A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Behavioral regulation of nutrient intake in the mealworm beetle, *Tenebrio molitor* (Coleoptera : Tenebrionidae)

갈색거저리의 영양섭식행동조절에 관한 연구

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갈색거저리의 영양섭식행동조절에 관한 연구

UNDER THE DIRECTION OF ADVISER KWANG PUM LEE
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

By
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Major in Entomology
Department of Agricultural Biotechnology
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February, 2018

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FOR THE DEGREE OF MASTER OF SCIENCE
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ABSTRACT

Mealworm beetle, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), is an important model organism widely used for studying physiology and behaviour in entomological researches. In recent years, there is also a growing interest in the possibility of using *T. molitor* for animal feed and human consumption. However, little research has been conducted on the nutritional biology of this potentially important insect.

In Chapter 1, the nutritional regulatory behaviour and post-ingestive nutrient utilisation efficiencies were described from adult *T. molitor* under controlled laboratory conditions. The beetles actively regulated their intake of protein and carbohydrate to a ratio of 1:1. When confined to a range of single suboptimal diets, they exhibited a strategy of nutrient balancing similar to that

of other omnivorous insects with a broad range of diets.

Chapter 2 tested whether *T. molitor* can fully recover from the negative consequences of ingesting nutritionally imbalanced diets by adjusting their nutrient preferences. When offered a choice between two nutritionally complementary diets after being exposed to an extreme nutritional imbalance, the beetles that had been previously deprived of protein preferentially selected a protein-rich diet and this compensatory feeding led them to quickly reinstate their optimal nutritional state.

Chapter 3 investigated the effect of dietary protein:carbohydrate balance on the two important components of fitness (longevity and reproduction) in *T. molitor*. Both longevity and lifetime fecundity were maximized on a diet with equal ratio of protein to carbohydrate (1:1), a ratio which was preferentially selected by *T. molitor* when given a food choice.

Collectively, these results provide experimental support to the prevailing notion that the optimal foraging has evolved in insects to maximize the Darwinian fitness through balancing the intake of multiple nutrients.

Key words : Diet selection, Fitness, Nutrient regulation, Post-ingestive nutrient utilization, *Tenebrio molitor*

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TABLE OF CONTENTS

CHAPTER 2.

Nutrient-specific food selection buffers the effect of nutritional imbalance in the mealworm beetle, *Tenebrio molitor*

CHAPTER 3.

Balanced intake of protein and carbohydrate maximizes lifetime reproductive success in the mealworm beetle, *Tenebrio molitor*

LIST OF TABLES

CHAPTER 1.

Geometric analysis of nutrient balancing in the mealworm beetle, *Tenebrio molitor*

CHAPTER 2.

Nutrient-specific food selection buffers the effect of nutritional imbalance in the mealworm beetle, *Tenebrio molitor*

LIST OF FIGURES

GENERAL INTRODUCTION

Experimental evidence collected over the past decades has demonstrated that insects are highly capable of regulating their intake of macronutrients to a species-specific mixture that supports optimal performance (reviewed by Simpson and Simpson, 1990; Waldbauer and Friedman, 1991; Simpson and Raubenheimer, 2012). The current understanding of nutrient balancing in insects has been greatly advanced by the use of the Geometric Framework (GF) for nutrition, an integrative state-space modelling platform that was designed by Stephen Simpson and David Raubenheimer (Simpson and Raubenheimer, 1993a, 2012; Raubenheimer and Simpson, 1993, 1997, 1999). The GF has been proved as a powerful tool for identifying the behavioural and physiological mechanisms underlying the nutritional regulatory responses in insects. There is a wealth of studies that used the GF to identify the nutrient balancing in a wide range of insects, including grasshoppers (Raubenheimer and Simpson, 1993, 2003; Simpson et al., 2002; Behmer and Joern, 2008), caterpillars (Lee et al., 2002, 2003, 2004, 2006, 2010), crickets and katydids (Simpson et al., 2006; Maklakov et al., 2008; Goeriz Pearson et al., 2011), cockroaches (Jones and Raubenheimer, 2001; Raubenheimer and Jones, 2006; South et al., 2011), carnivorous beetles (Mayntz et al., 2005; Raubenheimer et al., 2007; Jensen et al., 2012), flies (Lee et al., 2008; Jensen et al., 2015) and ants (Dussutour and

Simpson, 2009; Cook et al., 2010).

The main theme of this thesis is to explore the nutritional regulatory responses of the mealworm beetle, *Tenebrio molitor* L. (Coleoptera : Tenebrionoidea), using the GF. *T. molitor* is a cosmopolitan pest scavenging on a variety of post-harvest grains. This species has long been used as a popular model organism for studying behaviour, physiology, and immunology in insects. In recent years, there has been an increasing interest of commercially using this species as animal feed and edible insect for human consumption. Despite its emerging importance, few studies have explicitly addressed the fundamental questions of nutritional biology in this species: 1) what is the balance of macronutrients optimally required by this insect? ; 2) what is the nutrient balancing strategy adopted by this species when forced to deal with nutritional imbalances?; and 3) Does this insect regulate their macronutrient intake and, if it does, what is the adaptive significance of doing so?

As a first step in understanding the basic nutritional biology of this species, in Chapter 1, I first performed a comprehensive geometric analysis on the nutritional regulatory responses of *T. molitor* using both choice and no-choice feeding assays. In order to test whether this beetle had the capacity to regulate the intake of protein and carbohydrate independently, I conducted food choice assay where beetles of both sexes were allowed to mix their diet from two nutritionally imbalanced but complementary foods (protein-biased food: p35:c7 or p28:c5.6; carbohydrate-biased food: p7:c35 or p5.6:c28). Once the

regulated position of the target protein and carbohydrate intake was established, a no-choice feeding assay was subsequently carried out to identify the pattern of ingestive trade-off employed by *T. molitor* when forced to balance the costs of over-ingesting one nutrient and those of under-ingesting the other. This no-choice assay was carried out by confining the beetles to one of seven nutritionally imbalanced foods (p0:c42, p7:c35, p14:c28, p21:c21, p28:c14, p35:c7 or p42:c0). Since the adults of *T. molitor* are known to feed on a wide range of animal- and plant-derived materials (Ramos-Elorduy et al., 2002), I anticipate that *T. molitor* beetles will follow a nutrient balancing strategy that is normally adopted by many species experiencing a high degree of nutritional heterogeneity in the natural conditions (Simpson and Raubenheimer, 2012). In this chapter, the patterns of post-ingestive nutrient utilization efficiencies were also examined by plotting two-dimension utilization plots.

