then, I determined the feeding pattern of my model organism in the field (i.e., the number, specific taxa, and types of food plants) and studied how plant nutrient and allelochemical content, at the species level, can affect the insect performance. Finally, I tested a possible mechanism to explain why nutrient background mediates the effect of plant toxins, by measuring the production of detoxification enzymes.

CHAPTER II

FOOD PROTEIN-CARBOHYDRATE EFFECTS ON THE FEEDING BEHAVIOR AND PHYSIOLOGY OF AN INSECT HERBIVORE: A FITNESS LANDSCAPE APPROACH

2.1 Introduction

All animals eat to acquire the nutrients needed for growth, maintenance and reproduction. Animals require multiple nutrients, but protein and carbohydrates are two that are particularly important. Dietary protein provides the amino acids needed to build new tissues, enzymes and proteins, while carbohydrates are commonly used as the key energy source needed to fuel this biosynthesis. Foods vary in their protein and carbohydrate content, but generally variation in plants is much great than in animals (Schoonhoven et al. 2005, Clissold et al. 2006, Clissold et al. 2009, Behmer and Joern 2012). For plants, protein-carbohydrate variation exists at a number levels, including between species (Yeoh et al. 1992), within species (Sattelmacher et al. 1994), and within an individual plant (Mattson 1980), depending on tissue type (i.e. leaves, flowers, seeds and stems), and tissue age (i.e. young vs. old leaf). Additionally, plant protein-carbohydrate content can vary with environmental factors, including the amount of light a plant receives, soil chemical composition, and drought (Holt and Hilst 1969, Hendrix and Huber 1986, Chapin et al. 1987, Felton 1996, Walter et al. 2012). The nutrient density of plants is also generally low, compared to animal tissue (Bernays and Chapman 1994, Karasov and Martínez del Rio 2007). Thus, the challenge for herbivores is to find and eat foods that contain the best protein-carbohydrate blend, and at a high concentration.

Nutritionally optimal foods will often be rare, but herbivores can still meet their protein-carbohydrate requirements using a suite of mechanisms (Behmer 2009). For example, they can regulate their protein-carbohydrate intake through diet mixing, either by switching between plants or plant tissue (Singer et al. 2002, Villalba et al. 2002c, Wright et al. 2003, Villalba and Provenza 2005, Clements et al. 2009, Felton et al. 2009). When the opportunity to practice diet-mixing is limited, or constrained, herbivorous insects can also adjust to nutrient concentration and increase feeding in response to food dilution (Slansky and Wheeler 1992a, Raubenheimer and Simpson 1993, Yang and Joern 1994a, Lee et al. 2004, Berner et al. 2005, Fanson et al. 2012). However, in natural conditions these mechanisms are sometimes restricted. For instance, improving protein-carbohydrate balance by alternating host plant might not be possible because of the threat of predation (Beckerman et al. 1997, Schmitz and Suttle 2001, Danner and Joern 2003, Hawlena and Schmitz 2010) and compensatory feeding also increases the risk of predation (Stamp and Casey 1993, Bernays 1997).

The first attempt to broadly understand the effect of protein-carbohydrate variability led to the development of a fitness landscape approach (Simpson et al. 2004). In this study the generalist caterpillar (*Spodoptera littoralis*) was fed a range of diets that varied in two ways. First, diets differed in their protein-carbohydrate ratio (there were multiple food rails). Second, diets differed in their protein-carbohydrate concentrations (along each food rail there were diets that ranged from dilute to more concentrated).

Within this nutritional space, animal performance was used as a currency, and visualized as a response surface. This study revealed, for the first time for any herbivore, responses of an animal to food protein-carbohydrate content beyond a single slice of protein-carbohydrate nutritional space (Raubenheimer and Simpson 1993, Simpson and Raubenheimer 1993, 1995, Raubenheimer and Simpson 1999, Simpson and Raubenheimer 2000, Simpson and Raubenheimer 2001, Simpson et al. 2002, Lee et al. 2003, Raubenheimer and Simpson 2003), and best captured the range of protein-carbohydrate an insect herbivore is likely to encounter. However, caterpillars like *Spodoptera sp*. don't usually switch from plant to plant, and the variability they encounter at the individual level is clearly limited compare to grazing herbivores like woolly bear caterpillars (Arctiinae) or grasshoppers (Acrididae), that will feed on multiple plant species each day (Bernays and Minkenberg 1997, Bernays et al. 2004).

In this paper I conducted two experiments that examined how food protein-carbohydrate content affected performance and consumption in a generalist insect herbivore, the grasshopper *M. differentialis*. The first was a choice experiment, where grasshoppers were given three different food pairings (the paired foods were nutritionally complementary to one another); for each pairing I measured protein-carbohydrate consumption, mass gain and development time. In the second experiment, grasshoppers were constrained to one of nine diets, encompassing a range of protein-carbohydrate ratios and amounts found in plants typically eaten by this species. For each diet I recorded a suite of variable related to physiological performance (dry mass gain, development time, final lean mass, final lipid content) and consumption (food intake, calorie intake, protein and carbohydrate intake, frass production, and efficiency of digestion). I analyzed these data using response surface methods, and visualized then as fitness landscapes plotted over my defined protein-carbohydrate nutrient space. This approach allowed me to disentangle the relative effect of protein-carbohydrate amounts and ratios. My results show, in a comprehensive manner, how food protein-carbohydrate content affects herbivores, and reveal novel insights concerning nutrients related growth trade-offs.

2.2 Material and methods

2.2.1 Insects and experimental chambers

The generalist grasshopper *Melanoplus differentialis* occurs throughout much of the United States where it is recorded feeding on a wide range of herbaceous and successional plants (Capinera 2008). Insects used in this study came from a culture fed a diet of greenhouse-grown seedling wheat and wheat germ, and maintained in the Department of Entomology, Texas A&M University (College Station, TX); the colony was initiated in 2008 from a local population. They were maintained under standard laboratory conditions with a 12h: 12h L:D photoperiod, and under radiant heat of 35-37°C during the light phase (supplied by 60W full spectrum incandescent bulb), and at 24-26°C during the dark phase.

Nymphs of both sexes were removed from the culture within 4-6 hours after ecdysis to the sixth stadium (day 0), weighed (to the nearest 0.1 mg), and then transferred individually into clear plastic arenas (19 cm long x 14 cm wide x 9 cm high).

Each container contained either one food dish (experiment 1) or two food dishes (experiment 2), an expanded aluminum roost for perching, and a water source (a 30-mL Solo Cup®, fitted with a lid that had been modified to allow the use of a cotton wick for drinking); the food dishes were modified Petri dishes designed to minimized spillage (Raubenheimer and Simpson 1990). Insects were maintained throughout the experiment under a 14:10 light:dark regime in an incubator. Temperatures in the incubator varied between 32°C (light cycle) and 26°C (dark cycle).

2.2.2 Synthetic food composition

The chemically defined foods used in this study were based on those used by Simpson and Abisgold (1985). In total, nine foods differing in their absolute amounts and ratios of protein (p) and digestible carbohydrates (c) were prepared: p7:c7 (7% protein and 7% carbohydrate; expressed on a dry mass basis), p7:c14, p14:c7, p7:c35, p35:c7, p28:c14, p14:c28, p21:c21 and p35:c35. The protein component of all foods was a 3:1:1 mix of casein, peptone and albumen, while the digestible carbohydrate (henceforth carbohydrate) component was a 1:1 mix of sucrose and dextrin. All foods contained similar amounts of Wesson's salt (2.4%), cholesterol (0.5%), linoleic acid (0.5%), ascorbic acid (0.3%) and vitamin mix (0.2%) (Dadd 1961). The remainder of the diet was cellulose, a non-nutritive bulking agent.

2.2.3 Experimental treatments

Two separate experiments were performed. The first, a choice-experiment, had three treatments (each containing two food dishes): (1) p7:c35 paired with p35:c7, (2) p7:c35 paired with p28:c14, and (3) p7:c14 paired with p14:c7. The total macronutrient content, of each food, in the first two food pairings was 42%; for the third food pairing, the total macronutrient content of each food was only 21%. Previous work has shown that *M. differentialis* nymphs actively regulate their protein-carbohydrate intake when food macronutrient density is high (e.g., food pairings 1 and 2; see Behmer & Joern 2008). The third food pairing allowed us to test the extent to which *M. differentialis* nymphs regulate their protein-carbohydrate intake when food macronutrient density is low. For each treatment I measured food consumption from each dish, total protein and carbohydrate intake, dry mass gain, development time, and body lipid content.

The second was a no-choice experiment that included nine treatments. Here the idea was to test insects on a range of protein-carbohydrate that encompassed a range of protein-carbohydrate combination that might be encountered in the field (Fig. 2.1; see Behmer and Joern 2012). Specifically, these collective treatments allowed us to compare the relative importance of protein-carbohydrate balance and concentration. As in the choice experiments, I measured food consumption, total protein and carbohydrate intake, dry mass gain, development time, and body lipid content. In this experiment I also measured fecal production, which allowed me to examine various aspects of food utilization.

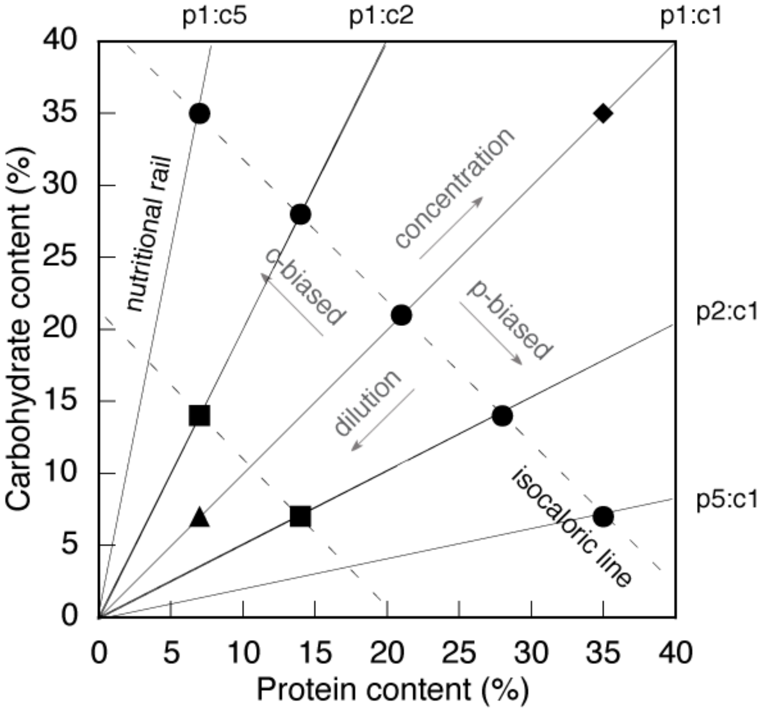

Figure 2.1 Protein (p) and digestible carbohydrate (c) content of the nine artificial diets used in the study. In total, five different p:c ratios were tested, each shown as a “nutritional rail” (the fine grey lines arising from the origin). Multiple foods are represented on three of these nutritional rails (p1:c2, p1:c1, and p2:c1); in each instance food nutrient concentration increases as the distance between the origin and the food increases. Additionally, two groups of diets were isocaloric (indicated by the dashed lines). In the first group, two diets (shown as squares) were isocaloric at 84 cal/mg. In the second group, five diets (shown as circles) were isocaloric at 168 cal/mg.

2.2.4 Protocol

In both experiments, similar protocols were followed. First, foods were scooped into individual dishes and then weighed to the nearest 0.1 mg after allowing food dishes to equilibrate under stable ambient room humidity for 24 h. On the first day of the 6th-stadium (day 0), individuals were placed into the experimental arena, and allowed to feed for 72 hours; at the end of this time food dishes were then replaced with fresh, pre-weighed dishes of the same food, every three days, until grasshoppers molted to the adult stage. All food that was pulled from the test arenas was left to equilibrate to room humidity for 24 before it was weighed (to the nearest 0.1 mg). Upon molting, grasshoppers were weighed (to the nearest 0.1 mg) and then placed into a freezer and stored; upon molting I also recorded total fecal production (to the nearest 0.1 mg). After the experiment ended, grasshoppers were removed from the freezer and dried at 40°C until they reached a constant mass and weighed to the nearest 0.1 mg. Lipids were extracted in three, 24-h changes of chloroform. At the end of the third chloroform wash grasshoppers were re-dried and re-weighed to calculate lipid content. To calculate dry mass gain, I estimated the initial dry mass with a regression equation that was derived from the dry mass of 20 newly molted 6th instar grasshoppers from the lab colony.

2.2.5 Statistical analyses and data presentation

In the choice experiment the amount of protein and carbohydrate eaten, for each treatment, was compared using MANCOVA techniques (with starting mass as a covariate to correct for size differences between individuals); I used Pillai's test statistic. Where significant effects were observed, post hoc comparisons were performed using contrasts following the techniques employed by Scheiner (1993), with α levels adjusted with Bonferroni correction methods. I also used univariate tests (ANOVA) to compare protein and carbohydrate intake independently. Dry mass gain and lipid content were analyzed using ANCOVA technique. Starting mass and sex were used as covariate for dry mass gain as female grasshoppers are usually bigger than male grasshoppers. Lean mass was used as a covariate in lipid content analysis. Development time across treatments was compared using failure-time analysis.

In the no-choice experiments I used response surface methodology to analyze how insects responded to different protein-carbohydrate food profiles. I also used ANCOVA techniques to compare the different diet treatments (with Tukey HSD post-hoc when appropriate). Starting mass and sex were used as covariate; in the case of lipid content analysis, lean mass was used as a covariate. All data from this second experiment were mapped over the designed nutrient landscape (Fig. 2.1), and presented as fitness surfaces. Because these surfaces are best visualized with nonparametric techniques that do not constrain the shape of the surface (Blows and Brooks 2003), thin-plate splines were used within the fields package of R (version R 2.11.1), following the approach developed by Simpson et al. (2004).

2.3 Results

2.3.1 Choice experiment

2.3.1.1 Nutrient intake

The protein-carbohydrate intake is shown in Fig. 2.2. Regardless of the treatment all grasshoppers ingested the same amount of protein (≈200mg) (ANCOVA: DF=2, F=0.91, p=0.417). On the most concentrated treatments (1 and 2), the insects converged to follow a similar protein–carbohydrate intake trajectory (Table 2.1), corresponding to a carbohydrate-biased ratio of p1:c1.4. However insects that were tested on the third -more diluted treatment (21% macronutrient)- ate about a third less carbohydrate (≈ 200mg *vs.* ≈ 300mg for treatment 1 and 2) (ANCOVA: DF=2, F=8.91, p=0.001*).

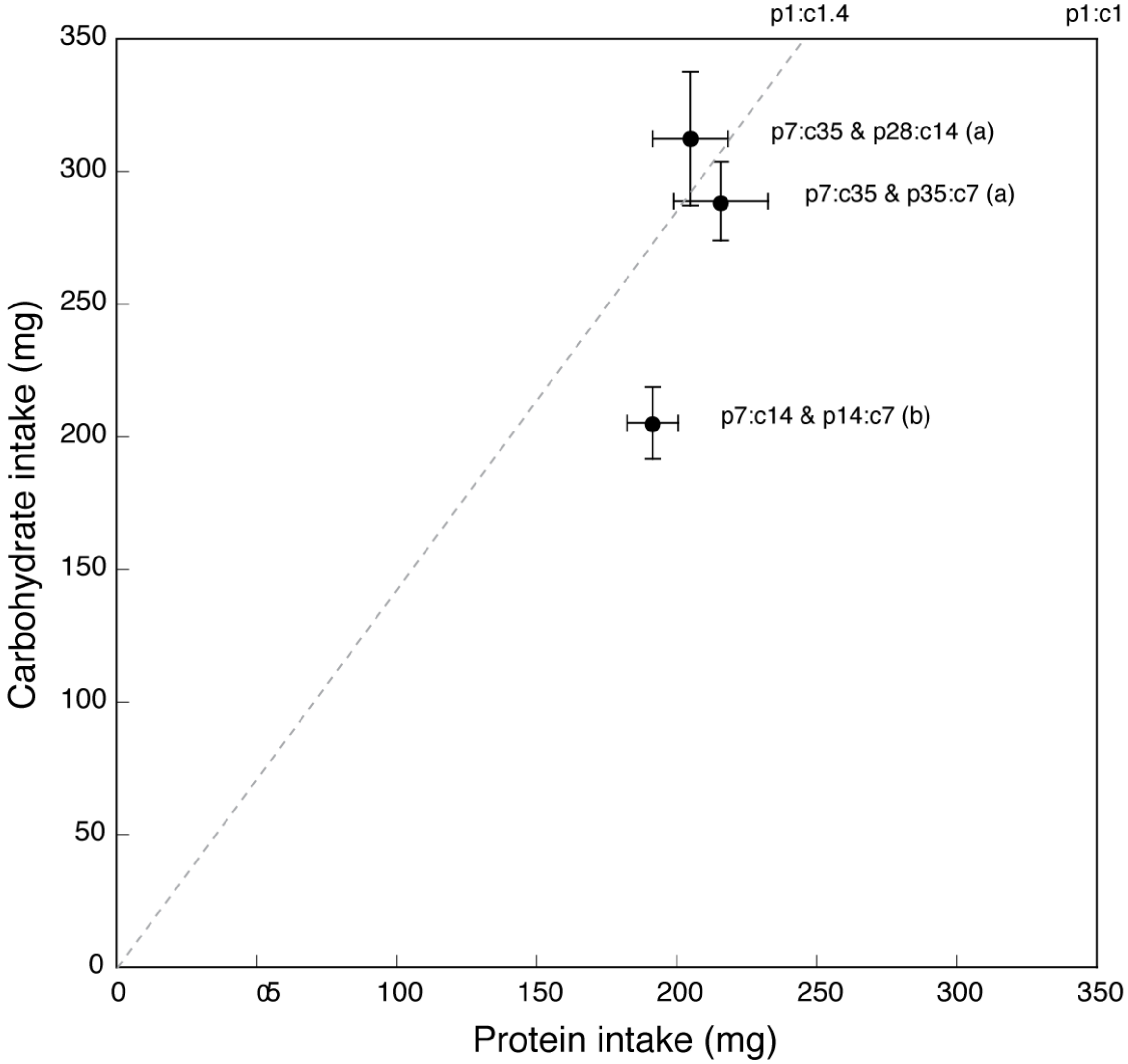

Figure 2.2 Protein-carbohydrate consumption for grasshoppers from the choice experiment. Different letters adjacent to treatments indicate statistically significant differences ($P < 0.05$). The dashed line represents the mean protein-carbohydrate ratio of the two high nutrient density treatments.

TABLE 2.1 Results of MANCOVA for protein and carbohydrate intake by grasshoppers from the choice experiment.

Source	df	*F* values
A) Protein and carbohydrate intake		
Treatment	4	3.15**
Sex	2	2.20
Initial mass	2	1.74
B) Contrast		
(1) p7:c35 & p35:c7 *vs* (2) p7:c35 & p28:c14	2	0.61
(2) p7:c35 & p28:c14 *vs* (3) p7:c14 & p14:c7	2	7.69†
(1) p7:c35 & p35:c7 *vs* (3) p7:c14 & p14:c7	2	4.09†

Notes: Part (A) reports overall *F* values (Pillai's Trace). Treatment refers to the P:C ratio; initial mass was used as a covariate to adjust for size differences among insects. Part (B) reports *F* values for multivariate contrasts between specific treatments.
* *P* , 0.05; ** *P* , 0.01.
† Significant difference with a $\alpha = 0.05/3$.

2.3.1.2 Dry mass gain and development time

Dry mass gain and development time are reported in Fig 2.2. No

No differences were observed between treatment 1 and 2 but the grasshoppers from treatment 3 gained about a third less body weight (ANCOVA: DF=2, F=5.57, p=0.010; Fig. 2.3 panel a). Developmental time varied with sex but wasn't affected by food composition (Fig. 2.3 panel b, Survival analysis, Df=2, ChiSquare: 1.08, p=0.583).

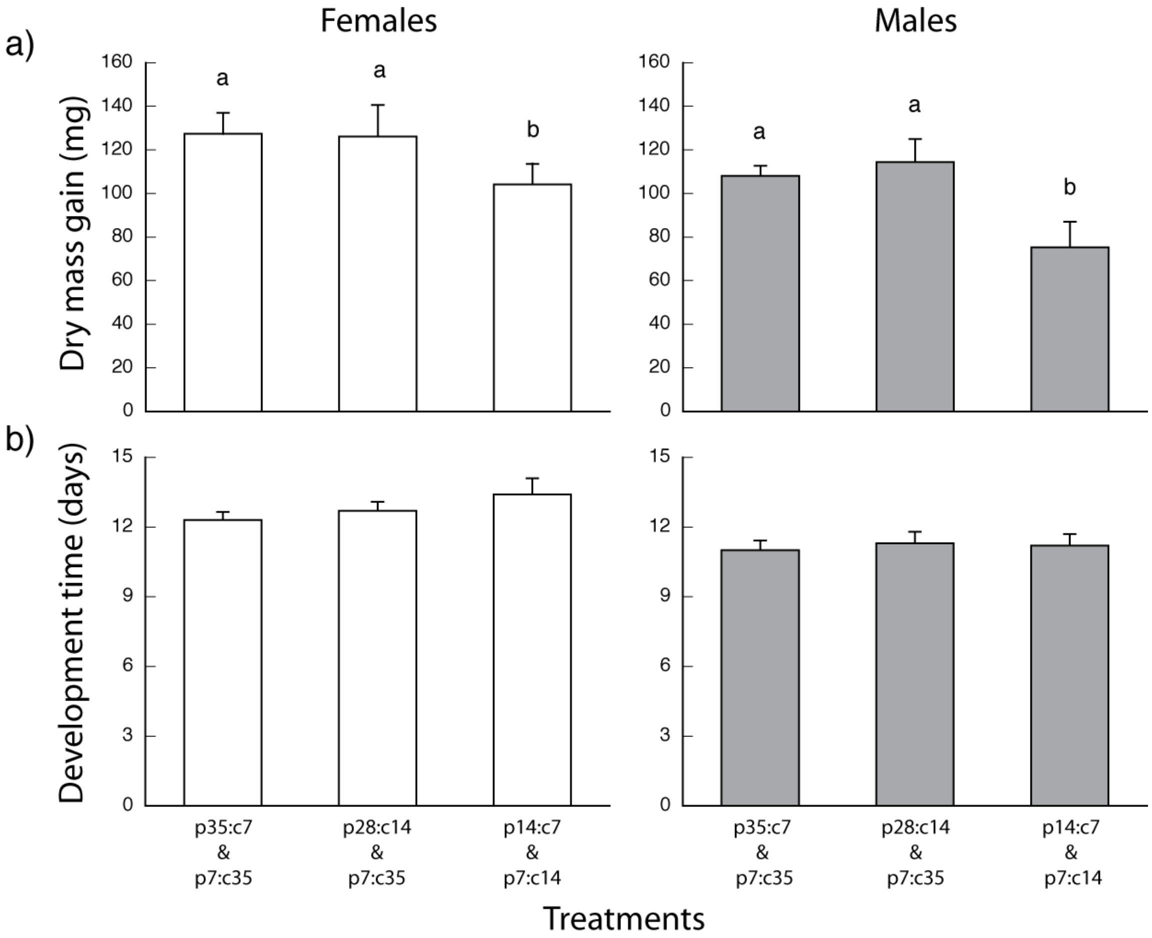

Figure 2.3 Grasshopper performance from the choice experiment. Panel (a) shows mean dry mass gain (±SEM) for females and males. Panel (b) shows mean development time (±SEM) for females and males. Different letters above the bars indicate statistically significant differences between treatments ($P < 0.05$).