It is well established that insects suffer significant performance losses when ingesting protein and carbohydrate in imbalanced quantities and ratios (Simpson et al., 2004). The detrimental consequences of nutritionally imbalanced diets can be reduced by insects using a variety of post-ingestive mechanisms (Zanotto et al., 1994, 1997; Clissold et al., 2010), but insects can easily overcome this nutritional stress by selectively consuming a diet that redresses the previous nutritional imbalance (Waldbauer and Friedman, 1991; Behmer, 2009; Simpson and Raubenheimer, 2012). In Chapter 2, I addressed the question of whether *T. molitor* beetles had the capacity to redress the

adverse effects of ingesting nutritionally imbalanced diets by actively adjusting the patterns of their nutrient selection. To this end, I experimentally manipulated the nutritional state of *T. molitor* beetles by feeding them for 16 days on one of three synthetic diets that varied in protein and carbohydrate content (p0:c42, p21:c21, or p42:c0). Thereafter, I allowed these beetles to self-compose their preferred protein and carbohydrate intake by freely mixing two nutritionally complementary diets (p0:c42 vs. p42:c0) over the next 18 days. I predicted that beetles that had been previously deprived of a specific nutrient (protein or carbohydrate) would exhibit a preference for a diet rich in that deficient nutrient. To confirm whether the nutritional conditions of the beetles really recovered from the previous nutrient imbalance through this compensatory feeding, I compared the body composition of beetles that were allowed to select between the diets with that of those that were fed only the imbalanced ones.

Recent advances in insect gerontological research suggested that the dietary P:C balance is the most critical determinant of lifespan and reproduction in many insects, but most studies investigating the dietary intervention to lifespan have been carried out using dipterans and orthopterans (e.g., Lee et al., 2008; Maklakov et al., 2008; Fanson et al., 2009). The aim of Chapter 3 was thus to examine the effects of dietary protein: carbohydrate (P:C) balance on longevity and fecundity in *T. molitor* beetles. In this chapter, I measured the longevity and the total number of eggs laid by *T. molitor* beetles over the lifetime as a

consequence of feeding on one of three synthetic foods differing in P:C ratio (p7:c35, p21:c21, or p35:c7). In addition to lifetime fecundity, the age-specific egg laying at each 7-day age interval was also quantified to test how dietary P:C ratio influenced the pattern of reproductive ageing in this insect. Having established the nutritional optima for lifespan and fecundity, I then conducted a food choice assay to test the long-held hypothesis that *T. molitor* beetles select protein and carbohydrate to a ratio that maximizes their Darwinian fitness (Simpson et al., 2004; Simpson and Raubenheimer, 2012; Jensen et al., 2012). To the best of my knowledge, this study is the first to report the effects of macronutrients on the expression of lifespan and reproduction in any coleopteran species.

CHAPTER 1.

Geometric analysis of nutrient balancing in the mealworm beetle, *Tenebrio molitor*

Abstract

Geometric analysis of the nutritional regulatory responses was performed on an omnivorous mealworm beetle, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) to test whether this beetle had the capacity to balance the intake of protein and carbohydrate. I also identified the pattern of ingestive trade-off employed when the insect was forced to balance the costs of over- and under-ingesting macronutrients. When allowed to mix their diet from two nutritionally imbalanced but complementary foods (protein-biased food: p35:c7 or p28:c5.6; carbohydrate-biased food: p7:c35 or p5.6:c28), beetles of both sexes actively regulated their intake of protein and carbohydrate to a ratio of 1:1. When confined to one of seven nutritionally imbalanced foods (p0:c42, p7:c35, p14:c28, p21:c21, p28:c14, p35:c7 or p42:c0), beetles over-ingested the excessive nutrient from these foods to such an extent that all the points of protein–carbohydrate intake aligned linearly in the nutrient space, a pattern that is characteristic of generalist feeders and omnivores. Under the restricted feeding conditions, males ate more nutrients but were less efficient at retaining their body lipids than females. Body lipid content was higher on carbohydrate-rich foods and was positively correlated with starvation resistance. These results are consistent with the prediction based on the nutritional heterogeneity hypothesis, which links the nutritional regulatory responses of insects to

their diet breadth and feeding ecology.

1. Introduction

Protein and carbohydrate are the two major macronutrients essential for survival, growth and reproduction in insects (Chapman et al., 2013). Self-selection of foods that provide an optimal amount and blend of these nutrients is an ultimate aim of foraging insects (Simpson et al., 2004; Lee et al., 2008). There has been a growing body of evidence that insects use a variety of behavioural and physiological mechanisms to balance the acquisition of multiple nutrients (Simpson and Simpson, 1990; Waldbauerand Friedman, 1991; Behmer, 2009; Clissold et al., 2010; Simpson and Raubenheimer, 2012). Much of these current knowledge about nutrient balancing in insects owes substantially to the development of the Geometric Framework (Simpson and Raubenheimer, 1993a, 2012; Raubenheimer and Simpson, 1993, 1997, 1999). The Geometric Framework is an integrative, state-space modelling program that has provided a powerful analytical method for identifying the key variables in nutritional regulatory responses in insects, including the self-regulated position of nutrient intake (the intake target) and the rule of compromise employed when insects are forced to trade-off between macronutrient excesses and deficits.

Until recently, the Geometric Framework has been used to explore the nutritional regulatory responses of taxonomically and functionally diverse

groups of insects, including herbivores (Raubenheimer and Simpson, 1993, 2003; Simpson et al., 2002; Lee et al., 2002, 2003, 2004, 2006; Telang et al., 2001; Behmer and Joern, 2008; Warbrick-Smith et al., 2009; Lee, 2010), omnivores (Jones and Raubenheimer, 2001; Raubenheimer and Jones, 2006; Simpson et al., 2006; Maklakov et al., 2008; South et al., 2011; Goeriz Pearson et al., 2011), carnivores (Mayntz et al., 2005; Raubenheimer et al., 2007; Jensen et al., 2012), liquid feeders (Abisgold et al., 1994; Lee et al., 2008, 2013; Fanson et al., 2012) and social insects (Dussutour and Simpson, 2009; Cook et al., 2010).

The main objective of this chapter is to conduct a geometric analysis on the nutritional regulatory responses of the mealworm beetle, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), an important model organism used for a wide range of research in insects. The larvae of *T. molitor* are commercially available as one of the most common protein sources for pets and captive animals worldwide (Ramos-Elorduy et al., 2002) and there has been a growing interest in the use of *T. molitor* as a novel protein source for aquaculture (Ng et al., 2001), aviculture (Ramos-Elorduy et al., 2002) and even human consumption (Ghaly and Alkoaik, 2009). So far, several studies have documented the nutrient balancing in *T. molitor* beetles (Ponton et al., 2011; Catalan et al., 2011; Morales-Ramos et al., 2011). These studies have shown that the larvae and adults of *T. molitor* actively regulate their food intake. *T.*

molitor beetles have also shown to regulate the nutrient utilisation efficiency post-ingestively (Ponton et al., 2011).

The ingestive responses of insects to macronutrient imbalance are known to correlate with the degree of nutritional heterogeneity to which they have evolved to adapt in natural conditions (Raubenheimer and Simpson, 1997, 1999, 2003; Lee et al., 2002, 2003, 2004; Simpson et al., 2002). For example, when restricted to single nutritionally imbalanced foods, insects with broader diet breadth (e.g., generalist herbivores and omnivores) tended to over-ingest excessive nutrients in the food to a greater extent than did those with narrower diet breadth (e.g., specialist herbivores). The nutritional heterogeneity hypothesis posits that this difference between the generalist and the specialist species in their responses to macronutrient imbalance arises from the fact that the generalist species have evolved to adapt to an environment where the probability of encountering nutritionally complementary foods is high (Raubenheimer and Simpson, 1997, 1999, 2003). Both the adults and larvae of *T. molitor* scavenge on a wide range of animal- and plant-derived materials, including dead plant and animal tissues, feces and grains (Ramos-Elorduy et al., 2002), indicating a high degree of nutritional heterogeneity. Based on this nutritional heterogeneity hypothesis, I predict that *T. molitor* beetles will balance the costs of over- and under-ingesting the two nutrients following the regulatory strategy that is employed by other omnivores and generalist

herbivores (Lee et al., 2002; Raubenheimer and Simpson, 1999, 2003; Raubenheimer and Jones, 2006). Since nutrient balancing is relatively understudied in omnivorous insects compared with their herbivorous and carnivorous counterparts, this chapter can provide a valuable basis for understanding the physiological and behavioural responses of omnivores to nutritional imbalances.

The first tested whether the adults of *T. molitor* could balance their intake of protein and carbohydrate through complementary food selection. To do so, I offered beetles to select between two nutritionally complementary foods and identified the regulated target of protein and carbohydrate intake defended by beetles from different food pairings. This chapter then investigated the nutrient balancing responses of beetles restricted to single foods that were nutritionally imbalanced with respect to protein and carbohydrate. I also examined the consequences of the different macronutrient intake for body composition and other proxies of fitness in *T. molitor* beetles, such as body mass and survival under starvation. An additional aim of this chapter is to compare the nutritional regulatory responses of male and female *T. molitor* beetles, an aspect that has never been investigated in this species before. Compared with the previous studies on *T. molitor* (Ponton et al., 2011; Catalan et al., 2011; Morales-Ramos et al., 2011), the novel results of chapter are the demonstration of the way that *T. molitor* beetles responded to nutritional imbalances, the diet effects on

starvation resistance via energy retention and sex-specific differences in nutritional responses.

2. Materials and methods

2.1. Experimental insects

T. molitor beetles were derived from a laboratory culture maintained at Seoul National University and were reared on wheat bran in an incubator set at 25℃ under a L:D 12 h:12 h photoregime. Larvae were kept in groups of 300–400 individuals per clear plastic container (40 × 18 × 8 cm^3 , L × W × H) where water-soaked cotton was provided as a water source thrice a week. Pupae were collected from the stock colony and sexed by inspecting the morphology of the eighth abdominal segment (Bhattacharya et al., 1970). Collected pupae were kept in a Petri dish (90 mm diameter) and allowed to complete their pupal stage in the same incubator used for culturing larvae as described above.

2.2. Synthetic foods

Dry, granular, synthetic foods were produced following the protocol

outlined by Simpson and Abisgold (1985). A total of nine foods differing in protein (p) and digestible carbohydrate (c) content were prepared: 0% protein with 42% digestible carbohydrate (p0:c42), p5.6: c28, p7:c35, p14:c28, p21:c21, p28:c5.6, p28:c14, p35:c7 and p42:c0 (% dry mass). Proteins consisted of a 3:1:1 mixture of casein, peptone and albumen and the sucrose was the digestible carbohydrate. All foods contained a fixed content of Wesson's salt (2.4%), cholesterol (0.5%), linoleic acid (0.5%), ascorbic acid (0.3%) and a vitamin mix (0.2%). The remainder of the food was filled with the non-nutritive bulking agent, indigestible cellulose.

2.3. Experimental protocol

On the day of eclosion into adulthood (day 0), a total of 220 newly emerged beetles (110 males and 110 females) were weighed to the nearest 0.1 mg using a microbalance (Ohaus Co., Parsippany, NJ, USA) and randomly allocated to one of 11 diet treatments. Four of them were the choice treatments, in which one of two protein-biased foods (P:C = 5:1; p35:c7 or p28:c5.6) was paired with one of two carbohydrate-biased foods (P:C = 1:5; p7:c35 or p5.6:c28). These two foods were individually imbalanced but were complementary to one another. The rest were the seven no-choice treatments, each with differing ratio of protein to carbohydrate (p0:c42, p7:c35, p14:c28, p21:c21, p28:c14, p35:c7 and p42:c0). These seven no-choice foods comprised

the same concentration of protein plus carbohydrate (P + C = 42% dry mass) and were near isocaloric because protein and carbohydrate are similar in caloric density (4 kcal per gram). During the entire experimental period (days 0–24), each individual was confined to its own feeding arena (90 mm diameter Petri dish) with ad libitum access to water (a water-filled 1.5 mL Eppendorf tube each capped with a cotton plug) and food. Before being introduced to insects, food dishes (the upturned lid of 1.5 mL Eppendorf tube, 9 mm diameter, 5 mm depth) were filled with dry granular foods, dried to constant mass at 40 C for 24 h and weighed to the nearest 0.1 mg. Food dishes were removed and replaced by new ones every two days. To minimise any measurement error in food intake, each food dish was placed in a Petri dish (diameter 40 mm) and any food that was spilled over this platform during feeding was added back to the food dish. Removed food dishes containing uneaten foods were dried at 40℃ for 24 h to remove moisture before being weighed. Food consumption was determined as the difference in dry mass before and after the two days of feeding. Protein and carbohydrate intakes were calculated as the product of food consumption and the known concentrations of respective nutrients in the foods. At the end of the experiment (day 24), beetles were freeze-killed at 80℃, dried to constant mass at 50 ℃ for 72 h and weighed to the nearest 0.1 mg. Lipids were extracted from dried carcasses by individually soaking them in 15 mL of pure chloroform for three days. During

this chloroform wash, the chloroform was refreshed every 24 h. Lipid-extracted carcasses were re-dried and re-weighed. Total extractable lipid content was calculated as the mass change before and after the three 24-h chloroform washes. Following lipid-extraction, the proteinaceous component of lean carcasses was digested and removed by individually immersing them in 15 mL of sodium hydroxide solution (NaOH, 0.35 M) at 33 ℃ for three days (Marden, 1987). The samples went through three successive, sodium hydroxide solution 24-h washes, after which they were re-dried and re-weighed. Body protein content was estimated as the difference in dry mass before and after the three days of sodium hydroxide wash.

To quantify the body composition of beetles at adult emergence (day 0), newly enclosed beetles of both sexes (N = 20 per sex) were weighed, killed and subjected to lipid and protein extraction following the identical protocol as described above. I found that the dry body mass of the newly emerged beetles (day 0) was similar between the two sexes (ANOVA: $F_{1,57} = 0.09$, $P = 0.767$). Nor was there any significant sex difference in the body lipid ($F_{1,57} = 0.96$, $P = 0.332$) and protein ($F_{1,57} = 0.03$, $P = 0.858$) content of this beetles at adult emergence (day 0). To investigate the effect of dietary P:C ratio on the ability of *T. molitor* beetles to survive prolonged periods of food deprivation, I conducted an additional experiment using 280 freshly emerged beetles (140 males and 140 females). Experimental insects were weighed, individually

confined in their feeding arena and then apportioned randomly among seven no-choice foods differing in their P:C ratio (p0:c42, p7:c35, p14:c28, p21:c21, p28:c14, p35:c7 and p42:c0) as previously described. Over the first 14 days post-eclosion, individual insects had ad libitum access to food and water and thereafter were subjected to a complete food depriving condition, receiving only water for the rest of their lives. Starvation- induced death was tallied twice a day and starvation resistance was determined as the number of days that insects remained alive under food deprivation. Throughout the experiment, all experimental insects were maintained in an incubator set at 25 ℃ under a L:D 12 h:12 h photoregime.