2.3.2 No-choice experiment

I reported the effect of food protein:carbohydrate content on performance and consumption using a fitness landscape approach. Specifically I indicated whether or not there was a maximum/minimum in the nutrient landscape (response surface analysis, abbreviated as R.S.) and I compared this to the self-selected ratio obtained in the choice experiment (p1:c1.4). Then, I teased apart theses effects by comparing across diet treatments (ANCOVA analysis). Here, I specifically asked three key questions: 1) When concentrations are similar (diets lie on the same isocaloric line, see Fig. 2.1), what is the effect of nutrient balance? 2) When p:c ratios are similar (diets lie on the same nutritional rail, see Fig. 2.1) what is the effect of macronutrient concentration? 3) Is absolute amount better than balance? For this, I compared diets that have a fixed concentration of a given macronutrient (e.g. 7% protein) and are increasingly concentrated but also unbalanced toward the other macronutrient (p7:c7, p7:c14 and p7:c35) and compared them with the diets that all contained 7% of carbohydrate (p7:c7; p14:c7 and p35:c7). I conducted a similar comparison for diet containing 14% of protein and 14% carbohydrates.

2.3.2.1 Performance

Nutrient landscapes. For mass gain no maximum was found in the nutrient landscape but there was a positive linear effect of carbohydrates (R.S., DF=1, F=23.51, p<0.0001*) and the highest mass gains were recorded near the preferred ratio from the choice experiment (yellow dotted line) (Fig. 2.4 panel a). However for development time we found that insects developed fastest on the most concentrated food (p35:c35), and the extreme nutrient ratio (p7:c35 and p35:c37) (R.S., DF=1, F= 4.54, p= 0.037*), which do not overlap with the self-selected nutrient intake (Fig. 2.4 panel b). Regarding the insect final lean mass, we found a minimum (negative quadratic effect) that was due to carbohydrates (R.S., DF=1, F=4.36, p=0.041*). The preferred self-selected nutrient intake is located at the edge of this minimum (Fig. 2.4 panel c). Similarly, there was also quadratic effect of carbohydrates for body lipid content, however corresponding to a maximum this time (R.S., Df=1, F=8.73, p=0.005*) and the preferred nutrient ratio was located at the edge of this maximum (Fig. 2.4 panel d).

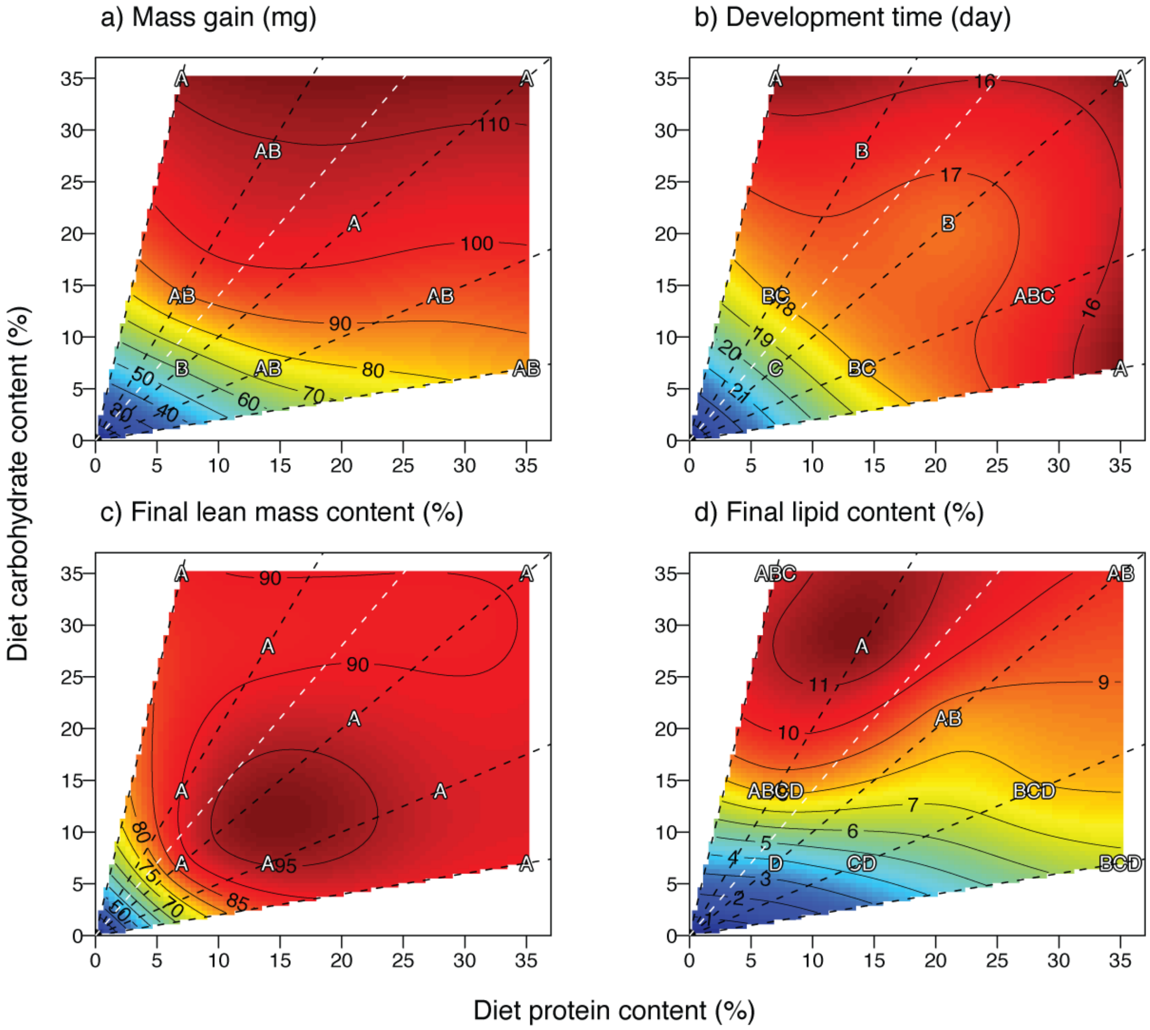

Figure 2.4 Last instar grasshopper performance, represented as fitness landscape plots, from the no-choice experiment. Panel (a) shows mean dry mass gain; panel (b) shows mean development time; panel (c) shows lean mass as a percent; panel (d) shows lipid content as a percent. The five p:c ratios tested are shown as black-dashed lines (nutritional rails) arising from the origin; the self selected p:c ratio calculated in the first experiment is indicated by the yellow-dashed line. Performance increases as colors become warmer and decreases as colors become cooler. Different letters indicate statistically significant differences between the nine diet treatments (as shown in Figure 2.1).

Nutrient balance. At similar concentration (along a single isocaloric line) but different nutrient ratio, there was no difference on dry mass gain among treatments (Fig. 2.4 panel a). For development time however I found that at high concentrations (42%) insects developed faster on the most unbalanced ratio (p7:c35 and p35:c7). No effects of nutrient balance were observed at lower concentration (21%) (Fig. 2.4 panel b). For lean mass there was no effect of nutrient balance at either concentration (Fig. 2.4 panel c). At high concentration (42%), lipid content was highest on the moderately carbohydrate-biased diet (p14:c28) than on the protein-biased diets. Although not significant, the same trend was observed at the lower concentration (21%) (Fig. 2.2 panel c).

Nutrient concentration. At similar nutrient ratio but different concentration (along a single nutritional rail), mass gain was smaller on the balanced but most diluted treatment (p7:c7) (Fig. 2.4 panel a). For development time, differences were also observed on the balanced ratio and insects grew faster as concentration increased (p7:c7 > p21:c21> p35:c35) (Fig. 2.4 panel b). Concentration had no effects on lean mass (Fig. 2. 4 panel c). Lipid content was also lower on the balance but most diluted diet (p7:c7) (Fig. 2.4 panel d).

Nutrient balance versus nutrient concentration. Insects fed on diets containing 7% protein achieved higher dry mass gain as the diet grew unbalanced toward carbohydrate (p7:c35 ≥ p7:c14 ≥ p7:c7) whereas the ones that consumed diets containing 7% carbohydrate did not benefit from an increase in protein concentration. No differences between diets were observed with 14% of protein or carbohydrate (Fig. 2.4 panel a). For development time, I found a symmetric pattern around the balanced nutritional rail for both the 7% and 14% level, with always a positive effect of nutrient concentration on development time (Fig. 2.4 panel b). For lean mass, on carbohydrate-biased diets, it was lower as concentration increased. However there was no effect of balance or concentration for protein-biased diets (Fig. 2.4 panel c). There was a strong positive effect of increasing unbalance toward carbohydrate for lipid content on diets containing 14% of protein (p14:c7 vs. p14:c28), while for diet containing 14% carbohydrate increase in protein concentration had no effects (p7:c14 vs p28:c14) (Fig. 2.4 panel d).

2.3.2.2 Consumption

Nutrient landscapes. The amount of food consumed is shown in Fig. 2.5 panel a. I found a minimum for the strongly protein-biased ratio p35:c10 (R.S., DF=1, F= 31.42, $p<0.0001$*) and the highest food intake was on the carbohydrate-biased area and encompassed the self-selected nutrient intake (yellow dotted line) (Fig. 2.5 panel a). For energy intake there was no maximum in the surface but carbohydrates had a positive linear effect (R.S., DF=1, F=10.31, $p<0.0021$*) and the highest intake was found near the preferred nutrient ratio (Fig. 2.5 panel b). For protein consumption there was a positive quadratic effect of protein (R.S., DF=1, F=6.09, $p<0.0162$*), this maximum was located at the edge of the self-selected nutrient ratio (Fig. 2.5 panel c). Similarly for carbohydrate consumption there was a quadratic effect of carbohydrate (R.S., DF = 1, F= 18.59, p=0.0001*). This maximum was centered around a p1:c2 ratio but encompassed the preferred p1:c1.4 ratio (Fig. 2.5 panel d). For frass production there was a minimum in the nutrient surface (R.S., DF = 1, F= 39.55, p=0.0001*). This minimum was near the most concentrated diet (p35:c35) and conversely the highest frass production was found for the most diluted diet (p7:c7) (Fig. 2.5 panel e). Regarding the amount of food digested, there was a maximum in the nutrient surface (R.S., DF = 1, F= 13.77, p=0.001*) centered at a p1:c2 ratio but encompassing the preferred nutrient ratio (Fig. 2.5 panel f).

Nutrient balance. At similar concentrations (isocaloric line), there was no effect of nutrient ratio on food consumption, however at high concentration (42%) there was a trend for consumption to be higher on the balanced diet and on the carbohydrate-biased diet when the food was more diluted (21%) (Fig. 2.5 panel a). There was no significant effect of nutrient ratio on energy intake, but there was a trend for it to be higher at moderately carbohydrate-biased foods (Fig. 2.5 panel b). Protein intake was always higher on protein-biased food (Fig. 2.5 panel c) and carbohydrate intake on carbohydrate-biased diets (Fig. 2.5 panel d). Nutrient balance had no effects on frass production (Fig. 2.5 panel e). The amount of food digested was higher on moderately carbohydrate-biased food for both concentrations (42% and 21%) (Fig. 2.5 panel f).

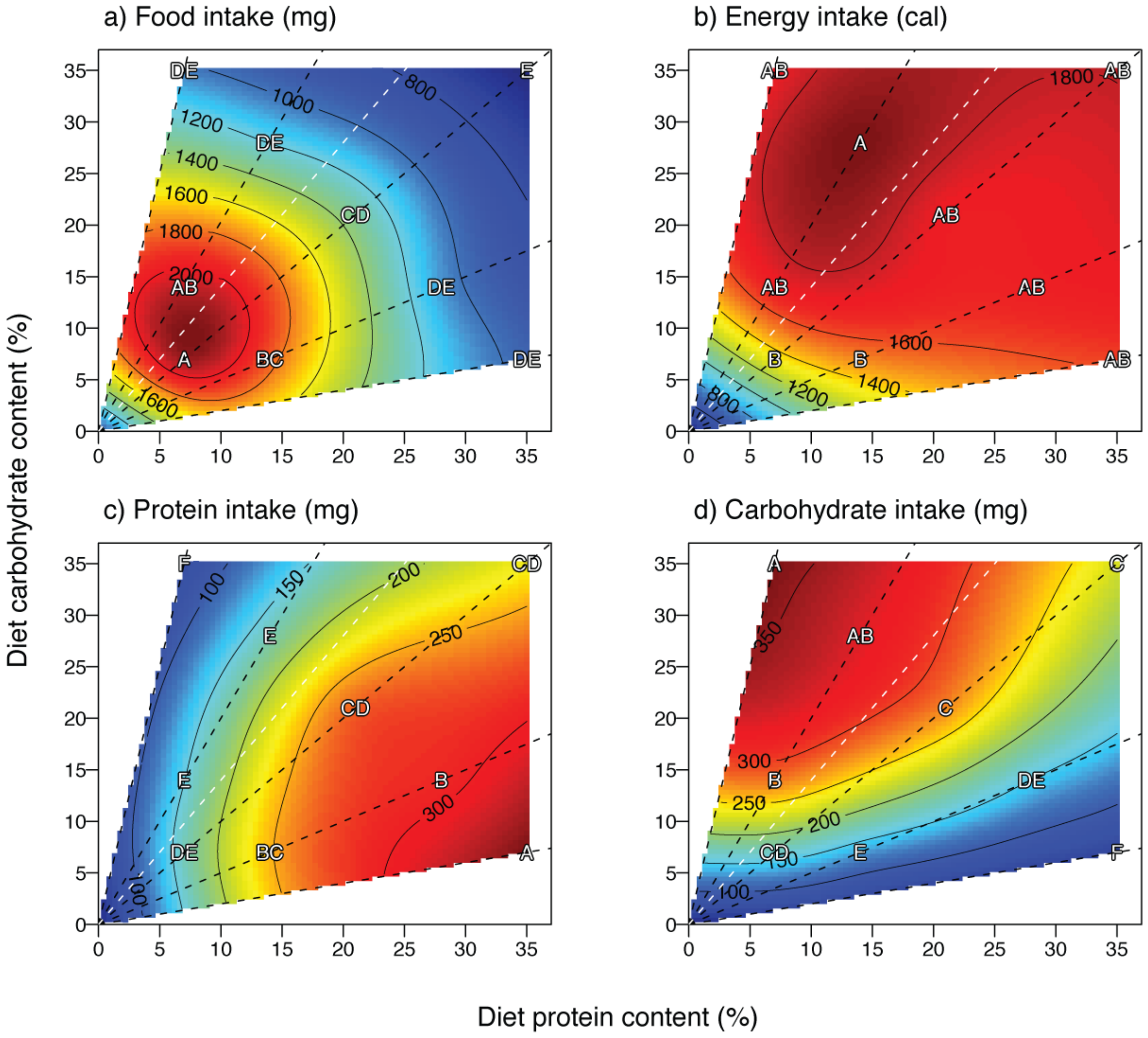

Figure 2.5. Last instar grasshopper consumption, represented as fitness landscape plots, from the no-choice experiment. Panel (a) shows mean food intake; panel (b) shows mean calorie intake; panel (c) shows mean protein intake; panel (d) shows mean carbohydrate intake. The five p:c ratios tested are shown as black-dashed lines (nutritional rails) arising from the origin; the self selected p:c ratio calculated in the first experiment is indicated by the yellow-dashed line. Performance increases as colors become warmer and decreases as colors become cooler. Different letters indicate statistically significant differences between the nine diet treatments (as shown in Figure 2.1).

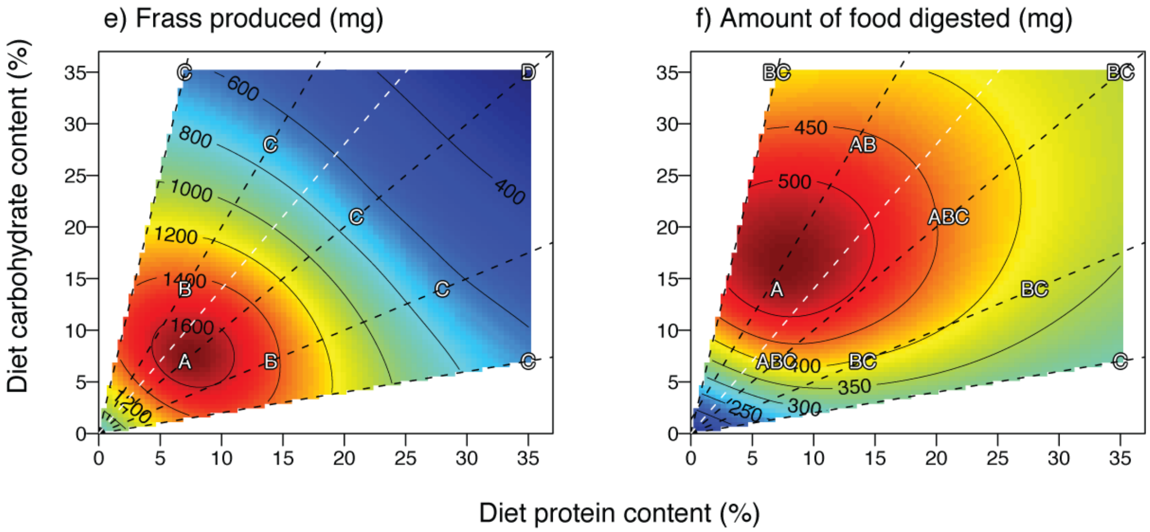

Figure 2.5 continued. Last instar grasshopper consumption, represented as fitness landscape plots, from the no-choice experiment. Panel (e) shows mean frass production; panel (f) shows mean amount of food digested. The five p:c ratios tested are shown as black-dashed lines (nutritional rails) arising from the origin; the self selected p:c ratio calculated in the first experiment is indicated by the yellow-dashed line. Performance increases as colors become warmer and decreases as colors become cooler. Different letters indicate statistically significant differences between the nine diet treatments (as shown in Figure 2.1).

Nutrient concentration. At similar nutrient ratio (nutritional rail), the amount of food consumed decreased with increasing concentration: 14% macronutrient ≥21% macronutrient>42% macronutrient≥70% macronutrients (Fig. 2.5 panel a) but had no effect on energy intake (Fig 2.5 panel b). For both protein and carbohydrate, the lowest intake was found on the balanced but most diluted diet (p7:c7) (Fig. 2.5 panel e & d). Frass production was always smaller as concentration increased (70% < 42% < 21% < 7%) (Fig. 2.5 panel e). but there was no effect of concentration on the amount of food digested (Fig. 2.5 panel f).

Nutrient balance versus nutrient concentration. For food consumption the negative effect of concentration was less important for carbohydrate-biased diet than for protein-biased diets (Fig 2.5 panel a). For energy intake however, concentration had a strong, positive effect, on carbohydrate-biased food containing 14% of protein (Fig. 2.5 panel b). For protein and carbohydrate intake, both concentration and nutrient balance affect nutrient intake (Fig. 2.5 panel c & d). Frass production is dependant on concentration only and balance has no effect on it (Fig. 2.5 panel e). For the amount of food digested unbalance had more effects than concentration for diets containing 7% of protein and diets containing 7% of carbohydrates. However, when looking at the 14% level, I found this to be true only for protein biased-diets (Fig. 2.5 panel f).

2.4 Discussion

When given the opportunity, most herbivorous insects will regulate their protein and carbohydrate intake by selectively eating foods that contain different blends of these nutrients (Behmer 2009). However, this has mostly been demonstrated using artificial diets at relatively high nutrient concentration (usually 42% macronutrients). In reality, insects usually encounter significant variation in total nutrient content due to genetic and environmental variables (i.e. drought, phenology, etc) (Felton 1996, Osier and Lindroth 2001, Schoonhoven et al. 2005, Walter et al. 2012). Interestingly, in my choice experiment, I found that concentration affected both performance and food selection behavior. Insects fed the diluted treatments ceased to regulate for a protein:carbohydrate ratio and prioritized protein intake solely. This plasticity in food selection behavior is most likely adaptive in field conditions, given that animals such as herbivorous insects pay substantial time costs to regulate nutrient intake, which increases predation risk (Bernays and Minkenberg 1997, Lima 1998), and that nutrient compensatory behavior is physiologically costly (Martin and Van't Hof 1988, Slansky and Wheeler 1989, Simpson and Simpson 1990, Slansky Jr et al. 1993). In the diluted choice treatment, grasshoppers achieved lower mass gain. Given that protein intake was regulated across all treatments, this establishes clearly the importance of carbohydrates in regard to growth. However the absence of carbohydrate regulation did not lead to any observable costs for development time, perhaps because protein can also be used to provide energy via gluconeogenesis (Thompson 1998, Thompson and Redak 2000).