2.4. Data analysis

Various aspects of nutrient consumption, body composition and starvation resistance were analysed using univariate and multivariate analysis of variance (ANOVA or MANOVA). For multivariate analysis, Pillai's trace statistic was used because it was considered the most robust to the violations of assumptions (Scheiner, 1993). When analysing the patterns of food selection by beetles in the food choice experiment, we used paired t tests to test for differences in consumption between the two choice foods. Utilisation plots and analysis of covariance (ANCOVA) were used to analyse the nutrient retention efficiencies following the method of Raubenheimer and Simpson (1992, 1994). Before the

analyses, the data were checked for their conformity to the underlying assumptions of these parametric statistical tests (the normal and homoscedasticity of the data). All statistical analyses were performed using SAS version 9.12 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Choice experiment

3.1.1. Nutrient intake

Cumulative protein and carbohydrate intake over the first 12 and 24 days post-eclosion (days 0–12 and 0–24) are presented in Fig. 1 for male and female beetles that were allowed to mix their diet from two nutritionally complementary foods in four choice treatments. Results from a two-way factorial MANOVA revealed that sex had little effect on the overall pattern of macronutrient intake (days 0–12: $F_{2,68} = 1.11$, $P = 0.335$; days 0–24: $F_{2,68} = 1.36$, $P = 0.264$), indicating that the two sexes consumed similar quantity of protein and carbohydrate. Further analyses using one-way MANOVA performed for each sex found that the effect of food pairing on the overall macronutrient intake over days 0–24 was not significant for both sexes (male: $F_{6,66} = 2.14$, $P = 0.060$; female: $F_{6,72} = 1.77$, $P = 0.118$). Hence, the intake target of each sex over days 0–24 was calculated as the mean nutrient intake

pooled over the four converging food pairings, which was 60.6 mg protein and 61.7 mg carbohydrate for males and 66.4 mg protein and 67.4 mg carbohydrate for females (Fig. 1). The mean self-selected ratio of protein to carbohydrate (P:C) was 0.999 for males and 0.997 for females. These ratios were not significantly different from 1 (one sample t-test, male: $t_{36} = 0.033$, $P = 0.974$; female: $t_{39} = 0.097$, $P = 0.923$) and from one another (unpaired t-test: $t_{75} = 0.050$, $P = 0.960$).

When p35:c7 was paired with p7:c35 food, beetles did not exhibit any significant difference in consumption between the two foods (paired t-test, males: $t_8 = 0.503$, $P = 0.629$; females: $t_9 = -1.015$, $P = 0.337$), but they consumed significantly more of carbohydrate-biased food when the two dilute foods were paired (p28:c5.6 vs. p5.6:c28) in the choice treatment (males: $t_9 = -3.549$, $P = 0.006$; females: $t_9 = -3.273$, $P = 0.010$; Fig. 2).

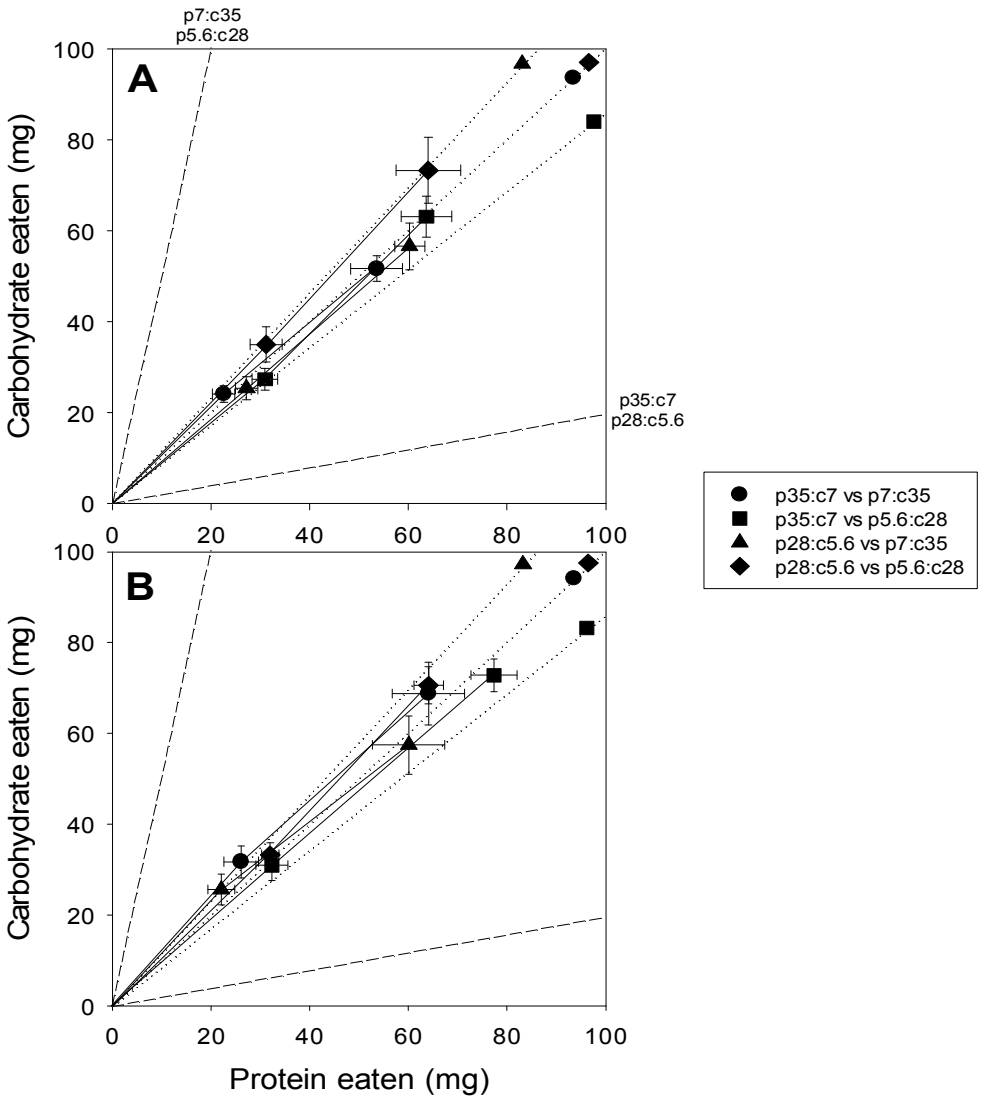

Figure 1. Bivariate means (±SE) of cumulative protein and carbohydrate intake by *Tenebrio molitor* beetles (A, male; B, female) in four food pairings. The solid lines represent the intake trajectories for beetles in four food pairings. The first and second sets of points along each trajectory represent the cumulative nutrient intake reached over the first 12 and 24 days post-eclosion, respectively. The two dashed lines are the nutritional rails of the two nutritionally complementary diets that were paired: protein-biased (p35:c7 or p28:c5.6) and carbohydrate-biased (p7:c35 or p5.6:c28) diets. The dotted lines are the hypothetic intake trajectories expected to be followed by beetles if they had fed indiscriminately between the two diets in four food pairings as indicated by symbols presented at the top right of the plot.

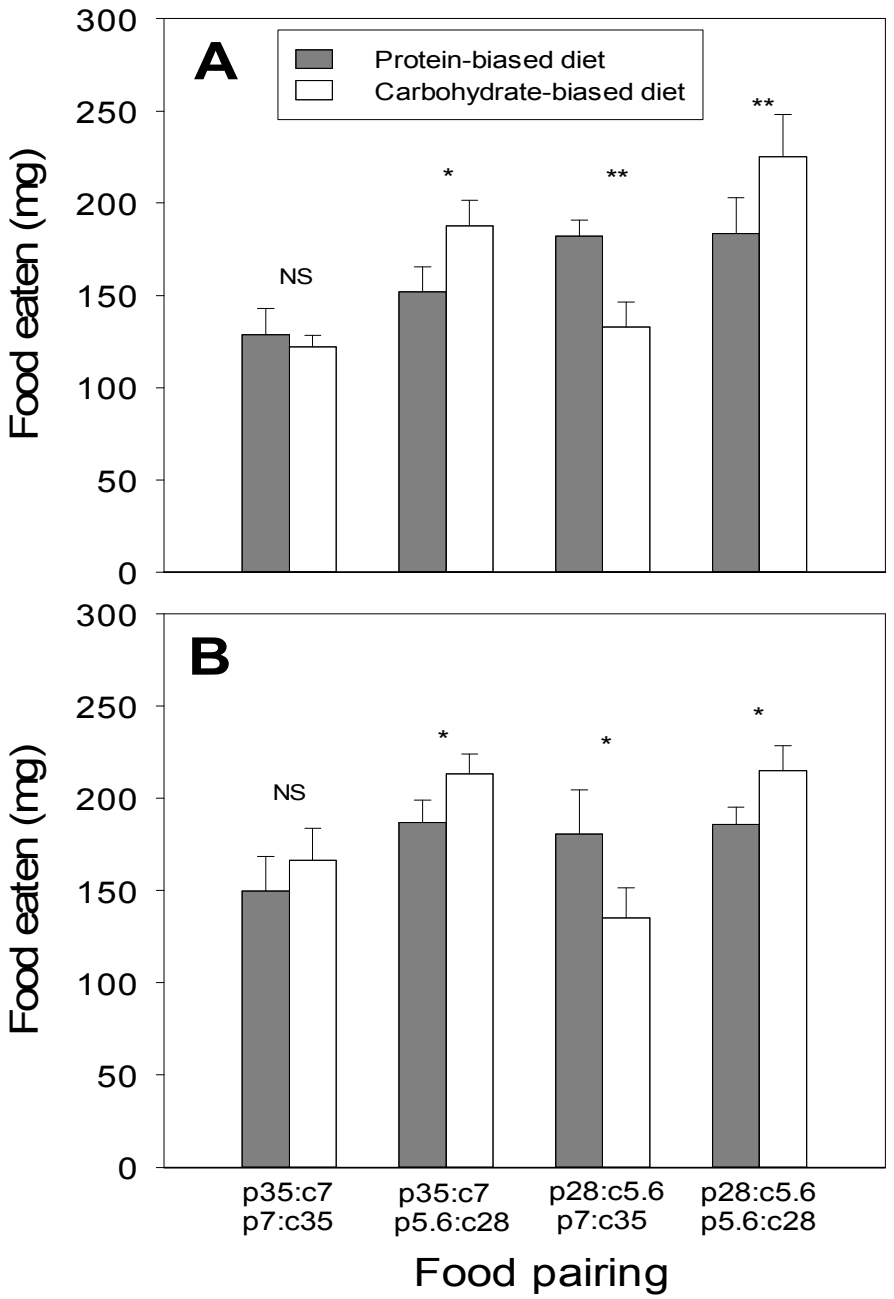

Figure 2. Means (+SE) of total amount of protein-biased and carbohydrate-biased diet eaten by *Tenebrio molitor* beetles (A, male; B, female) over the 24 days post-eclosion in four food pairings. Pair *t*-tests are performed to test for significant food preference (NS $P>0.05$, * $P<0.05$, ** $P<0.01$)

When a concentrated food (p35:c7 or p7:c35) was paired with a dilute one that was oppositely-balanced (p5.6:c28 or p28:c5.6), beetles consumed significantly more of the dilute food regardless of the balance (p35:c7 vs. p5.6:c28, male: t_9 = -2.415, P = 0.039; females: t_9 = -2.884, P = 0.018) (p28:c5.6 vs. p7:c35, male: t_7 = 3.904, P = 0.006; females: t_9 = 2.281, P = 0.048), indicating that foods were selected by beetles in a non-random manner (Fig. 2).

3.1.2. Body composition

A bicoordinate plot for body protein and lipid content is presented n Fig. 3 for male and female beetles in four food pairings. Results from a two-way factorial MANOVA demonstrated that the overall pattern of body nutrient composition was significantly affected by sex ($F_{2,68}$ = 9.86, P < 0.001), but neither by food pairing ($F_{6,138}$ = 0.37, P = 0.897) nor by the interaction between sex and food pairing ($F_{6,138}$ = 0.83, P = 0.551). Further analyses using one-way MANOVA performed for each sex confirmed that the protein–lipid points of all four food pairings converged on a sex specific growth target where they were statistically indistinguishable from one another (male: $F_{6,66}$ = 0.75, P = 0.611; female: $F_{6,72}$ = 0.65, P = 0.689). Both the mean protein and lipid content pooled over all these converging choice treatments were significantly greater for females (protein: 23.0 mg; lipid: 7.5 mg) than for males (protein: 21.4 mg;

lipid: 4.8 mg) (unpaired t-test, protein: t_{75} = -2.136, P = 0.036; lipid: t_{75} = -4.473, P < 0.001).