We have now established that in optimal conditions *M. differentialis* regulated for a carbohydrate-biased intake target and when we looked at the global effects of nutrients on performance and consumption we found that mass gain, food and energy intakes, as well as amount of nutrient digested were all high near the self-selected nutrient ratio (p1:c1.4). It thus appears that this nutrient ratio is optimal for adult weight. Adult body size is a correlate of fitness in many organisms and in insects large body sizes have been associated with greater survival and fecundity (Roff 1992, Stearns 1992, Nylin and Gotthard 1998). We found that both pre-and post ingestive mechanisms play an important role here as grasshoppers consumed more food on the preferred ratio, but they also digested more efficiently. A possible mechanism might be linked to optimal enzyme activity at the preferred nutrient ratio. It has already been shown that locusts can differentially release digestive enzyme in function of nutritional status to compensate for nutritional deficit (Clissold et al. 2010).

Reserves accumulated during the last instar are also important for numerous adult functions like dispersal, post-molting somatic growth (e.g. mass gain in internal tissues), reproduction, and maintenance (Hahn and Wheeler 2003, Hahn 2005). For example in the African migratory locust, nymphs fed carbohydrate-biased diets accumulated significant fat reserves and these locusts survived starvation in early adulthood better than leaner individuals fed balanced or protein-biased diets (Raubenheimer and Simpson 1997). Interestingly, we found that although the self-selected nutrient ratio did promote high body lipid content, it was not the highest. Most likely (as for humans) insects suffer fitness costs when lipid levels are too high. This has previously been demonstrated on caterpillars reared on carbohydrate-biased foods for multiple generations which progressively developed the ability to eat excess carbohydrate without laying it down as fat (Warbrick-Smith et al. 2006). Lipid deposition has important physiological cost, theoretical estimates suggest that the conversion of hexoses to storage fat can take up to 20–25% of the energy content of the food supplied (Westerterp 1994).

Development time is an important fitness parameter, because faster growing individuals reduce the risk of predation resulting in a higher probability of survivorship (Roff 1992). Development time was best on the concentrated and the extremely unbalanced food (p7:c35 and p35:c7), not on the preferred nutrient ratio. A possible explanation as to why extreme unbalanced lead to similar results might be because they both contributed to insects reaching the critical weight (as defined by Nijhout and

Williams 1974) necessary for molting to adults faster. On the extreme carbohydrate-biased diet because of the massive accumulation of lipids and on protein-biased diet because excess protein can be used for gluconeogenesis (Thompson 1998, Thompson and Redak 2000). Overall there was strong positive effect of nutrient concentration on development time. This is interesting because, as we saw in the choice experiment, when the food was diluted, insect opted for a behavioral strategy that favored development time over growth. There is thus an important trade-off for *M. differentialis.* When nutrients are readily available insects self-selected a nutrient ratio that promoted growth, but when nutrients were limited faster development time might was favored. Natural selection showed a tendency to increase size in 79% of studies (Kingsolver and Pfennig 2004), and 84% of studies show that natural selection acts to decrease development time (Kingsolver and Huey 2008). One possible reason for this physiological trade-off is that selection for both large size and short development time causes increased susceptibility to oxidative stress, as recently seen in caterpillars (Harrison et al. 2013).

Interestingly the part of the nutritional space that has previously being ignored (more diluted diets) showed very interesting results. I found that insects were rather resilient to food dilution at the notable exception of the ones fed the most diluted diet (p7:c7). This diet probably exceeds the insects capacities for compensation because of the direct physiological constraints associated with compensatory feeding such as reduced food retention time in the gut, or metabolic effects of ingesting excess cellulose (Martin and Van't Hof 1988, Slansky and Wheeler 1989, Simpson and Simpson 1990, Slansky Jr et al. 1993). In contrast insects were quite sensitive to nutrient balance and there was evidence for interactions between nutrient balance and concentrations. For instance the amount of food digested was best on carbohydrate-biased diet that were also diluted.

This study presents the first overview of insect responses to a large nutrient landscape and provided some insight into the physiology and behavior of a generalist herbivore when faced with unbalance and dilution altogether. I found that food intake behavior is important to understand the physiological basis of life-history trade-offs such as body size, fat reserve and development time.

CHAPTER III

EFFECTS OF NUTRIENT-ALLELOCHEMICAL INTERACTIONS ON A GENERALIST HERBIVORE

3.1 Introduction

The primary reason organisms eat is to acquire a suite of nutrients, with protein and carbohydrates being particularly important (Raubenheimer et al. 2009). Protein and carbohydrates provide the amino acids and energy necessary to sustain growth, maintenance and reproduction. Their importance is illustrated by the fact that it has been repeatedly shown that animals can adjust their food consumption to regulate protein and carbohydrate intakes, and that a failure to do so has major fitness consequences (Raubenheimer et al. 2009).

For herbivores, plant chemistry poses two major challenges. First, plant nutritional content is incredibly variable both in space and time (Hendrix and Huber 1986, Chapin et al. 1987, Felton 1996, Walter et al. 2012). There is variation in protein and carbohydrate content between plant species, but also within plant tissues as well as within plant tissues (i.e. young versus old leaf). Secondly, plants also contain secondary compounds that can act as toxins and affect consumption patterns as well as performance (Behmer et al. 2002, Wittstock and Gershenzon 2002, Harborne 2007).
Disentangling the relative effects of nutrient versus chemical defenses has proven difficult. This is particularly true for generalist herbivore that experience diet mixing and encounter a wide variety of plant chemical profiles. In fact, two hypotheses are usually advanced to explain the benefit of diet mixing in regard to plant chemistry (Bernays et

al. 1994). The first one states that the enhance performance observed on mixed diets are to be related with nutrient balance. The second hypothesis proposes that alternating between food sources allows keeping the intake of any given allelochemical low. Both hypotheses have received some support, but in reality they are not mutually exclusive.

Recent work in the field of nutritional ecology has demonstrated the existence and importance of interaction between nutrient and plant defenses on performance for a variety of herbivores ranging from a grass specialist locust (Simpson and Raubenheimer 2001) to grazing mammals (Provenza et al. 2003), although the exact mechanisms are still unknown. Despite these recent advances, diet quality is often defined as a single currency (often using plant induced defenses or nitrogen content) and little regard is paid to the interactions between diet components.

In this study I used a multifactorial approach to produce the first comprehensive study to examine the effect of nutrient-allelochemical interactions on a generalist insect herbivore. I used nymphs of the differential grasshopper *M. differentialis* and constrained them to nine different protein-carbohydrate ratio and concentrations. I recorded consumption, mass gain, development time and lipid content. I then measured how the addition of two different concentrations of an alkaloid (gramine) affected these variables using a fitness landscape approach. My results further the current understanding of how nutrient-allelochemical interaction might shape herbivore performance and physiology, and I discuss my results in the context of detoxification.

3.2 Material and Methods

3.2.1 Insects and experimental chambers

Melanoplus differentialis (Orthoptera: Acrididae), the ‘differential grasshopper’, feeds on a wide range of herbaceous early successional plants (Yang and Joern 1994b). Nymphs pass through six juvenile instars and adults are relatively large in size. The grasshoppers that were used come from a lab colony initiated in 2008 with individuals coming from College Station area. The colony is maintained in the Department of Entomology, Texas A&M University, USA, on a diet of greenhouse-grown seedling wheat and wheat germ. Nymphs of both sexes were collected from the culture at ecdysis to the sixth stadium (day 0), within an age range of 4–6 h. Insects from the final nymphal stadium were chosen because their large body size means that larger amounts of food are eaten, yet, since they are not adults there should be little feeding for reproductive development, only somatic growth. The mass of each newly molted grasshopper was recorded (to the nearest 0.1 mg), and then grasshoppers were placed individually into a 19 cm long x 14 cm wide x 9 cm high, clear plastic container. Each container was outfitted with an expanded aluminum perch, a water source, and one food dish (based on a 5.5-cm Petri dish, modified to minimize spillage, as described by Raubenheimer and Simpson [1990]). Insects were maintained throughout the experiment in a room where temperature varied daily from 37ºC during the day and 28ºC at night under a 14:10 light:dark regime.

3.2.2 Synthetic food composition

The chemically defined foods used in this study were based on those used by Simpson and Abisgold (1985). For convenience, foods were abbreviated in function of their macronutrient percentage. For instance a diet labeled p7:c21 contained 7% of protein and 21% of carbohydrate. In total, nine foods differing in their absolute amounts and ratios of protein and digestible carbohydrates were prepared:

3 had equal ratios (p7:c7, p14:c14 and p21:c21)

3 were protein-biased (p10.5:c21, p14:c7 and p21;c7)

3 were carbohydrate-biased (p21:c10.5, p14:c7 and p7:c21)

The protein content of all foods consisted of a 3:1:1 ratio of casein, peptone and albumen, and sucrose and dextrin (in a 1:1 ratio) as digestible carbohydrates. All foods contained fixed amounts of the following ingredients: Wesson's salt (2.4%), cholesterol (0.5%), linoleic acid (0.5%), ascorbic acid (0.3%) and 0.2% of a vitamin mix (Dadd 1961). The rest of the food was composed of a non-nutritive bulking agent, cellulose.

The allelochemical chosen was gramine. Gramine is an alkaloid present in grasses (Poaceae) with deterrent/toxic effects on herbivorous insects (Harley and Thorsteinson 1967, Hinks and Olfert 1999, Ishikawa and Kanke 2000). Gramine was added and mixed vigorously to obtain a homogeneous mixture, and because these diets were dry, the allelochemical remained inactive until it was consumed. Each of the nine diets was tested at three gramine concentrations: 0 (control), 0.5% (high) and 2% (very high) (Hanson et al. 1983, Bernays 1991).

3.2.3 Protocol

On the first day of the 6th instar (day 0), individuals received a dish of food from one of the nine treatments. For each treatment 10 grasshoppers were used (5 of each sex). Prior to be placed in the experimental arena, all food dishes were always allowed to equilibrate under stable ambient room humidity for ~24 h and then weighed to the nearest 0.1 mg. Every three days, the dishes were collected and replaced with fresh, pre-weighed food until the grasshoppers molted to the adult stage, or died. The food was changed and measured at these regular intervals to determine if consumption changed over time. The experiment ended when grasshoppers molted into adults, at which time they were removed and weighed to the nearest 0.1 mg. In order to measure the amount of food digested, frass production was also recorded. Following death, grasshoppers were freeze-dried and weighed to the nearest 0.1 mg. Lipid were extracted in three, 24-h changes of chloroform. At the end of the third chloroform wash grasshoppers were re-dried and re-weighed to calculate lipid content. Initial dry mass was estimated with a regression equation that was derived from the wet and dry mass of 20 newly molted 6th instar grasshoppers from the lab colony.

3.2.4 Statistical analyses

Consumption and various measures of performance (dry mass gain, development time and body lipid content) were mapped on the protein-carbohydrate landscape using response surface techniques. These fitness surfaces are best visualized by using nonparametric techniques that do not constrain the shape of the surface (Blows and

Brooks 2003); this was accomplished using thin-plate splines within the fields package in R (version R 2.11.1) (Simpson et al. 2004). Nutrient landscapes were compared using partial F-test and analyzed with a response surface approach where linear and quadratic effects of protein, carbohydrates, and gramine are given using start mass as a covariate.

3.3 Results

For all treatments gramine had a negative effect on the variables recorded (consumption, development time, mass gain and survival). However for some of these variables these effects were mediated by protein-carbohydrate content (quadratic effect) (Table 3.1).

3.3.1 Amount of food eaten at day 6 of the experiment

In the absence of gramine (Fig. 3.1, first panel), consumption was higher on diluted and on carbohydrate-biased diets (Fig. 3.1, left nutritional rails). When gramine was added this compensatory response was negatively affected (Fig. 3.1, second panel), and at the highest concentration (Fig. 3.1, third panel) insects were neither compensating for dilution, or for protein intake. Consumption is actually higher on the most concentrated equal ratio diet (Protein*gramine and carbohydrate*gramine interactions Table 3.1, Fig. 3.1).

Table 3.1 The full parametric model for the linear and quadratic effects of protein, carbohydrates, and gramine food content on consumption at day 6 and at the end of the experiment, development time, dry mass gain, and survival.

Variable	Consumption day 6	Total consumption	Development time	Dry mass gain	Survival
Start mass	F=31.0 P<0.001*	F=85.9 P<0.001*	F=2.6 P=0.111	F=37.1 P<0.001*	F=1.9 P=0.168
Protein	F=14.1 P<0.001*	F=174.0 P<0.001*	F=13.1 P<0.001*	F=15.5 P<0.001*	F=18.2 P<0.001*
Carbohydrates	F=0.1 P=0.730	F=108.8 P<0.001*	F=9.9 P=0.002*	F=29.4 P<0.001*	F=19.0 P=0.001*
Gramine	F=19.2 P<0.001*	F=9.2 P=0.003*	F=16.5 P<0.001*	F=8.5 P=0.004*	F=27.0 P=0.001*
Protein^2	F=3.6 P=0.059	F=12.1 P=0.001*	F=6.9 P=0.009*	F=4.5 P=0.035*	F=11.5 P=0.001*
Protein * carbohydrates	F=2.1 P=0.153	F=6.5 P=0.012*	F=1.2 P=0.273	F=0.1 P=0.710	F=2.9 P=0.090
Carbohydrates^2	F=0.5 P=497	F=0.1 P=0.722	F=0.1 P=0.714	F=9.7 P=0.002*	F=3.3 P=0.071
Protein * gramine	F=18.5 P<0.001*	F=0.1 P=0.795	F=4.5 P=0.035*	F=3.1 P=0.081	F=3.1 P=0.078
Carbohydrate * gramine	F=9.5 P=0.002*	F=3.5 P=0.064	F=3.0 P=0.090	F=0.1 P=0.746	F=1.8 P=0.175
Gramine^2	F=2.6 P=0.110	F=1.2 P=0.267	F=0.9 P=0.336	F=0.4 P=0.517	F=1.9 P=0.168

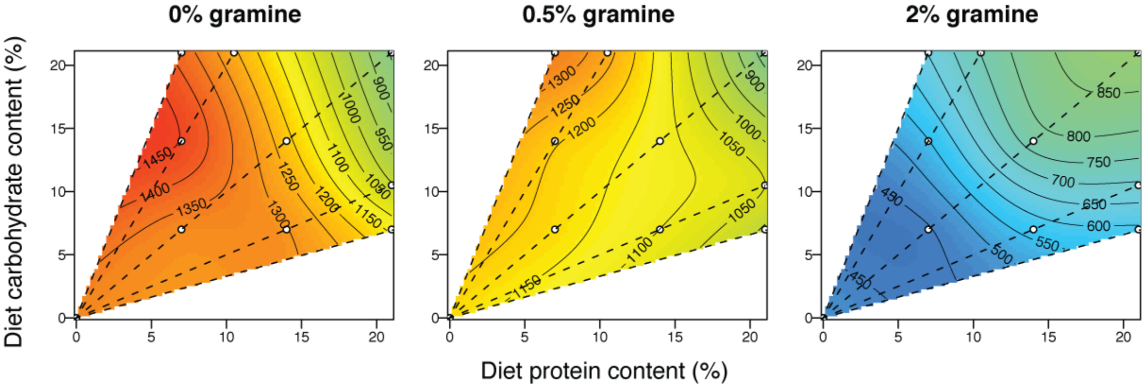

Figure 3.1 Amount of food eaten (mg) by last instar grasshopper at day 6 of the experiment in the absence of gramine, when 0.5% gramine is present in the diets, and when 2% of gramine is present in the diets. Data are represented as fitness landscape plots where amount of food eaten increases as colors become warmer and decreases as colors become cooler, full black lines (isoclines) indicate the amount eaten. White dots indicate the nine protein-carbohydrate treatments. The five p:c ratios tested are shown as black-dashed lines (nutritional rails) arising from the origin.

3.3.2 Amount of food eaten over the experiment

At the end of the experiment, regardless of the gramine content of the food, insects that were fed diluted diets ate more than those fed concentrated diets (Fig. 3.2). When concentration was similar, grasshoppers consumed more of the carbohydrate-biased food (Fig. 3.2, left nutritional rails); there is a general trend for this pattern to become stronger when gramine was added and the gramine*carbohydrate interaction was marginally significant (Table 3.1).

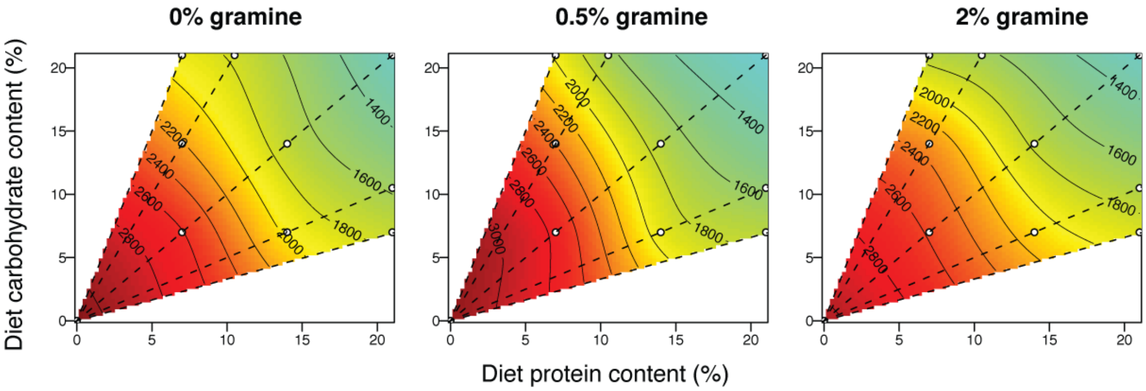

Figure 3.2 Amount of food eaten (mg) by last instar grasshopper at the end of the experiment in the absence of gramine, when 0.5% gramine is present in the diets, and when 2% of gramine is present in the diets. Data are represented as fitness landscape plots where amount of food eaten increases as colors become warmer and decreases as colors become cooler, full black lines (isoclines) indicate the amount eaten. White dots indicate the nine protein-carbohydrate treatments. The five p:c ratios tested are shown as black-dashed lines (nutritional rails) arising from the origin.

3.3.3 Development time

Without gramine, development time was best on concentrated foods, regardless of the nutrient ratio (Fig. 3.3, first panel). With 0.5% gramine in the diet, there was a moderate shift in this pattern (Fig. 3.3, second panel, isocline 15) and grasshoppers fed carbohydrate-biased foods (Fig. 3.3, left nutritional rails) took longer to develop. At 2% this pattern became stronger (Fig. 3.3, third panel), and insects fed diets that contained less than 12% of protein saw their development slow down considerably. For instance, grasshoppers on the p7:c14 diet developed in 15 days, on the 0.5% gramine in 15 to 20 days, and at 2% gramine development time was 25 to 30 days. By contrast insects fed the p14:c7 diet did not take any longer to develop at 0.5% gramine, and on the 2% gramine diet they developed in 20 to 25 days.

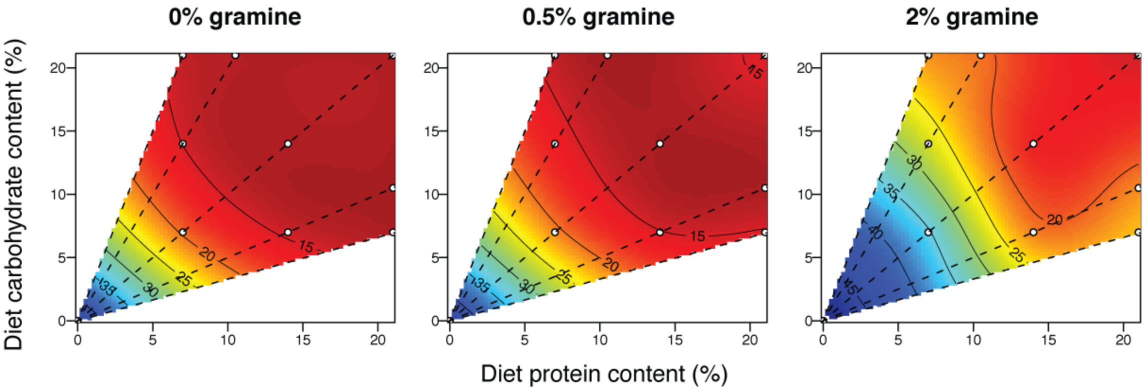

Figure 3.3 Development time (day) by last instar grasshopper in the absence of gramine, when 0.5% gramine is present in the diets, and when 2% of gramine is present in the diets. Data are represented as fitness landscape plots where the development time increases as colors become cooler and decreases as colors become warmer, full black lines (isoclines) indicate the number of days. White dots indicate the nine protein-carbohydrate treatments. The five p:c ratios tested are shown as black-dashed lines (nutritional rails) arising from the origin.

3.3.4 Dry mass gain

Grasshoppers were bigger when fed concentrated and carbohydrate-biased diets (Fig. 3.4, first panel, left nutritional rails). Mass gain was reduced by the addition of gramine (Table 3.1 linear effect). Interestingly there was a marginally significant carbohydrates*gramine interaction (Table 3.1); grasshoppers feeding on the concentrated and equal ratio protein:carbohydrate diets (Fig. 3.4, middle nutritional rail) were bigger than those fed diets with unbalanced ratios. Thus, there was a shift from carbohydrate-biased diet in the absence of gramine, to balanced diet in the presence of the alkaloid.