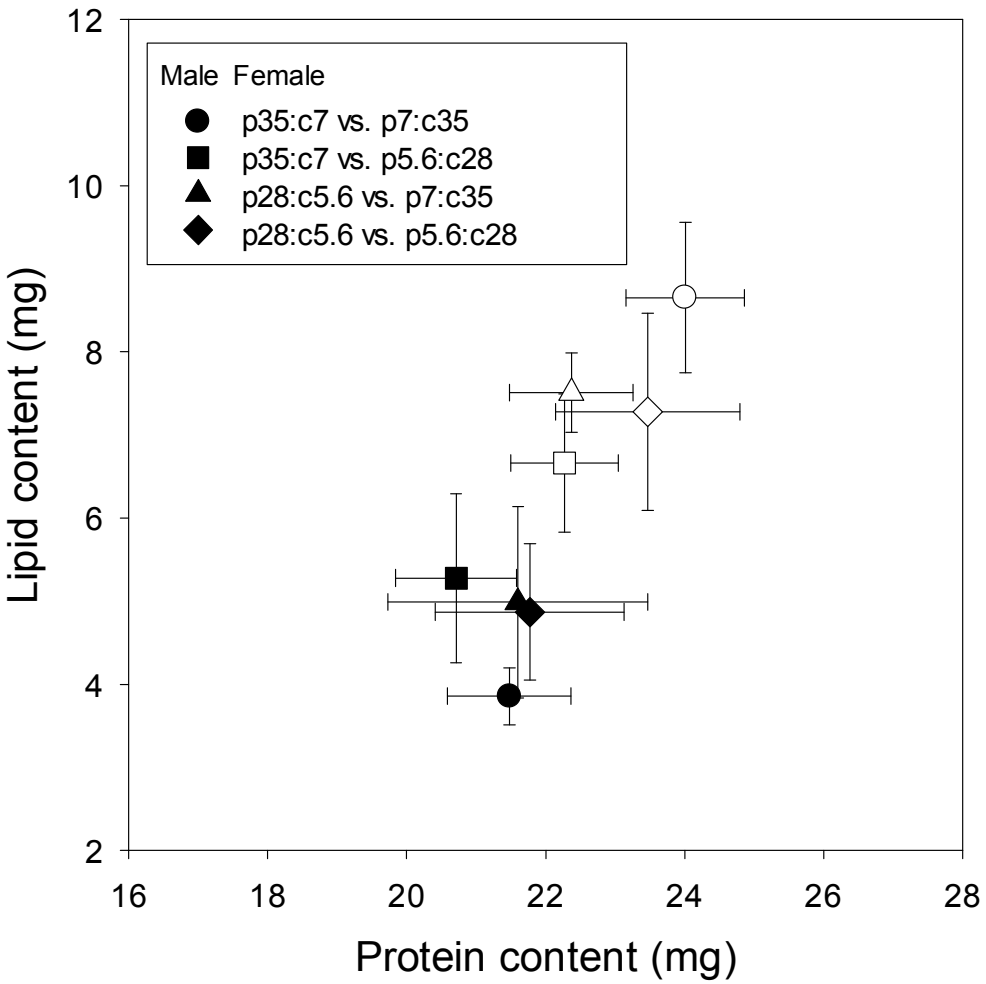

Figure 3. Bivariate means (±SE) of body protein and lipid content of *Tenebrio molitor* beetles measured at 24 days post-eclosion in four diet choice treatments.

3.2. No-choice experiment

3.2.1. Nutrient intake

Cumulative protein and carbohydrate intake over the first 12 and 24 days post-eclosion (days 0–12 and 0–24) are presented in Fig. 4 for male and female beetles restricted to one of seven isocaloric foods differing in their P:C ratio (p0:c42, p7:c35, p14:c28, p21:c21, p28:c14, p35:c7 and p42:c0). Results from a two-way factorial ANOVA indicated that the sum of protein plus carbohydrate eaten over the first 12 days (days 0–12) was not affected by food (Table 1), with all seven intake points aligning to form a linear intake array with a slope of 1 (Fig. 4). However, during the latter 12-day period of the experiment (days 12–24), beetles on the two protein-rich foods (p42:c0 and p35:c7) consumed significantly more macronutrients than did those on the others (Table 1; Tukey multiple comparison test: $P < 0.001$), causing the final intake points of these two protein-rich foods to move farther from the position expected if they would align along a slope of 1. The slope of the intake array over the experiment (days 0–24) was significantly shallower than 1 for both sexes (male: $F_{6,59} = 4.46$, $P < 0.001$; female: $F_{6,58} = 2.44$, $P = 0.036$). Males ingested significantly greater quantities of macronutrients than did females over each 12-day period of measurement (Table 1). Such sex-specific

difference in nutrient intake was greatly pronounced on the two protein-rich foods as indicated by a significant interaction between sex and food during the latter half of the experiment (days 12–24; Table 1).

3.2.2. Retention efficiency

Utilisation plots were plotted in Fig. 5 to demonstrate the relationship between the quantity of nutrients ingested and that of those retained in the body of insects at 24 days post-eclosion. In these utilisation plots, the mean of body lipid or protein content was plotted against that of carbohydrate or protein intake over days 0–24, respectively, for male and female beetles confined to one of seven isocaloric foods differing in their P:C ratio. ANCOVA with lipid content as the dependent variable and the carbohydrate intake as the covariate indicated that body lipid content increased significantly with carbohydrate intake in a linear fashion (covariate: $F_{1,128} = 103.31$, $P < 0.001$; Fig. 5A). Marginal mean of lipid content generated from this ANCOVA by adjusting for carbohydrate intake was significantly higher in females (5.32 mg) than n males (3.95 mg) (sex effect: $F_{1,128} = 16.92$, $P < 0.001$; Fig. 5A), suggesting that females were more efficient at retaining lipids than males. Body protein content exhibited a significant convex relationship with protein intake (quadratic effect of protein intake: $F_{1,127} = 3.92$, $P = 0.050$), with protein content being lower when protein was ingested either too much or too less (Fig. 5B). When both quadratic and linear terms of protein intake were included as

the covariates in ANCOVA for body protein content, I did not detect any significant sex effects on the retention efficiency of body protein ($F_{1,127} = 2.34$, $P = 0.129$).

Table 1. Summary of ANOVA testing the significance of the effects of diet, sex, and their interaction on the total quantity of protein plus carbohydrate eaten by *Tenebrio molitor* beetles over the two successive 12-day periods (days 0-12, 12-24) and over the whole 24 days (days 0-24).

Sources	Df	Days 0–12		Days 12–24		Days 0–24	
		F	*P*	*F*	*P*	*F*	*P*
Food	6	2.06	0.063	8.89	<0.001	5.86	<0.001
Sex	1	10.86	0.001	9.89	0.002	13.53	<0.001
Food × sex	6	0.63	0.706	2.42	0.031	1.66	0.137
Error	117						

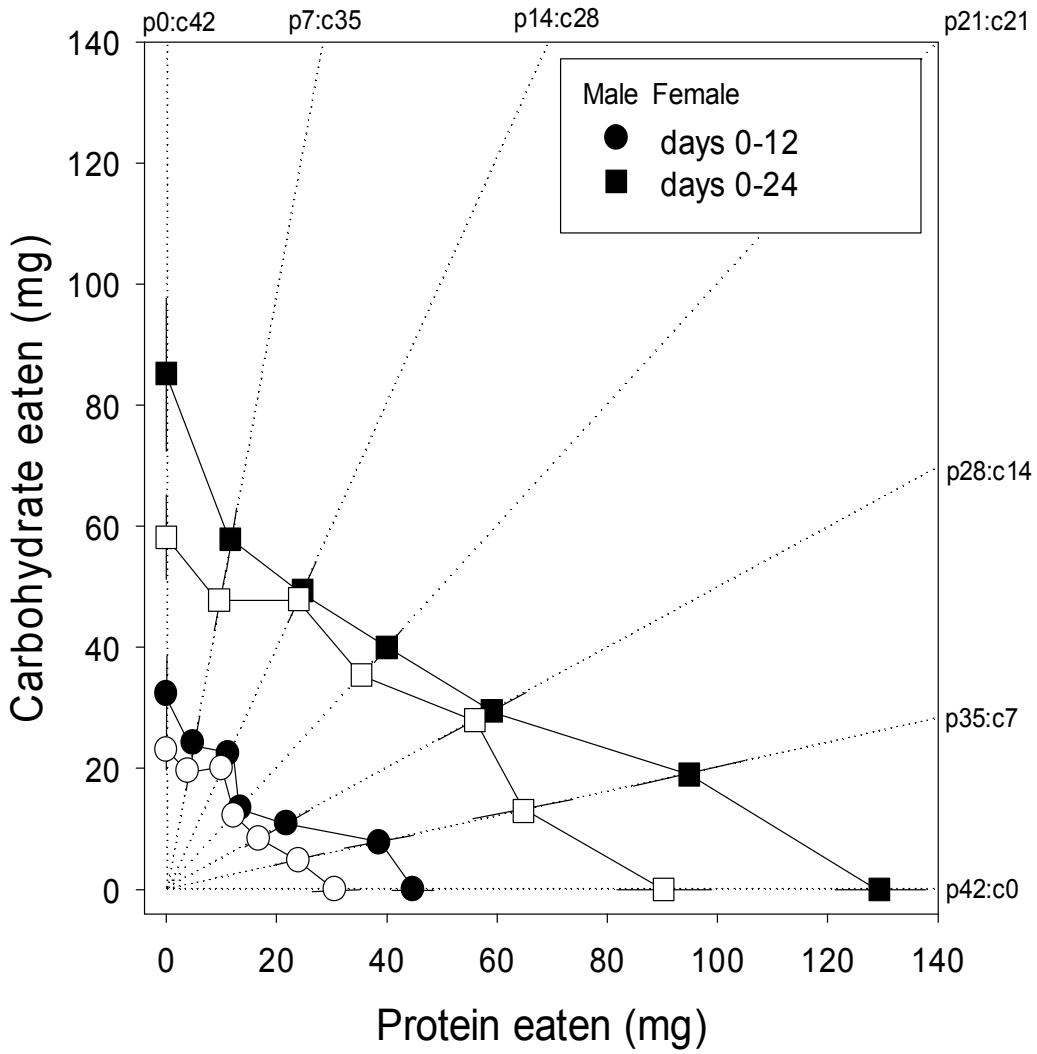

Figure 4. Means (±SE) of cumulative protein and carbohydrate intake by *Tenebrio molitor* beetles (males: filled symbols; females: open symbols) in seven no-choice diets differing in protein: carbohydrate ratio (p0:c42, p7:c35,

p14:c28, p21:c21, p28:c14, p35:c7 and p42:c0). The dotted lines are the nutritional rails of the seven diets as given in the margin. Seven cumulative intake points are connected with solid lines to demonstrate the pattern of the intake array at 12 and 24 days post-eclosion.

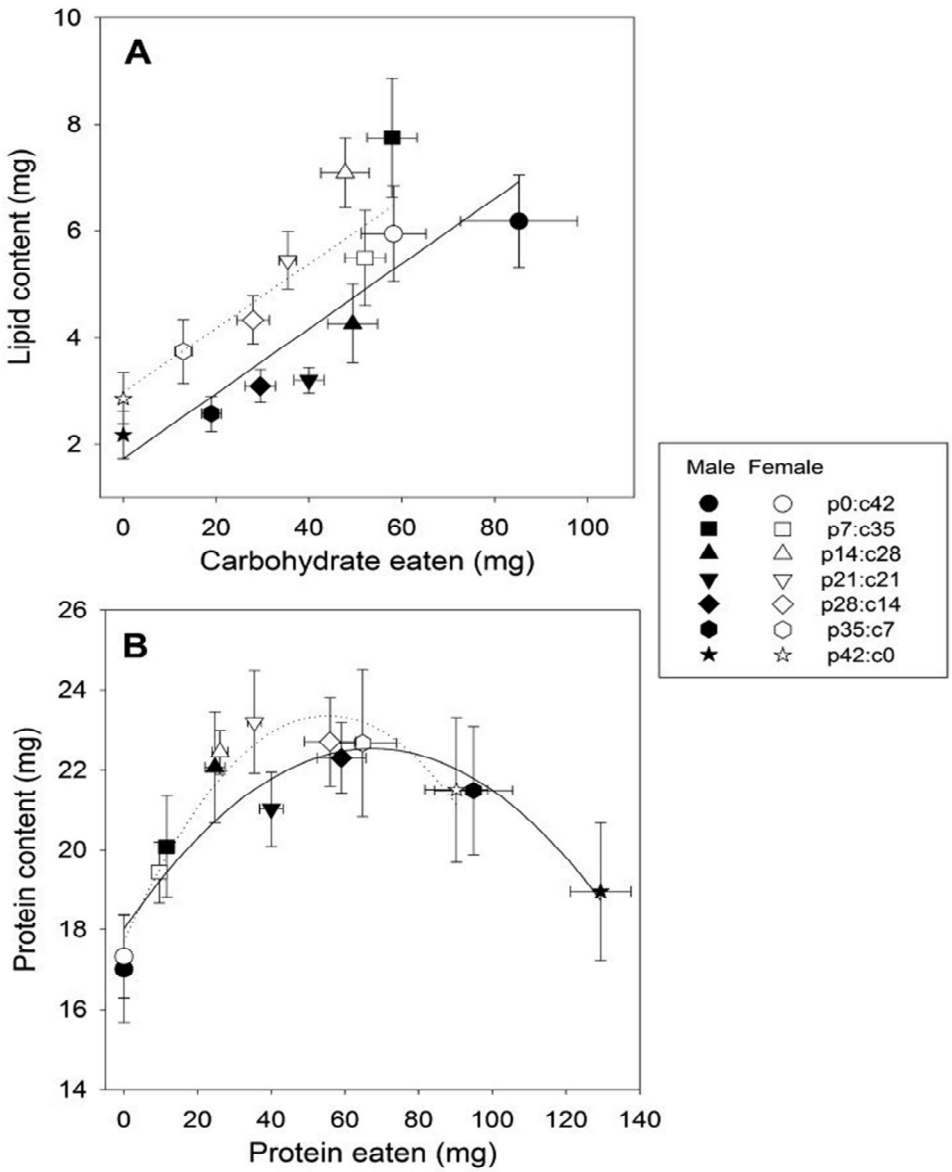

Figure 5. Bivariate means (±SE) of (A) carbohydrate intake and body lipid

content and of (B) protein intake and body protein content of *Tenebrio molitor* beetles (males: filled symbols; females: open symbols) measured at 24 days post-infection in seven no-choice diets differing in protein: carbohydrate ratio (p0:c42, p7:c35, p14:c28, p21:c21, p28:c14, p35:c7 and p42:c0). Linear and quadratic regressions are used to fit the relationships between carbohydrate intake and lipid content and between protein intake and protein content, respectively (males: solid line; females: dotted line).

3.2.3. Body composition

The dry body mass of beetles at 24 days post-eclosion was neither affected by food (ANOVA: $F_{6,118} = 1.61$, $P = 0.151$) nor by sex ($F_{1,118} = 0.40$, $P = 0.527$). Body lipid content was significantly higher on low P:C foods (food effect: $F_{6,118} = 12.86$, $P < 0.001$; Fig. 6A). Females retained a significantly greater body lipid content than did males (sex effect: $F_{1,118} = 5.21$, $P = 0.024$). Body protein content was not affected by sex (sex effect: $F_{1,118} = 1.74$, $P = 0.190$; Fig. 6B), but was significantly low on protein-deficient food (p0:c42) (food effect: $F_{6,118} = 4.69$, $P < 0.001$). For all measured parameters of body composition, the interaction between sex and food was not significant (body mass: $F_{6,118} = 0.52$, $P = 0.789$; protein: $F_{6,118} = 0.37$, $P = 0.894$; lipid: $F_{6,118} = 1.11$, $P = 0.360$).