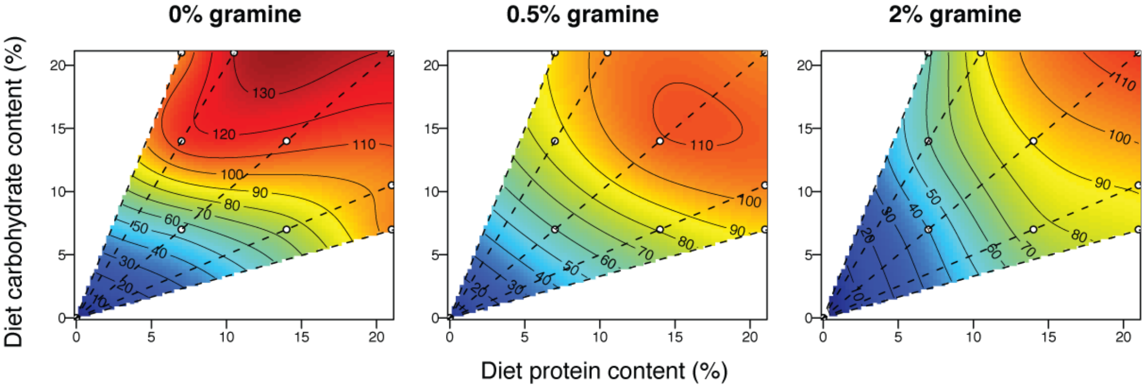

Figure 3.4 Dry mass gain (mg) by last instar grasshopper in the absence of gramine, when 0.5% gramine is present in the diets, and when 2% of gramine is present in the diets. Data are represented as fitness landscape plots where dry mass gain increases as colors become warmer and decreases as colors become cooler, full black lines (isoclines) indicate the amount of mass gained. White dots indicate the nine protein-carbohydrate treatments. The five p:c ratios tested are shown as black-dashed lines (nutritional rails) arising from the origin.

3.3.5 Survival

In the absence of gramine, survival was high on all treatments except the very diluted p7:c7 (Fig. 3.5, first panel). The addition of gramine shifted that pattern dramatically. There seems to be an optimum amount of protein (15%) in the food that promotes higher survival, although the realtionship is only marginally significant (Table 3.1, protein*gramine interaction).

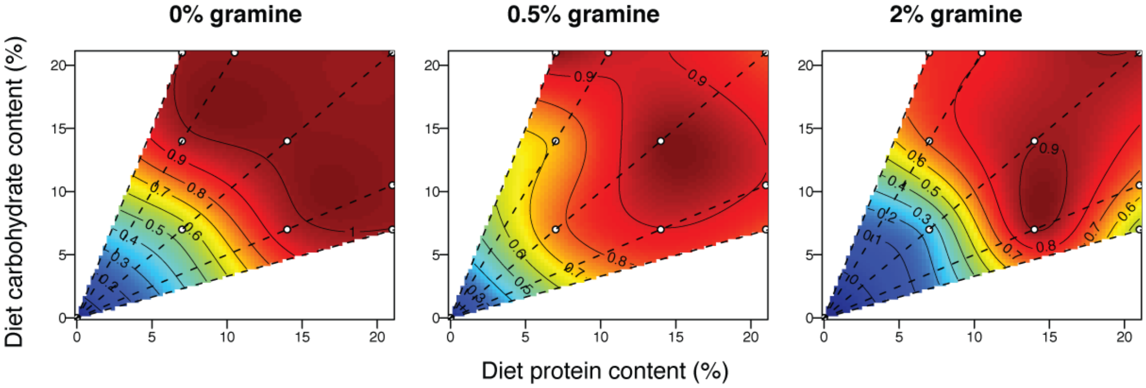

Figure 3.5 Survival (%) by last instar grasshopper in the absence of gramine, when 0.5% gramine is present in the diets, and when 2% of gramine is present in the diets. Data are represented as fitness landscape plots where survival increases as colors become warmer and decreases as colors become cooler, full black lines (isoclines) indicate the proportion of grasshopper surviving. White dots indicate the nine protein-carbohydrate treatments. The five p:c ratios tested are shown as black-dashed lines (nutritional rails) arising from the origin.

3.4 Discussion

In the absence of gramine *M. differentialis* compensated for dietary dilution by increasing feeding and ate slightly more of the carbohydrate-biased food. Mass gain was also best on carbohydrate-biased food. Development time, however, depended on nutrient density, and decreased as concentration increased. These results are consistent with what was described for this population in the previous chapter. Without gramine, survival was good for all treatments except for the extremely diluted p7:c7. My results also demonstrated that the addition of gramine negatively affected consumption (at least initially) as well as performance (development time, mass gain and survival). More importantly I showed, for the first time while using various nutrient ratio and concentration of protein and carbohydrates, how these effects are mediated by nutrient-allelochemical interactions.

It is worth noting that the gramine concentrations that I used in my experiments may be higher than the estimated gramine concentrations in grass leaves (Hanson et al. 1981, Hanson et al. 1983, Sun et al. 2013), although it is within the range of what has been used previously in grasshopper experiments (Bernays 1991). To make sure that gramine consumption leads to measurable effects, high concentrations of the compound in the artificial diets were necessary since the toxicity of gramine may be undetectable or not easy to detect if gramine concentration in the diets is as low as that in plant tissues. Importantly my results demonstrate that even at high concentrations food nutrient content can alleviate the toxic effects of gramine.

One of the strength of my approach was the use of protein-carbohydrates profiles that were less concentrated than most nutritional studies done with grasshoppers; most experiments use a total macronutrient concentration of 42% (Raubenheimer and Simpson 1993, Simpson and Raubenheimer 2000, Simpson and Raubenheimer 2001, Simpson et al. 2002, Raubenheimer and Simpson 2003, Behmer and Joern 2008). Increasing the concentration of protein and carbohydrates can increase the ability of animals to eat foods that contain toxins as diverse as lithium chloride (Wang and Provenza 1997), menthol (Illius and Jessop 1996), terpenes (Banner et al. 2000, Villalba et al. 2002a), tannins (Villalba et al. 2002b), and saponins (Williams et al. 1992, Martinez et al. 1993, Strickland et al. 1998). I provided the first study to examine if nutrient-allelochemical interactions are relevant for a range of nutrient concentrations that encompass the variation existing in plants (see Behmer and Joern 2012 for information on plant protein-carbohydrate content).

Similarly to what was found in *Locusta* with tannic acid (Simpson and Raubenheimer 2001), I found that the effect the allelochemical on consumption (at least initially) depended on food protein-carbohydrate content. I also found nutrient-allelochemical interaction to have strong effects on development time and marginal effects on mass gain and survival. Because gramine is a feeding deterrent (Bernays 1991) my results on performance (development time, mass gain and survival) could be a function of differential consumption. However I saw that: 1) initial deterrence was also function of the nutrient content of food (carbohydrate-biased food were more deterrent), and 2) the consumption patterns at the end of the experiment were actually similar showing that insects eventually overcame the initial deterrence and that amount of food eaten were similar.

In the 2001 study locusts performed better on diets that contained an equal ratio of protein:carbohydrate, which is close to the ratio that they self-select in choice-experiment (Simpson and Raubenheimer 1993, Raubenheimer and Simpson 1999). In my case, the laboratory population of *M. differentialis* self-select for a carbohydrate-biased ratio (p1:c1.4, see chapter 2), while there was a general trend for performance to improve when there was more protein in the diet (at least 14%). The fact that for generalist the nutrient background that promotes better performance is different that the one self-selected in the absence of toxin could be evidence that the intake target is in fact very plastic. One would predict that if the self-selected nutrient intake evolved to promote fitness (see previous chapter) then it should also be adaptive in the presence of toxins. Since it is not the case here, I can hypothesize that this intake is plastic (see chapter V). Additional evidence of this plasticity is the difference in intake target observed along a latitudinal gradient for another Melanoplinae grasshopper (Parsons 2011). This is an important result because it has tremendous implication for our understanding of the factors that govern animal foraging behavior and how plant chemistry can impact herbivorous insects.

There are at least two explanations as to why I see differences in the direction of the nutrient unbalance. First the nature of the toxin could be a factor. For instance, lambs infused with terpenes, nitrate, tannins, or lithium chloride preferred foods with higher ratios of protein:carbohydrate, but following infusions with cyanide, preferred foods with higher ratios of protein:carbohydrate (Villalba et al. 2002c). Sheep and goats fed diets high in protein, or allowed to select between concentrates high in either carbohydrates or protein, ate more of a high terpene diet (Villalba et al. 2002a), and of a high-tannin diet (Villalba et al. 2002b), than when they were fed diets high in carbohydrate.

The second explanation (not exclusive to the first one) is that *Locusta Migratoria*, a grass specialist (that does not naturally encounter tannic acid in its food), and *M. differentialis*, a generalist, use different detoxification mechanisms. Generalists usually depend on broad detoxification mechanisms such as the use of p450 or gluthatione S-transferase (Schuler 1996, Ramsey et al. 2010) enzymes. However, a key question to ask is whether the production of these enzymes occur at a high cost (metabolic load) (Appel and Martin 1992, Cresswell et al. 1992, Castaneda et al. 2011, Rivero et al. 2011)? Regardless, this could still be a possible mechanism for explaining nutrient-allelochemical interactions.

Gramine has been considered as a deterrent and/or toxicant to many herbivorous insects such as *Schizaphis graminum* (Corcuera 1984), *Rhopalosiphum padi* (Zuniga and Corcuera 1986), *Sitobion avenae* (Leszczynski et al. 1989, Rustamani 1992, Moharramipour et al. 1997), *Locusta migratoria* (Ishikawa and Kanke 2000), *Helicoverpa armigera* (Wang et al. 2006), and *Melanoplus sanguinipes* (Westcott et al. 1992). In vertebrates nutrient imbalances inhibit detoxification by affecting detoxification enzymes (Parke and Ioannides 1981) and protein deficits reduce their concentrations in humans (Guengerich 1984, Yang et al. 1992) but also shrimps (Xu and Pan 2014). Additionally it has been shown that glutathione synthesis can be enhanced by the dietary addition of amino acids in humans (Sekhar et al. 2011).

The glutathione-*S*-transferase enzyme system has been shown to be particularly important to detoxify gramine for aphids and planthoppers (Ni and Quisenberry 2003, Cai et al. 2009, Sun et al. 2013). It binds toxins to glutathione, a tripeptide of glutamate, glycine, and cysteine. The formation of glutathione conjugates is saturable, and high doses of toxins deplete glutathione in the cell (Shargel et al. 2012). These could explain why performance was overall better on diets that contained more protein. Additionally it has been shown in humans that diets too high in carbohydrates decrease activity of cytochrome P450 enzymes, another type of enzyme important in detoxification (Guengerich 1995). These results would support the idea of a metabolic load associated with detoxification in generalist herbivores.

My study provided the first overview of how important nutrient-allelochemical interactions are for generalist herbivore. Given how variable plant nutrient content is I believe these results are key to understanding what factors affect animal foraging behavior, as may also be relevant for addressing questions related to insecticide efficacy. This could explain why some barley line cultivated for high level of gramine are not deterrent to locust for instance (Ishikawa and Kanke 2000). However the question of the existence of a metabolic load associated with detoxification still need to get addressed in

CHAPTER IV

INSECT HERBIVORE DIET MIXING: PLANT NUTRIENT CONTENT, NOT TOXINS, EXPLAIN FOOD SELECTION BEHAVIOR FOR TWO POPULATIONS

4.1 Introduction

‘Jack of all trade is master of none’. This idea is the foundation for many theories regarding generalist adaptation (or lack of thereof) to their host plant chemistry (Ali and Agrawal 2012). Nevertheless many successful and ecologically important groups of generalist herbivores, such as grazing mammals (Provenza et al. 2003, Villalba et al. 2004) or Orthopterans (Bernays et al. 1994, Miura and Ohsaki 2004, Unsicker et al. 2008), show enhanced fitness when feeding on multiple plant species, yet relatively little is known about the basis of food mixing and its function for organisms (Singer and Bernays 2003).

Two hypotheses are usually advanced to explain why many generalists benefit from diet mixing (Bernays et al. 1994, Dearing et al. 2000). The first states that enhanced performance on mixed diets is to be tied to nutrients; by switching between different foods sources (different plants, and or plant species), herbivores secure a more balanced nutrient intake. The second hypothesis proposes that alternating between food sources keeps the intake of any given allelochemical low. Interestingly, both hypotheses have received some support (Bernays et al. 1994, Hägele and Rowell-Rahier 1999, Karban et al. 2010). In reality, though, these two hypotheses are not mutually exclusive (Behmer et al. 2002).

One major issue posed by the study of nutrition – and by diet mixing in particular – is the difficulty to disentangle the relative effects of each food components, not to mention the possible interactions. In order to address this problem a nutritional framework was developed about two decades ago: the Geometric Framework (Raubenheimer and Simpson 1993, Simpson and Raubenheimer 1993, Raubenheimer and Simpson 1999). Using this approach it was shown that herbivores can regulate their protein and carbohydrate intake (for a review on herbivore nutrient regulation see Behmer 2009). This approach has also shown that both protein-carbohydrate quantity and ratio are important, and species-specific (Behmer and Joern 2008). Recent work also suggests that nutrient regulation responses can differ between populations (Fielding and Brusven 1992, Parsons 2011). However less effort has been devoted to the toxin dilution hypothesis (Singer and Bernays 2003).

Here I analyze the effect of diet mixing in regard to plant chemistry (nutrients *and* toxins) by comparing two populations of the generalist grasshopper *M. differentialis*, using the experimental approach of the Geometric Framework. However, in contrast to much of the previous work, this study explores diet mixing using actual host plants, and for each host plant chemistry is documented, including nutrient profiles (protein and digestible carbohydrate content) and plant secondary compounds. Each host plant was offered in choice and no-choice treatments to test for single and interactive effects of nutrients and toxins. My results show how in two different populations, nutrient regulation is prioritized over toxin dilution.

4.2 Material and Methods

4.2.1 Organism

Melanoplus differentialis (Orthoptera: Acrididae), also called the ‘differential grasshopper’, feeds on a wide range of herbaceous early successional plants (Yang and Joern 1994b) but can be locally monophagous according to Lewis (1984). Nymphs pass through six juvenile instars and adults are relatively large in size. Grasshopper stadium can easily be assessed using wing pad morphology. I used final nymphal stadium (6th instar) throughout the entire study because their large body size means that larger amounts of food are eaten. Additionally, all nutrients are allocated towards somatic growth because reproductive tissues do not develop until after the final molt.

4.2.2 Study sites and collecting method

Grasshoppers were collected in May and June 2013 in privately owned land from two different ecoregions of Texas: 1) Brazos county: San Antonio prairie, post oak savannah (latitude: 30º 39' 56" N, longitude: 96º 22' 0" W, elevation: 114 m), and 2) Travis county: Balcones canyonlands, juniper-oak woodland (latitude: 30° 19' 30" N, longitude: 97° 27' 29" W, elevation: 177 m). Grasshoppers were collected as 4th or 5th instars and returned to the lab where they were fed a diet of seedling wheat and wheat bran upon molt into 6th instar at which the experiment began.

4.2.3 Plant collection and chemical assays

In June 2012, the most commonly eaten forb and grass species (as determined by crop content analysis (Le Gall, *unpublished data*)) were collected for each site. Plant samples were immediately placed in liquid nitrogen upon collection to preserve chemical integrity. In the lab, individual plants were freeze-dried and ground using a cutting-grinding mill (IKA, MF 10). Plant material belonging to the same species was pooled together and mixed thoroughly to insure that food quality was homogenous within a treatment. For all chemical assays three samples (technical replicates) of ground plant material were used. Protein and digestible carbohydrate content were estimated using the methodology described by Clissold et al. (2006). Total phenolic content was measured following Ainsworth and Gillespie's methods (2007). These compounds activity has been previously demonstrated both in sunflower and ragweed (Mullin et al. 1991, Maksimovic 2008). Sesquiterpenes lactones activity has also been reported in both plants (Mullin et al. 1991) and terpenoid content was also recorded accorded to the methodology described by Ghorai et al. (2012).

4.2.4 Treatments

For each site (Brazos and Travis), three different treatments were examined separately: 1) forbs only, 2) grass only and 3) a mixed plant treatment (2 sites x 3 treatments = 6 treatments). For each treatment, 10 grasshoppers were used (5 males and 5 females). Immediately following the final nymphal molt grasshoppers were weighed and placed individually into small plastic arenas (19 x 14 x 9 cm). Plastic arenas contained one (forb or grass) or two dishes (forb+grass), a small plastic container with water for drinking, and a small aluminium perch for roosting. Prior to being placed in the experimental arena, food dishes were allowed to equilibrate under stable ambient room humidity for ~24h and then weighed to the nearest 0.1mg.

Grasshoppers were allowed to feed for 3 days. At the end of this time, the original food was removed and replaced with fresh foods. The dishes that had been removed were allowed to equilibrate to ambient room humidity levels for ~24h prior to be weighed. This procedure was repeated every 3 days until grasshoppers molt to the adult stage. The food was changed at these regular intervals to insure that insects were fed ad libitum. When grasshoppers molted the date and time were recorded, as was the final mass. Individuals were then froze and stored for lipid analysis using the same methodology outlined in Boswell et al. (2008). The experiment was conducted in a room with temperature varying from 35-37°C during the day and 26-24°C at night under a 14:10 light: dark photoregime.

4.2.5 Statistical analyses

Protein-carbohydrate and terpenoid-phenolic intake was compared using MANCOVA techniques (with sex as a covariate because female are usually bigger than male in grasshoppers); using Pillai's test statistic. Where significant effects were observed, post hoc comparisons were performed using contrasts following the techniques employed by Scheiner (1993), with α levels adjusted with Bonferroni correction methods. Food intake and growth rate were analyzed using ANCOVA techniques (with population and sex as a covariates).

4.3 Results

4.3.1 Chemical assays

The mean (±SEM) protein-carbohydrate content of each plant, plus phenolic and terpenoid levels are shown in table 4.1. We found that grasses contained higher levels of carbohydrates (33-34%) than forbs (10-21%). However forb protein content was higher (13-15%) than grasses (7-8%). Terpenoid content was a little bit less than 1% for all plants (except for Paspalum 0.03%). Phenolic content was relatively similar for all plants (6-7%) except for ragweed that had much higher levels (12%).

Table 4.1 Results of the chemical assays for each plant. Results are expressed in percentage of dry mass.

Plant	Co.	% Protein (mean± S.E.)	% Carbohydrate (mean± S.E.)	% Terpenoid (mean± S.E.)	% Phenolic (mean± S.E.)
Johnson grass *(Sorghum halepense)*	Travis	7.42±0.02	33.16±0.04	0.08±0.00	6.46±0.00
Sunflower *(Helianthus annuus)*	Travis	14.86±0.01	10.94±0.02	0.09±0.00	6.41±0.00
Paspalum (*Paspalum plicatulum)*	Brazos	7.81±0.00	34.51±0.04	0.03±0.03	7.20±0.00
Ragweed *(Ambrosia sp)*	Brazos	12.62±0.01	21.10±0.02	0.09±0.00	12.25±0.00

4.3.2 Protein-carbohydrate intake

The protein-carbohydrate intake of grasshoppers differed significantly depending on the diet treatment (Table 4.2). Protein-carbohydrate intake for grasshoppers restricted to the two grasses (Johnson or Paspalum) was similar; on average these grasshoppers ingested about 4 times as much carbohydrate as protein (Fig. 4.1). Grasshoppers restricted to ragweed also ingested more carbohydrate than protein, but this ratio was less imbalanced (~8:5). In contrast, grasshoppers restricted to sunflower ate twice as much protein as carbohydrate (Fig. 4.1). When grasshoppers were given the possibility to mix their food, both populations ingested the same protein-carbohydrate intake (Table 4.2; Fig. 4.1, black symbols), yielding to a protein:carbohydrate intake of p1:c2.5.

Table 4.2 Results of MANCOVA for protein and carbohydrate intake by grasshoppers from all treatments.

Source	df	*F* values
A) Protein and carbohydrate intake		
Treatment	10	8.64***
Sex	2	54.90***
B) Contrast		
Johnson grass *vs* sunflower	2	107.51†
Paspalum *vs* ragweed	2	28.12†
Johnson grass vs. paspalum	2	1.90
Sunflower vs. ragweed	2	17.01†
Travis mix *vs* Brazos mix	2	0.16

Notes: Part (A) reports overall *F* values (Pillai's Trace). Sex was used as a covariate to adjust for size differences among insects. Part (B) reports *F* values for multivariate contrasts between specific treatments. *** $P < 0.001$.† Significant difference with a $\alpha = 0.05/3$.

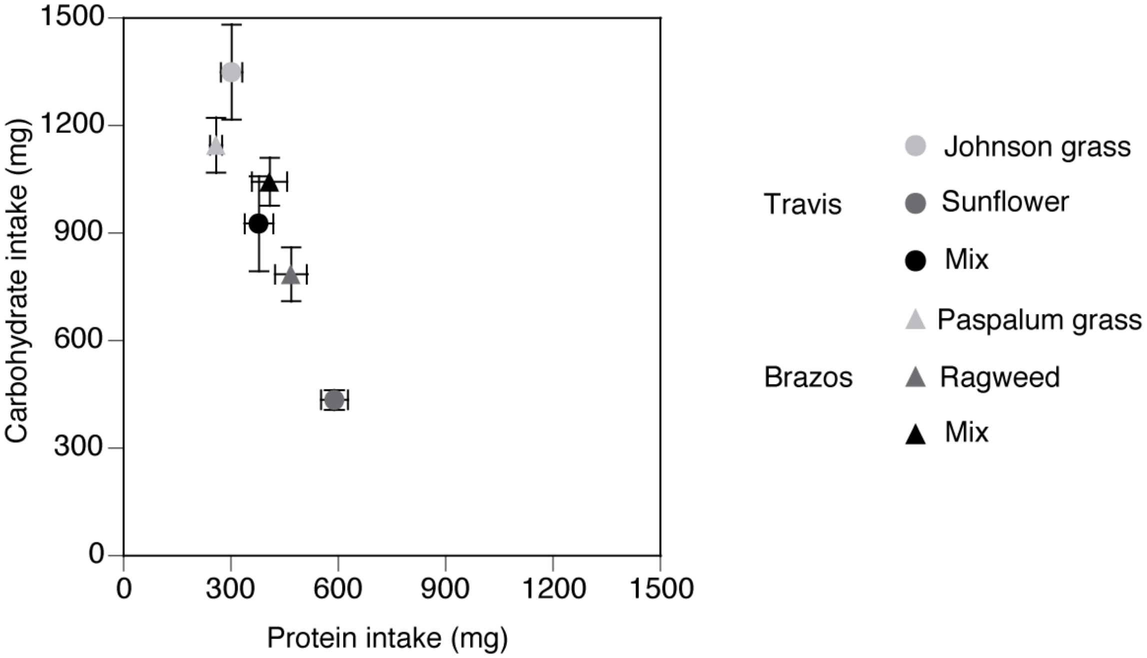

Figure 4.1 Protein-carbohydrate intake for all treatments.

4.3.3 Terpenoid-Phenolic intake

Results of toxin intakes showed that insects ingested different amount of terpenoid-phenolic depending on their plant treatment (Table 4.3; Fig. 4.2). For Travis County, grasshoppers fed Johnson grass ate more phenolic but less terpenoid than grasshoppers fed sunflower (Table 4.3; Fig. 4.2, round symbols). However in Brazos County insects that ate ragweed ingested high levels of both terpenoid and phenolic relative to the other treatments. Insects on the paspalum treatment ate generally less toxins (Fig. 4.2, triangle symbols). The amount of terpenoid-phenolic ingested on the mixed treatments was higher at the Brazos site (Table 4.3; Fig. 4.2).

Table 4.3 Results of MANCOVA for terpenoid and phenolic intake by grasshoppers from all treatments.

Source	df	*F* values
A) Protein and carbohydrate intake		
Treatment	10	21.86***
Sex	2	15.65***
B) Contrast		
Johnson grass *vs* sunflower	2	5.17†
Paspalum *vs* ragweed	2	28.11†
Johnson grass vs. paspalum	2	175.09†
Sunflower vs. ragweed	2	155.58†
Travis mix *vs* Brazos mix	2	144.48†

Notes: Part (A) reports overall *F* values (Pillai's Trace). Treatment refers to the P:C ratio; initial mass was used as a covariate to adjust for size differences among insects. Part (B) reports *F* values for multivariate contrasts between specific treatments. *** $P < 0.001$.† Significant difference with a $\alpha = 0.05/3$.

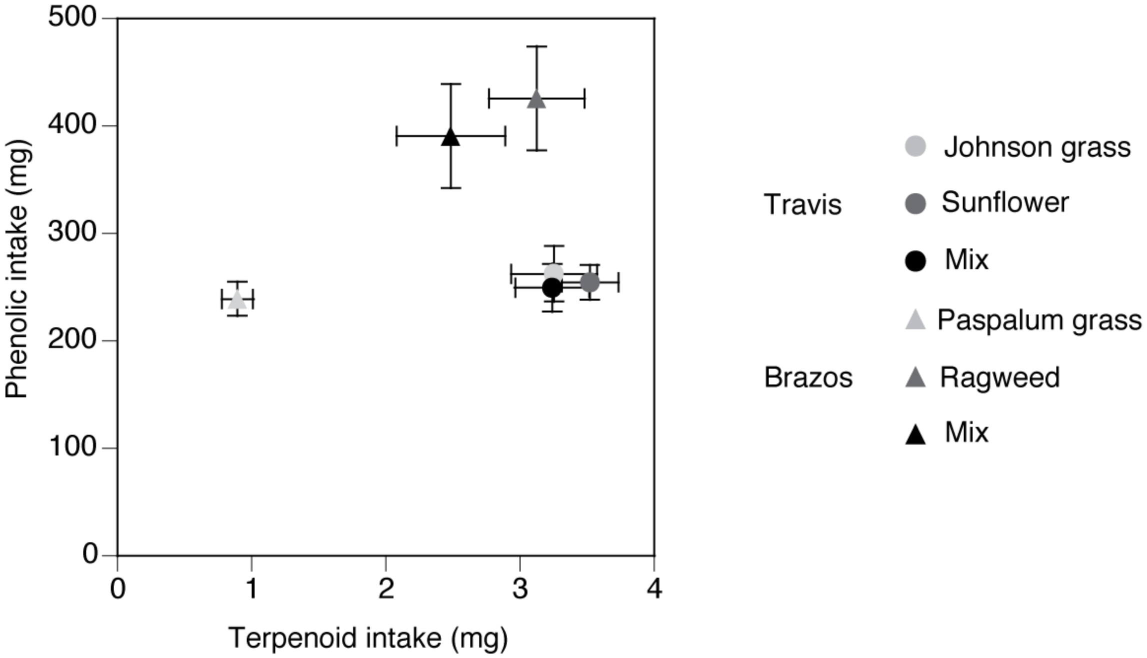

Figure 4.2 Terpenoid-phenolic intake for all treatments.

4.3.4 Food intake

Overall food intake was similar for all treatments (Table 4.4) but insects on the mix treatment from Travis that were offered a choice between sunflower and Johnson grass ate more of the latter, while insects from Brazos County consumed similar amount of ragweed and paspalum (ANCOVA, df=3, F= 3.10, p=0.042*) (Fig. 4.3).

Table 4.4 Results of ANCOVA for food intake and growth rate

Source	df	*F* values
A) Total food intake		
Treatment	2	1.02
Sex	1	107.79***
Population	1	2.52
B) Growth rate		
Treatment	2	7.49**
Sex	1	10.15**
Population	1	5.79*

Notes: Part (A) reports overall *F* values for food intake. Part (B) reports *F* values for growth rate. Treatment refers to the plant offered (forb, grass or forb + grass); sex and population (Travis or Brazos) were used as a covariates to adjust for differences among insects. *** $P < 0.001$; **$P<0.01$, *$P<0.05$.

4.3.5 Growth rate

In no-choice experiment, there was a trend for insects to have better growth rates on grass treatments than on forbs treatments (although the difference is not significant for Travis County) (Table 4.4, Fig. 4.4). Growth rates also tend to be higher on mix treatments than on forb treatments (although the difference is not significant for Brazos County) (Table 4.4, Fig. 4.4).

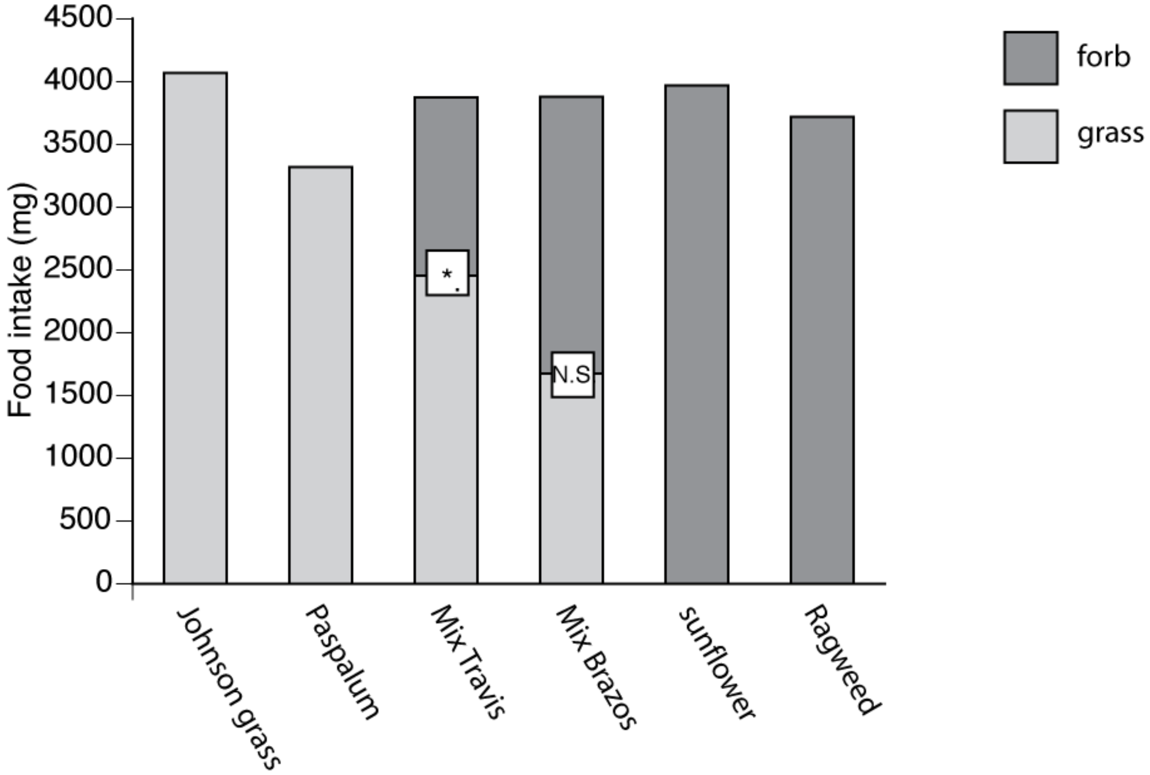

Figure 4.3 Total food intake and amount eaten of each plant in the mix treatment. No statistical differences were observed for overall amount (ANCOVA, DF=5, F=1.54, p=0.20). Posthoc results for the comparison in the mix treatment are reported in the white boxes (p<0.05).

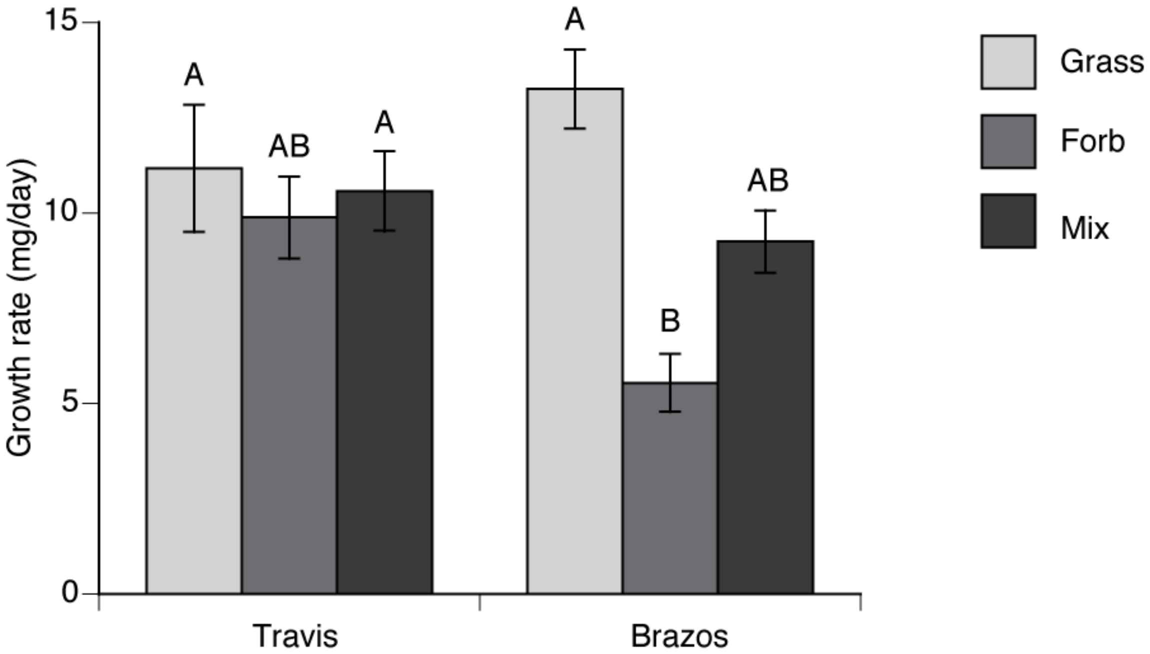

Figure 4.4 Growth rate for all treatments. Different letters indicate statistically significant differences ($P < 0.05$).

4.4. Discussion

Although many groups of generalist herbivores are ecologically successful little is known about the basis of food mixing and its function for organisms (Singer and Bernays 2003). This study was developed to analyze the effects of diet mixing on foraging behavior and performance while addressing the variation that might occur at population level.

First I wanted to know whether or not each population regulated their food intake to ingest a particular amount of protein-carbohydrate and if they did if they had similar requirements despite geographic isolation and the use of different host-plants. The regulatory behavior of the two populations was quite remarkable. The nutrient profiles of each plant was different and in the absence of choice so was grasshopper's protein-carbohydrate intake. However, when given the choice the two populations ate different amount of each plants (more grass in Travis, and equal amount of forb and grass in Brazos) and by doing so they ingested the exact same amount of protein and carbohydrate. That grasshoppers tightly regulate their protein-carbohydrate intake when given the choice is not new (see Behmer 2009 for a review on nutrient regulation in insect herbivores), however it has never been shown using actual plant nor for two different populations.

These results provide fundamental insight into the factors that govern animal foraging. Here I am clearly showing, and for the first time, that for both populations foraging decisions were made solely based on plant-nutrient content despite variations in plant defensive compounds. Interestingly both populations regulated for the same nutrient intake. This is important because most studies on plant-insect interaction use either nitrogen or plant defense as a proxy for plant quality and acceptability as a host (Awmack and Leather 2002). However, similarly as to what was shown with locusts and artificial diets by Behmer et al. (2002), my data showed that grasshoppers are willing to ingest toxins in order to reach an optimal protein-carbohydrate intake. Importantly they regulated equally tightly for both protein and carbohydrate, highlighting the importance of protein-carbohydrate balance.

It is worth noting that performance on the grass treatments was as high as on the mix treatment. A likely explanation for this lies on the grass defenses, recently a large body of works has highlighted the efficacy of silica as a defense of grasses against herbivores by limiting their access to nutrients (Hochuli 1993, Massey et al. 2006, Hunt et al. 2008). This is particularly true for C4 grasses like Johnson grass and Paspalum that have bundle-sheath cells that might be hard to break for a non-grass specialist like *M. differentialis* (Caswell and Reed 1976, Barbehenn 2005). However, in another study on grasshoppers it was shown that when grasses were grinded plant toughness was reduced and nutrient access improved (Clissold et al. 2009) which could explain the high performance on these carbohydrate-rich diets.

So do allelochemicals matter at all in diet mixing? I argue that if allelochemical dilution does enhance insect performance it is a consequence and not a cause of feeding behavior because nutrient regulation was not random and ultimately determined the amount consumed on each plant, not the amount of toxins.

Evolutionary theory predicts that animals should forage to maximize their fitness (MacArthur and Pianka 1966). In the second chapter, we saw that in the absence of toxin, insects selected a moderately carbohydrate-biased intake target (p1:c1.4), and in the third chapter we saw that with the addition of gramine in the diet insects performed better on diet containing higher levels of protein. However here, with plants full chemical profile, both populations selected a very carbohydrate-biased intake target (p1:c2.5). As discussed in the previous chapter, a possible explanation is the existence of nutrient-allelochemical interactions and the nutrient intake can change in function of the nature of the toxin (Villalba et al. 2002a, b, Villalba et al. 2002c). Interestingly both populations self-selected for a similar intake-target, even though they ate plants with different allelochemical profiles. It is possible that because the four plants belong to the same two families (Poaceae and Asteraceae), their allelochemical profiles are similar enough (Burrows and Tyrl 2012) that they trigger similar detoxification mechanisms. Another possible scenario is that detoxification does not lead to a metabolic load and that common abiotic factor such as temperature (Parsons 2011) are more important in shaping protein-carbohydrate intake regulatory behavior than plant allelochemical profiles.

A key problem in Ecology is to assess the factors that determine animal behavior. I believe these data provide direct evidence of the importance of plant protein and carbohydrate content in predicting herbivore foraging. In a growing world where 15% of crop is lost to herbivorous insects (Maxmen 2013) these result have tremendous applications. However to understand what factors shape nutrient intake, I believe that study on detoxification costs associated with nutrient availability are much needed.

CHAPTER V

PROTEIN AND CARBOHYDRATES IN DETOXIFICATION PROCESSES OF A GENERALIST HERBIVORE

5.1 Introduction

Herbivorous insects possess a variety of physiological mechanisms for tolerating toxic plant compound (Brattsten 1992). Of these, detoxification by cytochrome P450 enzymes is generally considered to be the most important (Feyereisen 1999). These enzymes are a crucial line of defense against the myriad chemicals of natural origin that insects have to fend off. Furthermore, P450 metabolism of certain plant chemicals is often the key to the adaptation of insect herbivores to their host plants (Schuler 1996, Snyder and Glendinning 1996, Feyereisen 1999). Insect cytochrome P450s can metabolize a large diversity of toxic plant compounds (Schuler 1996, Li et al. 2004) and endogenous substrates such as hormones, lipids, and pheromones (Feyereisen 1999). The inducibility of cytochrome P450 monooxygenases and other xenobiotic-metabolizing enzymes is thought to reflect material and energy costs of biosynthesis.

This idea is an old one, in fact as early as 1971 it was suggested that because generalists encounter more types of chemicals, the metabolic load associated with detoxification processes is more costly for them than for specialists and explains diet breadth (Krieger et al. 1971, Whittaker and Feeny 1971). While the evidences for high detoxification costs remain equivocal (Castaneda et al. 2010, Cogni et al. 2012), it is still the basis for most theories concerning dietary specialization among insect herbivores (but see Bernays and Graham 1988, Dyer and Floyd 1993, Bernays 2001, Singer et al.

2004, Smilanich et al. 2009). Many early studies reported cost of detoxification by measuring efficiency of conversion of ingested food (Waldbauer 1968, Schoonhoven and Meerman 1978, Scriber 1978). However, as noted by later studies, the problem of indices is that metabolic and toxicological impacts are confounded as toxins can reduce feeding rate (Appel and Martin 1992, Cresswell et al. 1992, Berenbaum and Zangerl 1994). The importance of nutrients in detoxification processes, particularly protein, was also pointed out (Lindroth et al. 1990, Berenbaum and Zangerl 1994).

In parallel, the early 90's saw the emergence of a new framework of nutrition: the Geometric Framework (GF) (Raubenheimer and Simpson 1993, Simpson and Raubenheimer 1993). Using this approach much was learned about the multidimensional nature of nutrition. For instance we now know that herbivorous insects simultaneously regulate their intake of protein and carbohydrate and that nutrient balance (i.e. protein:carbohydrate ratio) is a key element to both herbivore foraging decision and fitness (for a review on insect herbivore nutrient regulation see Behmer 2009). A couple of studies have also used the GF to investigate how nutrient balance affects consumption and performance in the presence of plant toxins, e.g., tannic acid (Simpson and Raubenheimer 2001, Behmer et al. 2002). In the first study it was shown that the effect of tannic acid on locust performance depended on protein: carbohydrate balance. The second one demonstrated that the effectiveness of tannic acid as a feeding deterrent was a function of food protein:carbohydrate content. In both instances, the focus herbivore was the African migratory locust (*Locusta migratoria*), a grass specialist.

Here I investigated the effect of protein-carbohydrate balance on detoxifications processes in a generalist herbivore. *Melanoplus differentialis* is a highly polyphagous grasshopper and there is evidence in this species that detoxification enzyme activities are affected by plant diet (Snyder et al. 1998). I perform two experiments. The first is a choice-experiment to examine whether the addition of a toxin (gramine, an alkaloid present in grasses) to diets that vary in their protein: carbohydrate ratios affects nutrient regulation behavior. In the second experiment (no-choice) I give grasshoppers a range of diets that vary in their protein:carbohydrate ratio with or without gramine. I measure consumption, mass gain and microsomal proteins.

5.2 Material and Methods

5.2.1 Insects and experimental chambers

The generalist grasshopper *Melanoplus differentialis* occurs throughout much of the United States where it is recorded feeding on a wide range of herbaceous and successional plants (Capinera 2008). Insects used in this study came from a culture fed a diet of greenhouse-grown seedling wheat and wheat germ, and maintained in the Department of Entomology, Texas A&M University (College Station, TX); the colony was initiated in 2008 from a local population. They were maintained under standard laboratory conditions with a 14h:10h L:D photoperiod, and under radiant heat of 35-37ºC during the light phase (supplied by 60W full spectrum incandescent bulb), and at 24-26ºC during the dark phase.

Nymphs of both sexes were removed from the culture within 4-6 hours after ecdysis to the sixth stadium (day 0), weighed (to the nearest 0.1 mg), and then transferred individually into clear plastic arenas (19 cm long x 14 cm wide x 9 cm high). Each container contained either one food dish (experiment 1) or two food dishes (experiment 2), an expanded aluminum roost for perching, and a water source (a 30-mL Solo Cup®, fitted with a lid that had been modified to allow the use of a cotton wick for drinking); the food dishes were modified Petri dishes designed to minimized spillage (Raubenheimer and Simpson 1990). Insects were maintained throughout the experiment under the same temperature and light conditions than the lab colony.

5.2.2 Synthetic food composition

The chemically defined foods used in this study were based on those used by Simpson and Abisgold (1985). We used a carbohydrate-biased diet containing 7% of protein (p) and 14% of carbohydrate (c) abbreviated as p7:c14, and a protein-biased diet, abbreviated p14:c7. Both dishes contained 21% of nutrient (7+14) and were isocaloric.

The protein component of all foods was a 3:1:1 mix of casein, peptone and albumen, while the digestible carbohydrate (henceforth carbohydrate) component was a 1:1 mix of sucrose and dextrin. All foods contained similar amounts of Wesson’s salt (2.4%), cholesterol (0.5%), linoleic acid (0.5%), ascorbic acid (0.3%) and vitamin mix (0.2%; Dadd, 1961). The remainder of the diet was cellulose, a non-nutritive bulking agent.

5.2.3 Experimental treatments

Two separate experiments were performed. The first, a choice-experiment, had four treatments, each containing the carbohydrate-biased diet (p7:c14) and the protein-biased diet (p14:c7), but varying in the presence or absence of gramine in neither, one, or both diets. Gramine was chosen because it is an alkaloid present in grasses (Poaceae) with deterrent/toxic effects on herbivorous insects (Harley and Thorsteinson 1967, Hinks and Olfert 1999, Ishikawa and Kanke 2000). When used, gramine was added at a 0.5% concentration and mixed vigorously to obtain a homogeneous mixture, and because the diets were dry, the allelochemical remained inactive until it was consumed. The four treatments were as followed (an asterisk indicates the presence of gramine):

1) p7:c14 & p14:c7
2) p7:c14* & p14:c7*
3) p7:c14* & p14:c7
4) p7:c14 & p14:c7*

For each treatment I measured food consumption from each dish, total protein and carbohydrate intake and mass gain.

The second was a no-choice experiment that included four treatments (the presence of gramine is indicated by an asterisk):

1) p7:c14
2) p7:c14*
3) p14:c7
4) p14:c7*

Again, for each treatment I recorded food consumption, mass gain and microsomal protein content.

5.2.4 Protocols

In both experiments, similar protocols were followed. First, foods were scooped into individual dishes and then weighed to the nearest 0.1 mg after allowing food dishes to equilibrate under stable ambient room humidity for 24 h. On the first day of the 6th-stadium (day 0), individuals were placed into the experimental arena, and allowed to feed for 6 days; at the end of this time the grasshoppers were weighed (to the nearest 0.1mg) and frozen. Food was pulled from the test arenas and left to equilibrate to room humidity for 24 before it was weighed (to the nearest 0.1 mg). After the experiment ended, grasshoppers from the no-choice experiment were removed from the freezer and dissected for microsome preparation.

5.2.5 Microsome preparation

Microsome are artificial vesicles made mostly of endoplasmic reticulum and concentrated by differential centrifugation (De Duve et al. 1955). Microsomal preparations contain high levels of p450 enzymes and are regularly used to study the latter (Feyereisen 1999). Microsome preparation followed the procedure described in Snyder et al. (1998). Grasshoppers were pooled into groups of three to four individuals. Dissections were carried on ice to prevent clotting and melanization. After removal of heads and legs, midguts, Malpighian tubules and fat body were collected. The pooled tissues were homogenized in 0.1M $NaPO_4$ buffer (pH7.5) containing 10% glycerol, 1mM EDTA, and 0.1mM dithiothreitol. The total homogenate for tissue was 2ml and was centrifuge at 10,000g for 15 minutes. The resulting supernatants were filtered through glass wool and centrifuged at 100,000g for an hour to pellet the microsomes. The microsomal pellets were individually re-suspended in the homogenization buffer. Total microsome protein level was assayed using the Bicinchoninic acid procedure (Sigma) for microtiter plates with bovine albumin as the standard. Three technical replicates per microsome, and tissue type, were used.

5.2.5 Statistical analyses

In the choice experiment the total amount of food eaten (food intake), the amount of food eaten in each dish and mass gain were analyzed using ANCOVA technique (with starting mass as a covariate to correct for size differences between individuals). The total amount of protein and carbohydrate eaten, for each treatment, was compared using MANCOVA techniques (with starting mass as a covariate), using Pillai's test statistic. Where significant effects were observed, post hoc comparisons were performed using contrasts following the techniques employed by Scheiner (1993), with α levels adjusted with Bonferroni correction methods. In the no-choice experiment, food intake, mass gain, and protein levels in the microsomal preparations were analyzed by ANCOVA (with average start mass as a covariate).

5.3 Results

5.3.1 Choice experiment

5.3.1.1 Total food intake and amount of food eaten in each dish

Grasshoppers from the choice experiments ate similar total amount of food on the four treatments (Fig. 5.1). They also ate similar amount of the protein-biased diet and carbohydrate biased diet except when gramine was present only in the protein-biased diet (p7:c14 & p14:c7*). In that case, grasshoppers ate considerably more of the gramine-free carbohydrate-biased diet (Fig. 5.1).

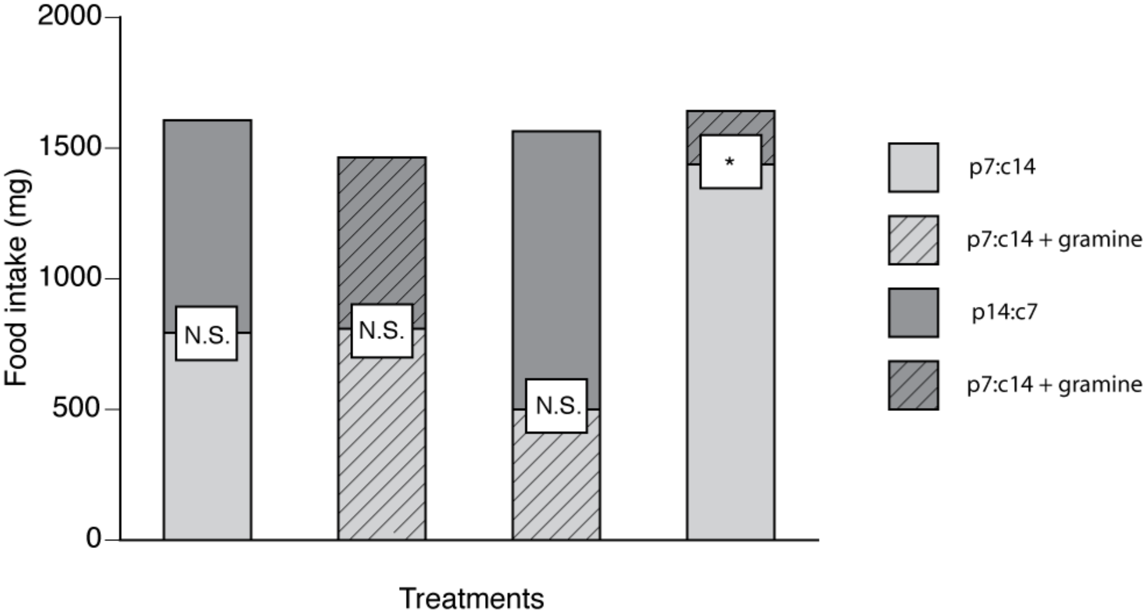

Figure 5.1 Total food intake and amount eaten for each dish. No statistical differences were observed for overall amount (ANCOVA, DF=3, F=0.74, p=0.535). Posthoc results for the comparison between the two dishes are reported in the white boxes (p<0.05).

5.3.1.2 Protein-carbohydrate intake

Protein-carbohydrate intake were similar for all treatments (equal protein: carbohydrate ratio p1:c1) except for the treatment where gramine was present only in the protein-biased diet (p7:c14 & p14:c7*); here the intake was more carbohydrate-biased (p1:c1.7) (Table 5.1, Fig. 5.2).

5.3.1.3 Mass gain

Despite differences in protein-carbohydrate intake, insects from the choice experiment had similar mass gain (ANCOVA, DF=3, F=1.24, p=0.31).

Table 5.1 Results of MANCOVA for protein and carbohydrate intake by grasshoppers from the choice experiment.

Source	df	*F* values
A) Protein and carbohydrate intake		
Treatment	6	4.75***
Start mass	2	1.02
B) Contrast		
p7:c14 & p14:c7* *vs* p7:c14 & p14:c7	2	10.78†
p7:c14 & p14:c7* *vs* p7:c14* & p14:c7*	2	8.25†
p7:c14 & p14:c7* *vs* p7:c14* & p14:c7	2	18.89†
p7:c14* & p14:c7* *vs* p7:c14 & p14:c7	2	0.81
p7:c14* & p14:c7* *vs* p7:c14* & p14:c7	2	3.20
p7:c14 & p14:c7 *vs* p7:c14* & p14:c7	2	1.59

Notes: Part (A) reports overall *F* values (Pillai's Trace). Treatment refers to the P:C ratio; initial mass was used as a covariate to adjust for size differences among insects. Part (B) reports *F* values for multivariate contrasts between specific treatments.
*** *P* , 0.001.† Significant difference with a $\alpha = 0.05/6$.

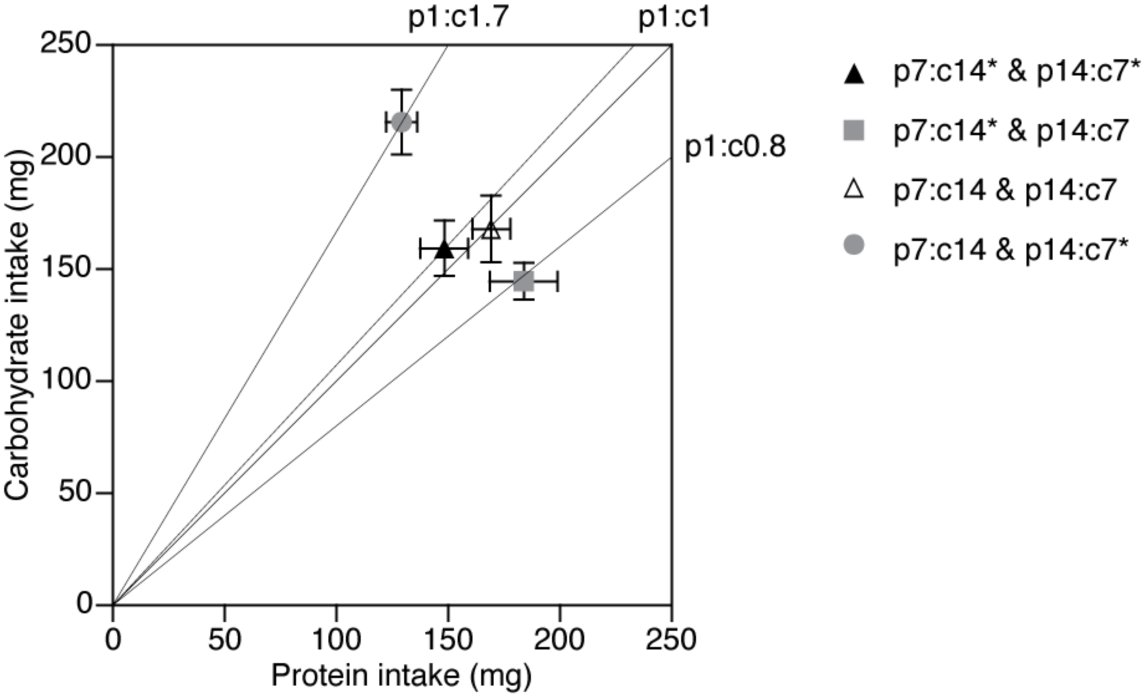

Figure 5.2 Protein-carbohydrate intake for the choice experiment.

5.3.2 No-choice experiment

5.3.2.1 Total food intake

Insects consumed more of the carbohydrate-biased diet (p7:c14) than of the protein-biased diet (p14:c7). Gramine had no effect on consumption (Table 5.2; Fig. 5.3).

5.3.2.2 *Mass gain*

Nutrient balance had opposite effects on mass gain when gramine was added to the diet as shown by the significant interaction between nutrient content and presence of gramine (Table 5.2; Fig. 5.4). Insects fed carbohydrate-biased diets that contained gramine gained more mass than the one eating gramine-free diet. However the addition of gramine to the protein-biased diet had a negative effect on mass gain.

Table 5.2 Results of ANCOVA for food intake and mass gain by grasshoppers from the no-choice experiment.

Source	df	*F* values
A) Food intake		
Nutrient	1	8.21**
Gramine	1	1.01
Nutrient x Gramine	1	0.90
Start mass	1	33.73***
B) Mass gain		
Nutrient	1	0.35
Gramine	1	0.75
Nutrient x Gramine	1	4.24*
Start mass	1	21.71***

Notes: Part (A) reports overall *F* values for food intake. Part (B) reports *F* values for mass gain. Nutrient refers to the P:C ratio; gramine referes to the presence of the toxin in the food, initial mass was used as a covariate to adjust for size differences among insects. *** $P < 0.001$; **P<0.01, *P<0.05.

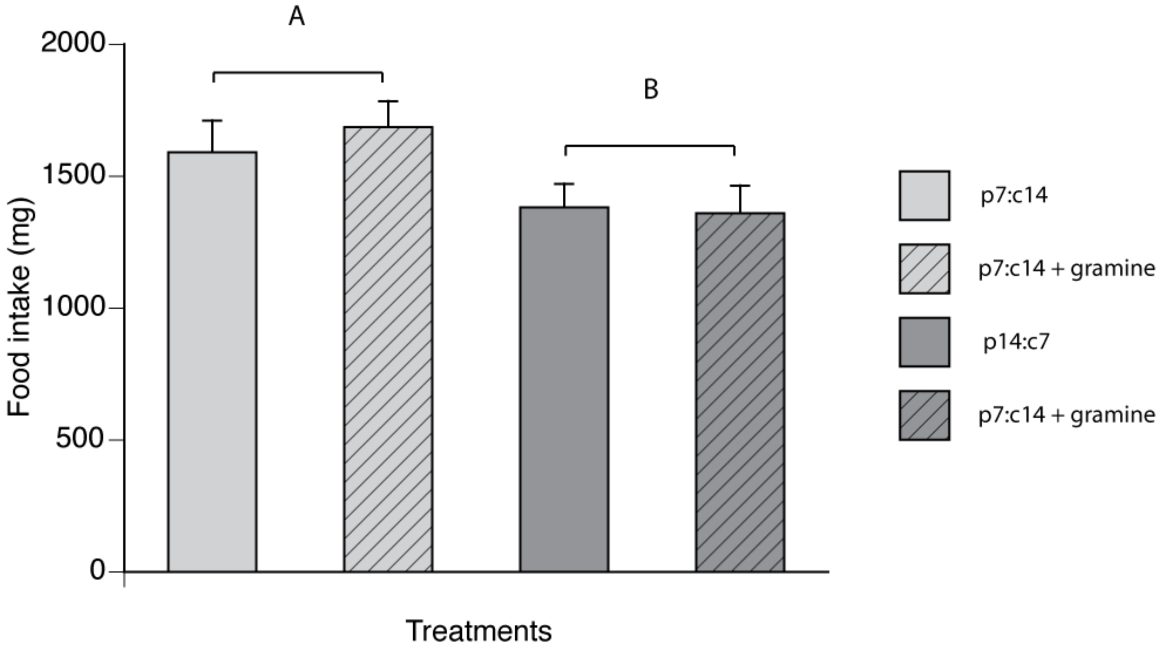

Figure 5.3 Food intake for the no-choice experiment. Different letters indicate statistically significant differences ($P < 0.05$).

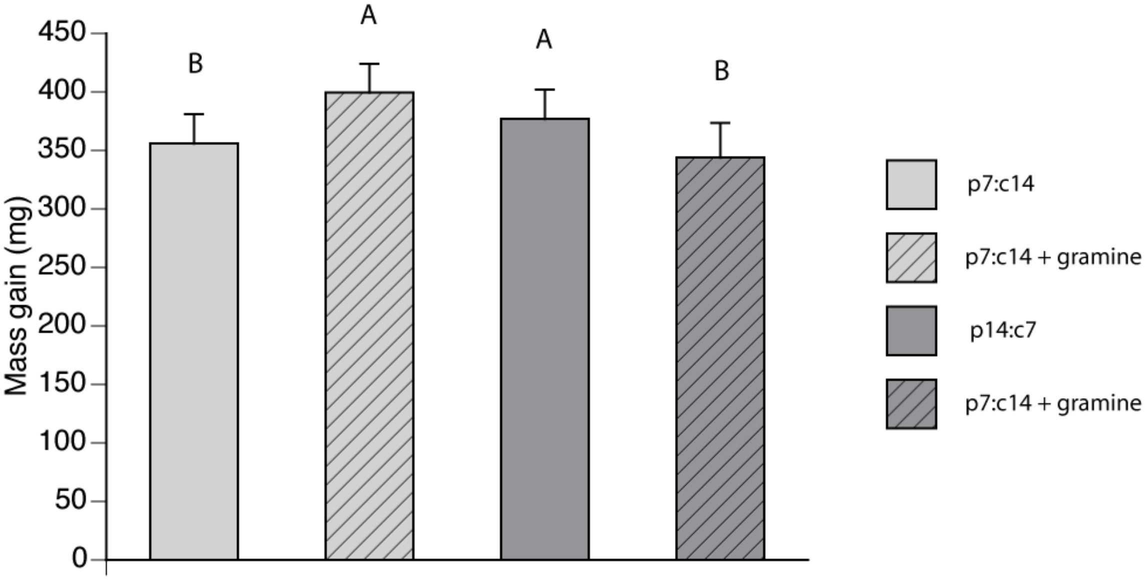

Figure 5.4 Mass gain for the no-choice experiment. Different letters indicate statistically significant differences ($P < 0.05$).

5.3.2.3 Microsomal protein

We found a significant interaction between tissue type and gramine (Table 5.3; Fig. 5.5). In other words, there was more protein in the fat bodies of grasshoppers that were fed gramine.

Table 5.3 Results of ANCOVA for microsomal protein from the no-choice experiment.

Source	df	*F* values
Amount of protein		
Nutrient	1	2.13
Gramine	1	6.41*
Tissue type	2	8.27**
Nutrient x gramine	1	0.07
Tissue type x gramine	2	8.5**
Start mass	1	4.72*

Notes: Nutrient refers to the P:C ratio; gramine refers to the presence of the toxin in the food, Tissue type refers to gut, Malpighian tubules or fat body ,initial mass was used as a covariate to adjust for size differences among insects. $^{**}P<0.01$, $^{*}P<0.05$.

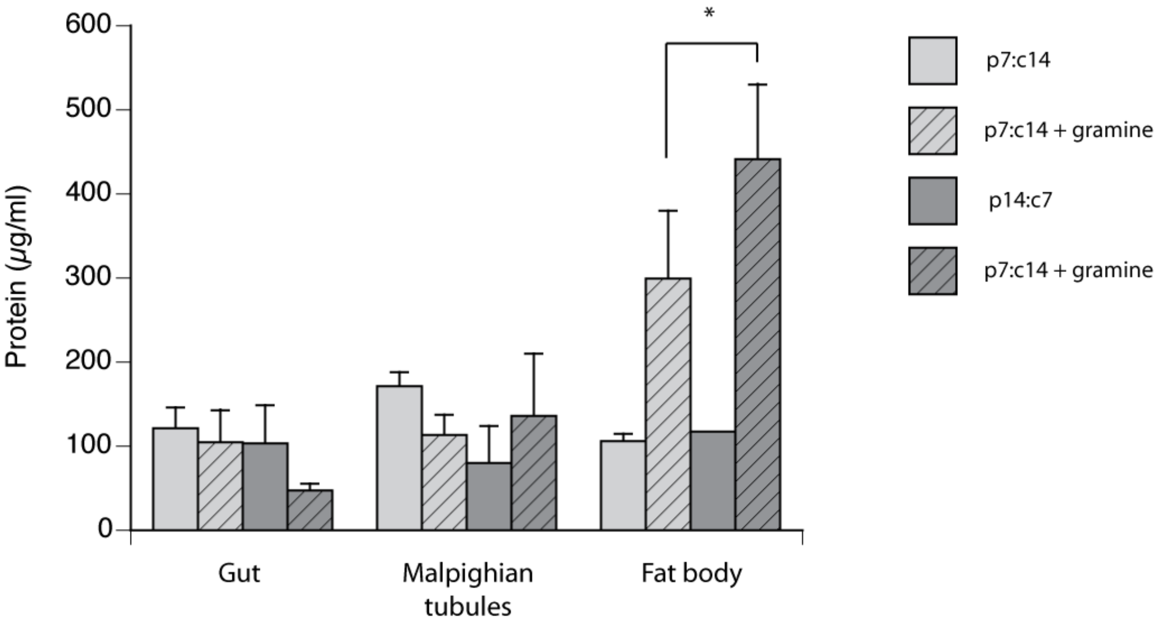

Figure 5.5 Amount of protein in the microsomal preparations. Statistically significant difference is indicated by an asterisk ($P < 0.05$).

5.4 Discussion

Foraging generalist insects often must balance nutrient regulation against limiting the intake of toxins (Bernays et al. 1994, Karban et al. 2010). The extent to which detoxification processes are costly, particularly for generalist herbivore, remains largely unknown. Although the importance of nutrients in detoxification process has already been established, it was often done by varying the concentration of a single nutrient (protein) (Lindroth et al. 1990, Berenbaum and Zangerl 1994). A major issue with that type of approach is that modifying protein concentration de facto alter carbohydrate concentration and we now know that nutrient balance is very important (Behmer 2009). Only two studies thus far have examined nutrient-allelochemical interactions with respect to protein:carbohydrate balance (Simpson and Raubenheimer 2001, Behmer et al. 2002). Both studies found that toxin effects on performance and consumption are mitigated by protein:carbohydrate balance.

In this study I found that nutrient regulation behavior was affected by the presence of gramine, and this effect was also mediated by protein: carbohydrate balance. Interestingly, when gramine was present in the protein-biased diet, insects consumed more of the gramine-free carbohydrate diet. It is important to note that this cannot be attributed to the antifeedant effect of gramine alone, as when gramine was present only in the carbohydrate-biased diet, or in both diets, insects presented similar regulatory responses as in the absence of gramine. Conversely Simpson and Raubenheimer (2001) found that carbohydrate-biased diet containing tannic acid were more repellent to locust. A possible explanation relates to how tannic acid interacts with protein – when protein content is low, tannic acid binds to protein in the oral cavity of the locusts, making the food less palatable. So why this reduced consumption of the protein-biased food in the presence of gramine?

A possible reason lies in compensatory limitations. It has previously been demonstrated that insects have physiological mechanism to deal with the overingestion of carbohydrates, for instance they can respire excess carbohydrate (Zanotto et al. 1997). However plant protein is almost always very tightly regulated, even when nutrient content decreases (see chapter II). It has even been shown that high nitrogen content in plant and artificial diets can affect negatively the size and viability of *Oedaleus asiaticus*, a dominant locust of North Asian grasslands (Cease et al. 2012). This could explain why when given the choice, insects avoid diets containing gramine if they are protein-biased but not if they are carbohydrate-biased. Although the strategy to overeat the gramine-free carbohydrate biased diet did not lead to perfect protein compensation, I did not find evidence of cost in term of mass gain.

Surprisingly, when I constrained insects to a single type of food I found that insects fed carbohydrate-biased diets that contained gramine tended to eat more, but they also gained more weight than insects fed gramine-free carbohydrate-biased diets. Conversely insects that were fed gramine-free but protein-biased diet ate more and gained more weight than the ones fed gramine. It has been suggested that the production of detoxification enzymes is costly (Coustau and Chevillon 2000), and we verified here that gramine is linked to the production of new microsomales protein, at least two or three fold in the fat body. Fat body is an important organ for the detoxification of nitrogen-based compounds (Arrese and Soulages 2010) and increase in p450 enzymes and gluthatione S-transferase have ben shown following exposure to xenobiotics in *Manduca sexta* (Snyder et al. 1995). So did I observe detoxification costs in our study?

There is at least one occasion where this is the case: when gramine is added to protein-biased diet in the absence of choice. Here I saw that for protein-biased diets consumption was similar with and without gramine, however mass gain was lower when gramine was present – this could potentially represent the cost of the production of detoxification protein in the fat body. This is interesting because the glutathione-*S*-transferase enzyme system has been shown to be particularly important to detoxify gramine for aphids and planthoppers (Ni and Quisenberry 2003, Cai et al. 2009, Sun et al. 2013), and it has been shown that glutathione synthesis can be enhanced by the dietary addition of amino acids in humans (Sekhar et al. 2011). Furthermore although it is not significant (worth noting that our replication are low because grasshoppers must be pooled to prepare microsomes), we did observe more protein in the microsomes produced on protein-biased diets. So why is mass gain lower in the protein-biased diet?

An important parameter of this study is nutrient density. Previous work has shown that *M. differentialis* nymphs actively regulate their protein-carbohydrate intake when food macronutrient density is high (i.e. 42%) (Behmer and Joern 2008). However in the field nutrient concentration vary wildly and few plants actually contain 42% total macronutrient content (see Behmer and Joern 2012 for a nutrient landscape available to insect herbivores). For instance, the nutrient concentrations of *M. differentialis* host-plants varied from 7 to 14% for protein and 11 to 33% for carbohydrate, highlighting the realism of the concentration chosen. The diets in this study are thus reasonably concentrated, and compensatory mechanisms might be limited in this case. However, in the case of carbohydrate, there is more room for compensatory feeding and we can see that insects do so and have higher mass gain. It might be because the excess energy can be allocated to detoxification mechanisms. It was recently demonstrated in the mosquito *Culex pipiens* insecticide resistance mediated by p450 enzymes affected glycogen and lipid content (Hardstone et al. 2010). Additionally gramine has negative effects on the lipid metabolism of locusts (Samaranayaka 1976).

In conclusion, I showed that the regulatory behavior of *M. differentialis* depends on nutrient allelochemical interactions and allows it to cope with presence of toxin in its diet. I also demonstrated the importance of using realistic nutrient concentration to study the limit of compensatory behavior, particularly in presence of toxin.

CHAPTER VI

CONCLUSIONS

Nutrient-allelochemical interactions underlie herbivore performance (Simpson and Raubenheimer 2001) and nutritional decisions (Behmer et al. 2002). But how this interaction affects diet mixing remains elusive. Thus far the two hypotheses – nutrient balance and allelochemical dilution – are usually advanced to explain why generalist herbivore exhibit enhanced performance. Both have received some support (Bernays et al. 1994, Hägele and Rowell-Rahier 1999, Karban et al. 2010), but Behmer et al. has shown that these two hypotheses are not mutually exclusive (2002).

A first key observation in my study was the importance of nutrient concentration. Most previous work performed in the context of the Geometric Frameworks was done using diets that contained 42% protein + carbohydrate (Raubenheimer and Simpson 1993, Simpson and Raubenheimer 1993, Raubenheimer and Simpson 1999, Simpson and Raubenheimer 2001, Behmer et al. 2002, Simpson et al. 2002). Interestingly, the part of the nutritional space that has previously being ignored (more diluted diets) showed the most interesting results. For instance, I saw that in terms of mass gain insects were tolerant to dilution if the food was carbohydrate-biased (near their self-selected intake target of p1:c1.4), illustrating the importance of protein:carbohydrate ratio for growth. However, regardless of the nutrient ratio, dilution increased development time.

When gramine was added to artificial diets, again, protein:carbohydrate ratio was incredibly important because it mitigated the negative effect of the toxin on mass gain, development time and survival. These results were similar to what Simpson and

Raubenheimer observed with tannic acid (2001) at the difference that they found that an equal ratio of protein:carbohydrate was best for locusts. In our case we found that higher protein concentration tend to have positive effects on performance. A possible explanation to this would be that, as seen in vertebrates herbivores (Villalba et al. 2002a, b, Villalba et al. 2002c), the nature of the toxin can alter the direction of the nutrient ratio. I hypothesized that the positive effect of protein could be linked to detoxification processes by glutathione S-transferase because this enzyme is particularly important to detoxify gramine for aphids and planthoppers (Ni and Quisenberry 2003, Cai et al. 2009, Sun et al. 2013) and it has been shown that its synthesis can be enhanced by the dietary addition of amino acids in humans (Sekhar et al. 2011). Regardless of the mechanism, these results are extremely important because they show that the effectiveness of toxin varies depending on nutrient availability.

Maybe one of the most original results of this work is the clear evidence using plant material, that for *M. differentialis* plant nutrient content, not allelochemicals, influence diet mixing. That said, nutrient regulation with plants was strongly carbohydrate-biased (p1:c2.5) compare to what I found with artificial diets that did not contain toxin (p1:c1.4). Perhaps this extra carbohydrate is used to fuel detoxification effort. Plant secondary chemistry also had important effects on performance. For instance, mass gain was lower on ragweed, which contained high level of phenolic compounds.

Interestingly, when given a choice, and when gramine was present in the protein-biased diet, insects also self-selected for a carbohydrate-biased ratio (p1:c1.7). This does not necessarily contradict what we found in the previous experiment using gramine where protein had positive effects on performance at high concentrations. Although protein might have positive effects on gramine detoxification by boosting gluthatione S-transferase, in the field, obtaining high quanties of dietary protein might prove difficult as plant tend to be carbohydrate-biased (Behmer and Joern 2012). Consequently when given the choice *M. differentialis* might have opted for a strategy that alleviates cost of detoxification by consuming more carbohydrates. This would also make sense in the light of the fact that *M. differentialis* produced more microsomal protein in the fat body when gramine was present and in the mosquito *Culex pipiens* insecticide resistance mediated by p450 enzymes affected glycogen and lipid content (Hardstone et al. 2010), both linked to carbohydrate metabolism.

Given that nutrient-allelochemical interactions affect foraging behavior and performance of the grass specialist *Locusta migratoria* (Simpson and Raubenheimer 2001, Behmer et al. 2002) and the generalist (*M. differentialis*), I predict that they are in fact prevalent for most insect herbivores, but additional studies are needed. A key question will be to determine which protein:carbohydrate ratio efficiently reduces the negative effects of toxin as this mechanism might influence the efficiency of insecticides. Comparative studies using different toxins, and the associated detoxification mechanisms, could provide some answers.

REFERENCES

Ainsworth, E. A. and K. M. Gillespie. 2007. Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent. Nature Protocols **2**:875-877.

Ali, J. G. and A. A. Agrawal. 2012. Specialist versus generalist insect herbivores and plant defense. Trends in Plant Science **17**:293-302.

Appel, H. M. and M. M. Martin. 1992. Significance of metabolic load in the evolution of host specificity of Manduca sexta. Ecology **73**:216-228.

Arrese, E. L. and J. L. Soulages. 2010. Insect fat body: energy, metabolism, and regulation. Annual Review of Entomology **55**:207.

Awmack, C. S. and S. R. Leather. 2002. Host plant quality and fecundity in herbivorous insects. Annual Review of Entomology **47**:817-844.

Banner, R. E., J. Rogosic, E. A. Burritt, and F. D. Provenza. 2000. Supplemental barley and charcoal increase intake of sagebrush by lambs. Journal of Range Management **53**:415-420.

Barbehenn, R. V. 2005. Grasshoppers efficiently process C4 grass leaf tissues: implications for patterns of host-plant utilization. Entomologia Experimentalis et Applicata **116**:209-217.

Beckerman, A. P., M. Uriarte, and O. J. Schmitz. 1997. Experimental evidence for a behavior-mediated trophic cascade in a terrestrial food chain. Proceedings of the National Academy of Sciences of the United States of America **94**:10735-10738.

Behmer, S. T. 2009. Insect herbivore nutrient regulation. Annual Review of Entomology **54**:165-187.

Behmer, S. T. and A. Joern. 2008. Coexisting generalist herbivores occupy unique nutritional feeding niches. Proceedings of the National Academy of Sciences of the United States of America **105**:1977-1982.

Behmer, S. T. and A. Joern. 2012 Insect herbivores viewed through a physiological framework: insights from Orthoptera. Pages 3-29 *in* P. Barbosa, D. K. Letourneau, and A. A. Agrawal, editors. Insect Outbreaks Revisited. Wiley-Blackwell, Chichester, U.K.

Behmer, S. T., S. J. Simpson, and D. Raubenheimer. 2002. Herbivore foraging in chemically heterogeneous environments: Nutrients and secondary metabolites. Ecology **83**:2489-2501.

Bennett, R. N. and R. M. Wallsgrove. 1994. Secondary metabolites in plant defence mechanisms. New Phytologist **127**:617-633.

Berenbaum, M. R. and A. R. Zangerl. 1994. Costs of Inducible Defense: Protein Limitation, Growth, and Detoxification in Parsnip Webworms. Ecology **75**:2311-2317.

Bernays, E. and M. Graham. 1988. On the evolution of host specificity in phytophagous arthropods. Ecology **69**:886-892.

Bernays, E. A. 1991. Relationship between deterrence and toxicity of plant secondary compounds for the grasshopper *Schistocerca americana*. Journal of Chemical Ecology **17**:2519-2526.

Bernays, E. A. 1997. Feeding by lepidopteran larvae is dangerous. Ecological Entomology **22**:121-123.

Bernays, E. A. 2001. Neural limitations in phytophagous insects: implications for diet breadth and evolution of host affiliation. Annual Review of Entomology **46**:703-727.

Bernays, E. A., K. L. Bright, N. Gonzalez, and J. Angel. 1994. Dietary Mixing in a Generalist Herbivore: Tests of Two Hypotheses. Ecology **75**:1997-2006.

Bernays, E. A. and R. F. Chapman. 1994. Host-Plant Selection by Phytophagous Insects. Chapman & Hall, New York.

Bernays, E. A. and O. Minkenberg. 1997. Insect herbivores: different reasons for being a generalist. Ecology **78**:1157-1169.

Bernays, E. A. and D. Raubenheimer. 1991. Dietary mixing in grasshoppers: Changes in acceptability of different plant secondary compounds associated with low levels of dietary protein (Orthoptera: Acrididae). Journal of Insect Behavior **4**:545-556.

Bernays, E. A., M. S. Singer, and D. Rodrigues. 2004. Foraging in nature: foraging efficiency and attentiveness in caterpillars with different diet breadths. Ecological Entomology **29**:389-397.

Berner, D., W. U. Blanckenhorn, and C. Korner. 2005. Grasshoppers cope with low host plant quality by compensatory feeding and food selection: N limitation challenged. Oikos **111**:525-533.

Blows, M. W. and R. Brooks. 2003. Measuring Nonlinear Selection. The American Naturalist **162**:815-820.

Boswell, A. W., T. Provin, and S. T. Behmer. 2008. The relationship between body mass and elemental composition in nymphs of the grasshopper *Schistocerca americana*. Journal of Orthoptera Research **22**:307-313.

Brattsten, L. B. 1992. Metabolic defenses against plant allelochemicals. Herbivores: their Interactions with Secondary Plant Metabolites **2**:175-242.

Broadway, R. M. and S. S. Duffey. 1986. Plant proteinase inhibitors: Mechanism of action and effect on the growth and digestive physiology of larval *Heliothis zea* and *Spodoptera exiqua*. Journal of Insect Physiology **32**:827-833.

Burrows, G. E. and R. J. Tyrl. 2012. Toxic Plants of North America. John Wiley & Sons.

Cai, Q.-N., Y. Han, Y.-Z. Cao, Y. Hu, X. Zhao, and J.-L. Bi. 2009. Detoxification of gramine by the cereal aphid *Sitobion avenae*. Journal of Chemical Ecology **35**:320-325.

Capinera, J. L. 2008. Differential Grasshopper, *Melanoplus differentialis* (Thomas)(Orthoptera: Acrididae). Encyclopedia of Entomology **2**:1218-1221.

Castaneda, L. E., K. Barrientos, P. A. Cortes, C. C. Figueroa, E. Fuentes-Contreras, M. Luna-Rudloff, A. X. Silva, and L. D. Bacigalupe. 2011. Evaluating reproductive fitness and metabolic costs for insecticide resistance in *Myzus persicae* from Chile. Physiological Entomology **36**:253-260.

Castaneda, L. E., C. C. Figueroa, and R. F. Nespolo. 2010. Do insect pests perform better on highly defended plants? Costs and benefits of induced detoxification defences in the aphid *Sitobion avenae*. Journal of Evolutionary Biology **23**:2474-2483.

Caswell, H. and F. C. Reed. 1976. Plant-herbivore interactions. Oecologia **26**:151-156.

Cease, A. J., J. J. Elser, C. F. Ford, S. Hao, L. Kang, and J. F. Harrison. 2012. Heavy livestock grazing promotes locust outbreaks by lowering plant nitrogen content. Science **335**:467-469.

Chambers, P. G., S. J. Simpson, and D. Raubenheimer. 1995. Behavioural mechanisms of nutrient balancing in *Locusta migratoria* nymphs. Animal Behaviour **50**:1513-1523.

Chapin, F. S., A. J. Bloom, C. B. Field, and R. H. Waring. 1987. Plant responses to multiple environmental factors. Bioscience **37**:49-57.

Chapman, R. F. 1998. The Insects: Structure and Function. Cambridge University Press, Cambridge.

Clements, K. D., D. Raubenheimer, and J. H. Choat. 2009. Nutritional ecology of marine herbivorous fishes: ten years on. Functional Ecology **23**:79-92.

Clissold, F. J., G. D. Sanson, and J. Read. 2006. The paradoxical effects of nutrient ratios and supply rates on an outbreaking insect herbivore, the Australian plague locust. Journal of Animal Ecology **75**:1000-1013.

Clissold, F. J., G. D. Sanson, J. Read, and S. J. Simpson. 2009. Gross vs. net income: how plant toughness affects performance of an insect herbivore. Ecology **90**:3393-3405.

Clissold, F. J., B. J. Tedder, A. D. Conigrave, and S. J. Simpson. 2010. The gastrointestinal tract as a nutrient-balancing organ. Proceedings of the Royal Society B: Biological Sciences **277**:1751-1759.

Cogni, R., J. R. Trigo, and D. J. Futuyma. 2012. A free lunch? No cost for acquiring defensive plant pyrrolizidine alkaloids in a specialist arctiid moth (*Utetheisa ornatrix*). Molecular Ecology **21**:6152-6162.

Corcuera, L. J. 1984. Effects of indole alkaloids from Gramineae on aphids. Phytochemistry **23**:539-541.

Coustau, C. and C. Chevillon. 2000. Resistance to xenobiotics and parasites: can we count the cost? Trends in Ecology & Evolution **15**:378-383.

Cresswell, J. E., S. Z. Merritt, and M. M. Martin. 1992. The effect of dietary nicotine on the allocation of assimilated food to energy metabolism and growth in fourth-instar larvae of the southern armyworm, *Spodoptera eridania* (Lepidoptera: Noctuidae). Oecologia **89**:449-453.

Dadd, R. H. 1961. The nutritional requirements of locusts : Observations on essential fatty acids, chlorophyll, nutritional salt mixtures, and the protein or amino acid components of synthetic diets. Journal of Insect Physiology **6**:126-145.

Danner, B. J. and A. Joern. 2003. Stage-specific behavioral responses of *Ageneotettix deorum* (Orthoptera : Acrididae) in the presence of lycosid spider predators. Journal of Insect Behavior **16**:453-464.

De Duve, C., B. Pressman, R. Gianetto, R. Wattiaux, and F. o. Appelmans. 1955. Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. Biochemical Journal **60**:604.

Dearing, M. D., A. M. Mangione, and W. H. Karasov. 2000. Diet breadth of mammalian herbivores: nutrient versus detoxification constraints. Oecologia **123**:397-405.

Dyer, L. A. and T. Floyd. 1993. Determinants of predation on phytophagous insects: the importance of diet breadth. Oecologia **96**:575-582.

Fanson, B. G., S. Yap, and P. W. Taylor. 2012. Geometry of compensatory feeding and water consumption in *Drosophila melanogaster*. The Journal of Experimental Biology **215**:766-773.

Felton, A. M., A. Felton, D. Raubenheimer, S. J. Simpson, W. J. Foley, J. T. Wood, I. R. Wallis, and D. B. Lindenmayer. 2009. Protein content of diets dictates the daily energy intake of a free-ranging primate. Behavioral Ecology **20**:685-690.

Felton, G. W. 1996. Nutritive quality of plant protein: sources of variation and insect herbivore responses. Archives of Insect Biochemistry and Physiology **32**:107-130.

Feyereisen, R. 1999. Insect P450 enzymes. Annual Review of Entomology **44**:507-533.

Fielding, D. J. and M. A. Brusven. 1992. Food and habitat preferences of *Melanoplus sanguinipes* and *Aulocara elliotti* (Orthoptera: Acrididae) on disturbed rangeland in southern Idaho. Journal of Economic Entomology **85**:783-788.

Ghorai, N., S. Chakraborty, S. Gucchait, S. K. Saha, and S. Biswas. 2012. Estimation of total Terpenoids concentration in plant tissues using a monoterpene, Linalool as standard reagent. Nature Partner Journal, Protocol Exchange

Guengerich, F. P. 1984. Effects of nutritive factors on metabolic processes involving bioactivation and detoxication of chemicals. Annual Review of Nutrition **4**:207-231.

Guengerich, F. P. 1995. Human cytochrome P450 enzymes. Pages 473-535 *in* P. R. Ortiz de Montellano, editor. Cytochrome P450. Springer.

Gusewell, S. and M. Koerselman. 2002. Variation in nitrogen and phosphorus concentrations of wetland plants. Perspectives in Plant Ecology Evolution and Systematics **5**:37-61.

Hägele, B. F. and M. Rowell-Rahier. 1999. Dietary mixing in three generalist herbivores: nutrient complementation or toxin dilution? Oecologia **119**:521-533.

Hahn, D. A. 2005. Larval nutrition affects lipid storage and growth, but not protein or carbohydrate storage in newly eclosed adults of the grasshopper *Schistocerca americana*. Journal of Insect Physiology **51**:1210-1219.

Hahn, D. A. and D. E. Wheeler. 2003. Presence of a single abundant storage hexamerin in both larvae and adults of the grasshopper, *Schistocerca americana*. Journal of Insect Physiology **49**:1189-1197.

Hanson, A. D., K. M. Ditz, G. W. Singletary, and T. J. Leland. 1983. Gramine accumulation in leaves of barley grown under high-temperature stress. Plant Physiology **71**:896-904.

Hanson, A. D., P. L. Traynor, K. M. Ditz, and D. A. Reicosky. 1981. Gramine in Barley Forage - Effects of Genotype and Environment1. Crop Sciences **21**:726-730.

Harborne, J. B. 2007. Role of Secondary Metabolites in Chemical Defence Mechanisms in Plants. John Wiley & Sons, Ltd.

Hardstone, M. C., X. Huang, L. C. Harrington, and J. G. Scott. 2010. Differences in development, glycogen, and lipid content associated with cytochrome P450-mediated permethrin resistance in *Culex pipiens quinquefasciatus* (Diptera: Culicidae). Journal of Medical Entomology **47**:188-198.

Harley, K. L. S. and A. J. Thorsteinson. 1967. The influence of plant chemicals on the feeding behavior, development, and survival of the two-striped grasshopper, *Melanoplus bivittatus* (Say), Acrididae: Orthoptera. Canadian Journal of Zoology **45**:305-319.

Harrison, J. F., A. J. Cease, J. M. VandenBrooks, T. Albert, and G. Davidowitz. 2013. Caterpillars selected for large body size and short development time are more susceptible to oxygen-related stress. Ecology and Evolution **3**:1305-1316.

Hawlena, D. and O. J. Schmitz. 2010. Physiological Stress as a Fundamental Mechanism Linking Predation to Ecosystem Functioning. American Naturalist **176**:537-556.

Hendrix, D. L. and S. C. Huber. 1986. Diurnal fluctuations in cotton leaf carbon export, carbohydrate content, and sucrose synthesizing enzymes. Plant Physiology **81**:584-586.

Hinks, C. F. and O. Olfert. 1999. Factors Influencing the Sensitivity of Binary Preference Assays for Determining Cereal Cultivar Resistance to Grasshoppers. Journal of Orthoptera Research **8**:1-7.

Hochuli, D. F. 1993. Does silica defend grasses against invertebrate herbivores? Trends in Ecology & Evolution **8**:418-419.

Holt, D. A. and A. R. Hilst. 1969. Daily variation in carbohydrate content of selected forage crops. Agronomy Journal **61**:239-242.

Howe, G. A. and G. Jander. 2008. Plant immunity to insect herbivores. Annual Review of Plant Biology **59**:41-66.

Hunt, J. W., A. P. Dean, R. E. Webster, G. N. Johnson, and A. R. Ennos. 2008. A novel mechanism by which silica defends grasses against herbivory. Annals of Botany **102**:653-656.

Illius, A. W. and N. S. Jessop. 1996. Metabolic constraints on voluntary intake in ruminants. Journal of Animal Science **74**:3052-3062.

Ishikawa, Y. and T. r. Kanke. 2000. Role of gramine in the feeding deterrence of barley against the migratory locust, *Locusta migratoria* (Orthoptera: Acrididae). Applied Entomology and Zoology **35**:251-256.

Jonas, J. L., G. W. T. Wilson, P. M. White, and A. Joern. 2007. Consumption of mycorrhizal and saprophytic fungi by Collembola in grassland soils. Soil Biology and Biochemistry **39**:2594-2602.

Karasov, W. H. and C. Martínez del Rio. 2007. Physiological ecology : how animals process energy, nutrients, and toxins. Princeton University Press, Princeton.

Karban, R., C. Karban, M. Huntzinger, I. A. N. Pearse, and G. Crutsinger. 2010. Diet mixing enhances the performance of a generalist caterpillar, *Platyprepia virginalis*. Ecological Entomology **35**:92-99.

Kessler, A. and I. T. Baldwin. 2002. Plant Response to Insect Herbivory: The Emerging Molecular Analysis. Annual Review of Plant Biology **53**:299-328.

Kingsolver, J. G. and R. B. Huey. 2008. Size, temperature, and fitness: three rules. Evolutionary Ecology Research **10**:251.

Kingsolver, J. G. and D. W. Pfennig. 2004. Individual-level selection as a cause of Cope's rule of phyletic size increase. Evolution **58**:1608-1612.

Krieger, R. I., P. P. Feeny, and C. F. Wilkinson. 1971. Detoxication enzymes in the guts of caterpillars: an evolutionary answer to plant defenses? Science **172**:579-581.

Lee, K. P., D. Raubenheimer, S. T. Behmer, and S. J. Simpson. 2003. A correlation between macronutrient balancing and insect host-plant range: evidence from the specialist caterpillar *Spodoptera exempta* (Walker). Journal of Insect Physiology **49**:1161-1171.

Lee, K. P., D. Raubenheimer, and S. J. Simpson. 2004. The effects of nutritional imbalance on compensatory feeding for cellulose-mediated dietary dilution in a generalist caterpillar. Physiological Entomology **29**:108-117.

Leszczynski, B., L. C. Wright, and T. Bakowski. 1989. Effect of secondary plant substances on winter wheat resistance to grain aphid. Entomologia Experimentalis et Applicata **52**:135-139.

Lewis, A. C. 1984. Plant Quality and Grasshopper Feeding: Effects of Sunflower Condition on Preference and Performance in *Melanoplus Differentialis*. Ecology **65**:836-843.

Li, X., J. Baudry, M. R. Berenbaum, and M. A. Schuler. 2004. Structural and functional divergence of insect CYP6B proteins: From specialist to generalist cytochrome P450. Proceedings of the National Academy of Sciences of the United States of America **101**:2939.

Lima, S. L. 1998. Nonlethal effects in the ecology of predator-prey interactions. Bioscience **48**:25-34.

Lindroth, R. L., B. D. Anson, and A. V. Weisbrod. 1990. Effects of protein and juglone on gypsy moths: growth performance and detoxification enzyme activity. Journal of Chemical Ecology **16**:2533-2547.

Lindroth, R. L., T. L. Osier, H. R. H. Barnhill, and S. A. Wood. 2002. Effects of genotype and nutrient availability on phytochemistry of trembling aspen (*Populus tremuloides Michx*.) during leaf senescence. Biochemical Systematics and Ecology **30**:297-307.

MacArthur, R. H. and E. R. Pianka. 1966. On optimal use of a patchy environment. American Naturalist **100**:603-609.

Maksimovic, Z. 2008. In vitro antioxidant activity of ragweed (*Ambrosia artemisiifolia* L., Asteraceae) herb. Industrial Crops and Products **28**:356-360.

Martin, M. M. and H. M. Van't Hof. 1988. The cause of reduced growth of *Manduca sexta* larvae on a low-water diet: Increased metabolic processing costs or nutrient limitation? Journal of Insect Physiology **34**:515-525.

Martinez, J. H., T. T. Ross, K. A. Becker, and G. S. Smith. 1993. Ingested dry snakeweed foliage did not impair reproduction in ewes and heifers during late gestation. Pages 32-35 *in* Proceedings Western Section American Society Animal Science

Massey, F. P., A. R. Ennos, and S. E. Hartley. 2006. Silica in grasses as a defence against insect herbivores: contrasting effects on folivores and a phloem feeder. Journal of Animal Ecology **75**:595-603.

Mattson Jr, W. J. 1980. Herbivory in relation to plant nitrogen content. Annual Review of Ecology and Systematics **11**:119-161.

Maxmen, A. 2013. Crop pests: Under attack. Nature **501**:S15-S17.

Miura, K. and N. Ohsaki. 2004. Diet mixing and its effect on polyphagous grasshopper nymphs. Ecological Research **19**:269-274.

Moharramipour, S., H. Tsumuki, K. Sato, S. Murata, and K. Kanehisa. 1997. Effects of leaf color, epicuticular wax amount and gramine content in barley hybrids on cereal aphid populations. Applied Entomology and Zoology **32**:1-8.

Mullin, C. A., A. A. Alfatafta, J. L. Harman, S. L. Everett, and A. A. Serino. 1991. Feeding and toxic effects of floral sesquiterpene lactones, diterpenes, and phenolics from sunflower (*Helianthus annuus* L.) on western corn rootworm. Journal of Agricultural and Food Chemistry **39**:2293-2299.

Ni, X. and S. S. Quisenberry. 2003. Possible roles of esterase, glutathione S-transferase, and superoxide dismutase activities in understanding aphid-cereal interactions. Entomologia Experimentalis et Applicata **108**:187-195.

Nijhout, H. F. and C. M. Williams. 1974. Control of moulting and metamorphosis in the tobacco hornworm, *Manduca sexta* (L.): cessation of juvenile hormone secretion as a trigger for pupation. Journal of Experimental Biology **61**:493-501.

Nylin, S. and K. Gotthard. 1998. Plasticity in life-history traits. Annual Review of Entomology **43**:63-83.

Oleksyn, J., P. B. Reich, R. Zytkowiak, P. Karolewski, and M. G. Tjoelker. 2002. Needle nutrients in geographically diverse *Pinus sylvestris* L. populations. Annals of Forest Science **59**:1-18.

Osier, T. L. and R. L. Lindroth. 2001. Effects of genotype, nutrient availability, and defoliation on aspen phytochemistry and insect performance. Journal of Chemical Ecology **27**:1289-1313.

Parke, D. V. and C. Ioannides. 1981. The role of nutrition in toxicology. Annual Review of Nutrition **1**:207-234.

Parsons, S. M. A. 2011. A generalist grasshopper species (*Melanoplus femurrubrum*) is adapted to variable environments along a latitudinal gradient. Kansas State University.

Paul, N. D., P. E. Hatcher, and J. E. Taylor. 2000. Coping with multiple enemies: an integration of molecular and ecological perspectives. Trends in Plant Science **5**:220-225.

Provenza, F. D., J. J. Villalba, L. E. Dziba, S. B. Atwood, and R. E. Banner. 2003. Linking herbivore experience, varied diets, and plant biochemical diversity. Small Ruminant Research **49**:257-274.

Ramsey, J. S., D. S. Rider, T. K. Walsh, M. De Vos, K. H. J. Gordon, L. Ponnala, S. L. Macmil, B. A. Roe, and G. Jander. 2010. Comparative analysis of detoxification enzymes in *Acyrthosiphon pisum* and *Myzus persicae*. Insect Molecular Biology **19**:155-164.

Raubenheimer, D. and S. J. Simpson. 1990. The effects of simultaneous variation in protein, digestible carbohydrate and tannic acid on the feeding behaviour of larval *Locusta migratoria* (L.) and *Schistocerca gregaria* (Forskal). I. Short term studies. Physiological Entomology **15**:219-233.

Raubenheimer, D. and S. J. Simpson. 1993. The Geometry of Compensatory Feeding in the Locust. Animal Behaviour **45**:953-964.

Raubenheimer, D. and S. J. Simpson. 1997. Integrative models of nutrient balancing: application to insects and vertebrates. Nutrition Research Reviews **10**:151-179.

Raubenheimer, D. and S. J. Simpson. 1999. Integrating nutrition: a geometrical approach. Entomologia Experimentalis et Applicata **91**:67-82.

Raubenheimer, D. and S. J. Simpson. 2003. Nutrient balancing in grasshoppers: behavioural and physiological correlates of dietary breadth. Journal of Experimental Biology **206**:1669-1681.

Raubenheimer, D., S. J. Simpson, and D. Mayntz. 2009. Nutrition, ecology and nutritional ecology: toward an integrated framework. Functional Ecology **23**:4-16.

Rivero, A., A. Magaud, A. Nicot, and J. Vezilier. 2011. Energetic cost of insecticide resistance in *Culex pipiens* mosquitoes. Journal of Medical Entomology **48**:694-700.

Roff, D. A. 1992. Evolution of life histories: theory and analysis. Chapman & Hall, New York. Springer.

Rustamani, M. A. 1992. Gramine and aconitic acid as resistant components against cereal aphids. Okayama University

Samaranayaka, M. 1976. Possible involvement of monoamines in the release of adipokinetic hormone in the locust *Schistocerca gregaria*. Journal of Experimental Biology **65**:415-425.

Sattelmacher, B., W. J. Horst, and H. C. Becker. 1994. Factors that contribute to genetic variation for nutrient efficiency of crop plants. Zeitschrift fur Pflanzenernahrung und Bodenkunde **157**:215-224.

Scheiner, S. M. 1993. MANOVA: Multiple response variables and multispecies interactions, Scheiner SM, Gurevitch J., Design and Analysis of Ecological Experiments, 1993, 94-112. Chapman & Hall, New York **30**:209-213.

Schmitz, O. J. and K. B. Suttle. 2001. Effects of top predator species on direct and indirect interactions in a food web. Ecology **82**:2072-2081.

Schoonhoven, L. M., T. Jermy, and J. J. A. v. Loon. 1998. Insect-plant biology: from physiology to evolution. Chapman & Hall Ltd, London.

Schoonhoven, L. M., J. J. A. v. Loon, and M. Dicke. 2005. Insect-plant biology. Oxford University Press, Oxford; New York.

Schoonhoven, L. M. and J. Meerman. 1978. Metabolic cost of changes in diet and neutralization of allelochemics. Entomologia Experimentalis et Applicata **24**:689-693.

Schuler, M. A. 1996. The role of cytochrome P450 monooxygenases in plant-insect interactions. Plant Physiology **112**:1411.

Scriber, J. M. 1978. Cyanogenic glycosides in *Lotus corniculatus*. Oecologia **34**:143-155.

Scriber, J. M. and F. Slansky Jr. 1981. The nutritional ecology of immature insects. Annual Review of Entomology **26**:183-211.

Sekhar, R. V., S. G. Patel, A. P. Guthikonda, M. Reid, A. Balasubramanyam, G. E. Taffet, and F. Jahoor. 2011. Deficient synthesis of glutathione underlies oxidative stress in aging and can be corrected by dietary cysteine and glycine supplementation. The American Journal of Clinical Nutrition **94**:847-853.

Shargel, L., A. Yu, and S. Wu-Pong. 2012. Applied Biopharmaceutics & Pharmacokinetics, Sixth Edition. Mcgraw-hill.

Simpson, S. J. and J. D. Abisgold. 1985. Compensation by locusts for changes in dietary nutrients: behavioural mechanisms. Physiological Entomology **10**:443-452.

Simpson, S. J. and D. Raubenheimer. 1993. A Multi-Level Analysis of Feeding Behaviour: The Geometry of Nutritional Decisions. Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences **342**:381-402.

Simpson, S. J. and D. Raubenheimer. 1995. The Geometric Analysis of Feeding and Nutrition - a Users Guide. Journal of Insect Physiology **41**:545-553.

Simpson, S. J. and D. Raubenheimer. 2000. The hungry locust. Advances in the Study of Behavior, Vol. 29 **29**:1-44.

Simpson, S. J. and D. Raubenheimer. 2001. The Geometric Analysis of Nutrient-Allelochemical Interactions: A Case Study Using Locusts. Ecology **82**:422-439.

Simpson, S. J., D. Raubenheimer, S. T. Behmer, A. Whitworth, and G. A. Wright. 2002. A comparison of nutritional regulation in solitarious- and gregarious-phase nymphs of the desert locust *Schistocerca gregaria*. Journal of Experimental Biology **205**:121-129.

Simpson, S. J., R. M. Sibly, K. P. Lee, S. T. Behmer, and D. Raubenheimer. 2004. Optimal foraging when regulating intake of multiple nutrients. Animal Behaviour **68**:1299-1311.

Simpson, S. J. and C. L. Simpson. 1990. The mechanisms of nutritional compensation by phytophagous insects. Insect-Plant Interactions **2**:111-160.

Singer, M. S. and E. A. Bernays. 2003. Understanding omnivory needs a behavioral perspective. Ecology **84**:2532-2537.

Singer, M. S., E. A. Bernays, and Y. Carriere. 2002. The interplay between nutrient balancing and toxin dilution in foraging by a generalist insect herbivore. Animal Behaviour **64**:629-643.

Singer, M. S., D. Rodrigues, J. O. Stireman Iii, and Y. Carriere. 2004. Roles of food quality and enemy-free space in host use by a generalist insect herbivore. Ecology **85**:2747-2753.

Slansky, F. and J. Rodriguez. 1987. Nutritional ecology of insects, mites, spiders, and related invertebrates. John Wiley, New York.

Slansky, F. and G. S. Wheeler. 1989. Compensatory increases in food consumption and utilization efficiencies by velvetbean caterpillars mitigate impact of diluted diets on growth. Entomologia Experimentalis et Applicata **51**:175-187.

Slansky, F. and G. S. Wheeler. 1992a. Caterpillars' compensatory feeding response to diluted nutrients leads to toxic allelochemical dose. Entomologia Experimentalis et Applicata **65**:171-186.

Slansky, F. and G. S. Wheeler. 1992b. Feeding and Growth Responses of Laboratory and Field Strains of Velvetbean Caterpillars (Lepidoptera: Noctuidae) to Food Nutrient Level and Allelochemicals. Journal of Economic Entomology **85**:1717-1730.

Slansky Jr, F., N. E. Stamp, and T. M. Casey. 1993. Nutritional ecology: the fundamental quest for nutrients. Caterpillars: Ecological and Evolutionary Constraints on Foraging. Chapman & Hall Ltd, London.

Smilanich, A. M., L. A. Dyer, J. Q. Chambers, and M. D. Bowers. 2009. Immunological cost of chemical defence and the evolution of herbivore diet breadth. Ecology Letters **12**:612-621.

Snyder, M. J., D. E. Champagne, M. B. Cohen, and J. J. Howard. 1998. Effects of plant diet on detoxification enzyme activities of two grasshoppers, *Melanoplus differentialis* and *Taeniopoda eques*. Journal of Chemical Ecology **24**:2151-2165.

Snyder, M. J. and J. I. Glendinning. 1996. Causal connection between detoxification enzyme activity and consumption of a toxic plant compound. Journal of Comparative Physiology A **179**:255-261.

Snyder, M. J., J. L. Stevens, J. F. Andersen, and R. Feyereisen. 1995. Expression of cytochrome P450 genes of the CYP4 family in midgut and fat body of the tobacco hornworm, *Manduca sexta*. Archives of Biochemistry and Biophysics **321**:13-20.

Stamp, N. E. and T. M. Casey. 1993. Caterpillars: ecological and evolutionary constraints on foraging. Chapman & Hall Ltd.

Stearns, S. C. 1992. The evolution of life histories. Oxford University Press Oxford.

Strickland, J. R., L. F. Gulino-Klein, T. T. Ross, S. Slate, M. K. Peterson, T. May, and J. B. Taylor. 1998. Effects of nutrient supplementation in beef cows of poor body condition fed snakeweed (*Gutierrezia spp*). Veterinary and Human Toxicology **40**:278-284.

Sun, X.-Q., M.-X. Zhang, J.-Y. Yu, Y. Jin, B. Ling, J.-P. Du, G.-H. Li, Q.-M. Qin, and Q.-N. Cai. 2013. Glutathione S-transferase of brown planthoppers (*Nilaparvata lugens*) is essential for their adaptation to gramine-containing host plants. PloS one **8**:e64026.

Thompson, S. N. 1998. Long-term regulation of glucogenesis by dietary carbohydrate and relevance to blood sugar level in an insect *Manduca sexta* L. The International Journal of Biochemistry & Cell biology **30**:987-999.

Thompson, S. N. and R. A. Redak. 2000. Interactions of dietary protein and carbohydrate determine blood sugar level and regulate nutrient selection in the insect *Manduca sexta* L. Biochimica et Biophysica Acta (BBA)-General Subjects **1523**:91-102.

Tilman, D. 1982. Resource competition and community structure. Princeton University Press, Princeton, N.J.

Unsicker, S. B., A. Oswald, G. n. Kohler, and W. W. Weisser. 2008. Complementarity effects through dietary mixing enhance the performance of a generalist insect herbivore. Oecologia **156**:313-324.

Villalba, J. J. and F. D. Provenza. 2005. Foraging in chemically diverse environments: Energy, protein, and alternative foods influence ingestion of plant secondary metabolites by lambs. Journal of Chemical Ecology **31**:123-138.

Villalba, J. J., F. D. Provenza, and R. E. Banner. 2002a. Influence of macronutrients and activated charcoal on intake of sagebrush by sheep and goats. Journal of Animal Science **80**:2099-2109.

Villalba, J. J., F. D. Provenza, and R. E. Banner. 2002b. Influence of macronutrients and polyethylene glycol on intake of a quebracho tannin diet by sheep and goats. Journal of Animal Science **80**:3154-3164.

Villalba, J. J., F. D. Provenza, and J. P. Bryant. 2002c. Consequences of the interaction between nutrients and plant secondary metabolites on herbivore selectivity: benefits or detriments for plants? Oikos **97**:282-292.

Villalba, J. J., F. D. Provenza, and G. Ä. Han. 2004. Experience influences diet mixing by herbivores: implications for plant biochemical diversity. Oikos **107**:100-109.

von Fircks, Y., T. Ericsson, and L. Sennerby-Forsse. 2001. Seasonal variation of macronutrients in leaves, stems and roots of *Salix dasyclados* Wimm. grown at two nutrient levels. Biomass and Bioenergy **21**:321-334.

Waldbauer, G. P. 1968. The consumption and utilization of food by insects. Advances in Insect Physiology **5**:229-288.

Waldbauer, G. P., R. W. Cohen, and S. Friedman. 1984. An Improved Procedure for Laboratory Rearing of the Corn-Earworm, *Heliothis-Zea* (Lepidoptera, Noctuidae). Great Lakes Entomologist **17**:113-118.

Walling, L. L. 2000. The Myriad Plant Responses to Herbivores. Journal of Plant Growth Regulation **19**:195-216.

Walter, J., R. Hein, H. Auge, C. Beierkuhnlein, S. L√∂ffler, K. Reifenrath, M. Schadler, M. Weber, and A. Jentsch. 2012. How do extreme drought and plant community composition affect host plant metabolites and herbivore performance? Arthropod-Plant Interactions **6**:15-25.

Wang, J. and F. D. Provenza. 1997. Dynamics of preference by sheep offered foods varying in flavors, nutrients, and a toxin. Journal of Chemical Ecology **23**:275-288.

Wang, Y., Q. Cai, Q. Zhang, and Y. Han. 2006. Effect of the secondary substances from wheat on the growth and digestive physiology of cotton bollworm *Helicoverpa armigera* (Lepidoptera: Noctuidae). European Journal of Entomology **103**:255.

Warbrick-Smith, J., S. T. Behmer, K. P. Lee, D. Raubenheimer, and S. J. Simpson. 2006. Evolving resistance to obesity in an insect. Proceedings of the National Academy of Sciences **103**:14045-14049.

Westcott, N. D., C. F. Hinks, and O. Olfert. 1992. Dietary effects of secondary plant compounds on nymphs of *Melanoplus sanguinipes* (Orthoptera: Acrididae). Annals of the Entomological Society of America **85**:304-309.

Westerterp, K. R. 1994. Energy expenditure. Food Intake and Energy Expenditure. Westerterp-Plantenga, Margriet S & Fredrix, Elisabeth W. H. M & Steffens, Anton B CRC Press, Boca Raton.

Whittaker, R. H. and P. P. Feeny. 1971. Allelochemics: chemical interactions between species. Science **171**:757-770.

Williams, J. L., D. Campos, T. T. Ross, K. A. Becker, J. M. Martinez, B. C. Oetting, and G. S. Smith. 1992. Snakeweed (*Gutierrezia spp.*) toxicosis in beef heifers. Pages 67-69 *in* Proceedings Western Section American Society Animal Science

Wittstock, U. and J. Gershenzon. 2002. Constitutive plant toxins and their role in defense against herbivores and pathogens. Current Opinion in Plant Biology **5**:300-307.

Wright, G. A., S. J. Simpson, D. Raubenheimer, and P. C. Stevenson. 2003. The feeding behavior of the weevil, *Exophthalmus jekelianus*, with respect to the nutrients and allelochemicals in host plant leaves. Oikos **100**:172-184.

Xu, W.-J. and L.-Q. Pan. 2014. Evaluation of dietary protein level on selected parameters of immune and antioxidant systems, and growth performance of juvenile *Litopenaeus vannamei* reared in zero-water exchange biofloc-based culture tanks. Aquaculture **426**:181-188.

Yang, C. S., J. F. Brady, and J.-Y. Hong. 1992. Dietary effects on cytochromes P450, xenobiotic metabolism, and toxicity. The FASEB Journal **6**:737-744.

Yang, Y. and A. Joern. 1994a. Compensatory feeding in response to variable food quality by *Melanoplus differentialis*. Physiological Entomology **19**:75-82.

Yang, Y. and A. Joern. 1994b. Influence of diet quality, developmental stage, and temperature on food residence time in the grasshopper *Melanoplus differentialis*. Physiological Zoology **67**:598-616.

Yeoh, H.-H., Y.-C. Wee, and L. Watson. 1992. Leaf protein contents and amino acid patterns of dicotyledonous plants. Biochemical Systematics and Ecology **20**:657-663.

Zanotto, F., S. Gouveia, S. Simpson, and D. Calder. 1997. Nutritional homeostasis in locusts: is there a mechanism for increased energy expenditure during carbohydrate overfeeding? Journal of Experimental Biology **200**:2437.

Zuniga, G. E. and L. J. Corcuera. 1986. Effect of gramine in the resistance of barley seedlings to the aphid *Rhopalosiphum padi*. Entomologia Experimentalis et Applicata **40**:259-262.