3.2.4. Starvation resistance

Starvation resistance, which is measured as the number of days that beetles survived under food deprivation, was significantly affected by food (ANOVA:

$F_{6,208}$ = 21.95, P < 0.001) and sex ($F_{1,208}$ = 152.10, P < 0.001). Starvation period before death was prolonged for beetles previously fed on carbohydrate-biased foods (p0:c42 and p7:c35; Fig. 6). Females lived significantly longer (ca. 7.1 days) than did males. The interaction between sex and food was not significant for starvation resistance ($F_{6,208}$ = 1.33, P = 0.245), indicating that both sexes followed a similar pattern of survival across diets.

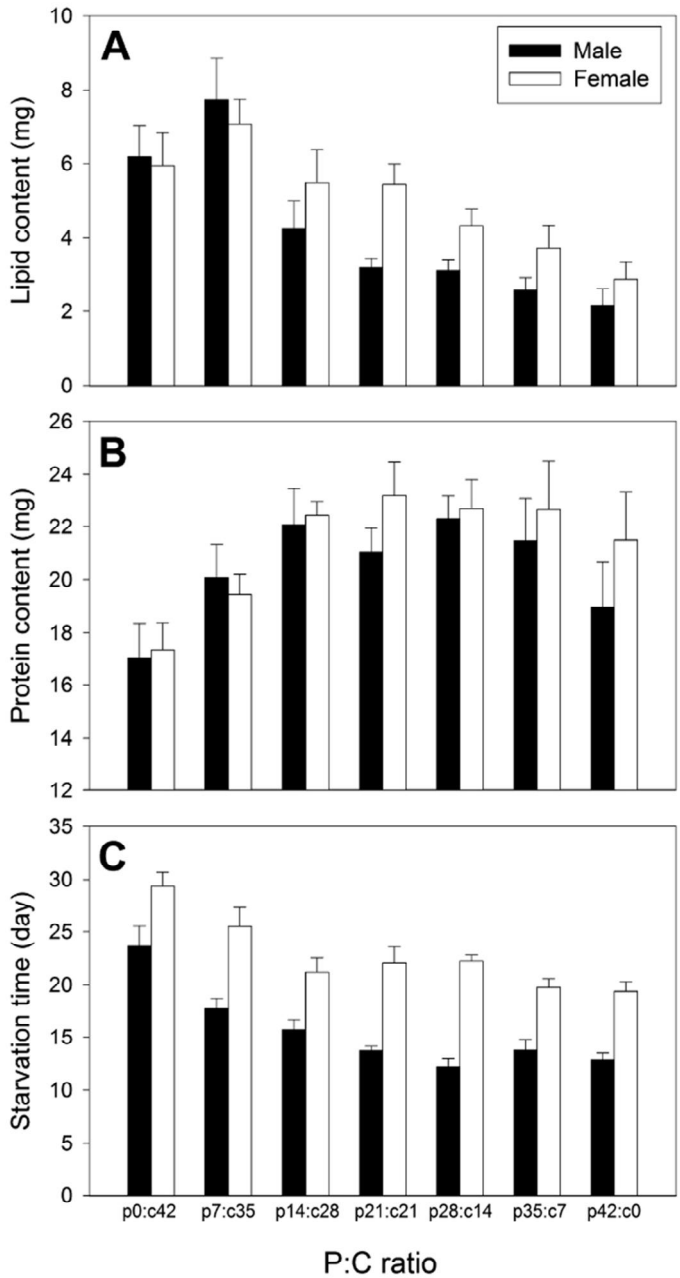

Figure 6. Means (+SE) of (A) body lipid content, (B) body protein content, and (C) starvation time of *Tenebrio molitor* beetles in seven no-choice diets differing in protein: carbohydrate ratio (p0:c42, p7:c35, p14:c28, p21:c21, p28:c14, p35:c7 and p42:c0).

4. Discussion

Much of the pioneering research on the nutritional regulatory responses of insects has been conducted on Lepidoptera and Orthoptera (reviewed in Behmer, 2009; Simpson and Raubenheimer, 2012). Despite some early investigations on dietary self-selection in Coleoptera (Waldbauer and Bhattacharya, 1973; Morales-Ramos et al., 2011; Catalan et al., 2011), it was not until recently that the nutrient balancing of some predatory beetles was analysed in such detail comparable to that of caterpillars and grasshoppers (Mayntz et al., 2005; Raubenheimer et al., 2007; Jensen et al., 2012). This chapter investigated various aspects of nutritional regulatory responses in an omnivorous beetle, *T. molitor*.

It is well established that many insects have the capacity to balance the intake of protein and carbohydrate by selectively mixing nutritionally complementary foods (Waldbauer and Friedman, 1991; Simpson et al., 2004; Behmer, 2009; Simpson and Raubenheimer, 2012). These data from the choice experiment showed that *T. molitor* beetles actively regulated their macronutrient intake, with the protein–carbohydrate intake points of all food pairings converging on a specific target position in the two dimensional nutrient space. When a concentrated food was paired with a dilute one that was imbalanced in an opposite direction (choice treatment: p35:c7 vs. p5.6:c28 and p28:c5.6 vs. p7:c35), the dilute food was ingested in significantly higher

quantities than the concentrated one, confirming that foods were selected non-randomly by beetles to compensate for food dilution. Physiological processes underlying such regulatory responses remain to be addressed in beetles, but may include the direct modulation of taste receptor responsiveness through blood-borne nutrient feedbacks, possible role of neuromodulators in the central nervous system and associative learning (Cohen et al., 1988; Simpson and Simpson, 1990; Simpson and White, 1990; Simpson and Raubenheimer, 1993b).

Comparative analyses over a range of insect groups have shown that the optimal balance of protein:carbohydrate (P:C) that maximises the insect performance correlates with various physiological, ecological and evolutionary characteristics of insects, such as diets, phylogeny, life-history and their evolutionary associations with symbiotic microbes (Simpson and Raubenheimer, 1993a). For example, the optimal diets of the larvae of many holometabolous insects (e.g., Lepidoptera and Diptera) are generally biased in favour of protein, reflecting their high protein requirement to support rapid tissue growth. By contrast, the slow-growing larvae of hemimetabolous insects (e.g., Orthoptera) prefer slightly carbohydrate- biased foods to meet high energy requirement for activity (reviewed in Waldbauer and Friedman, 1991; Behmer, 2009; Simpson and Raubenheimer, 1993a, 2012). Since *T. molitor* adults are active and do not undergo substantial body growth anymore, they

were expected to prefer a food relatively high in carbohydrates. However, these data showed that both male and female beetles of *T. molitor* selected protein and carbohydrate in a 1:1 ratio, which is not very different from the slightly carbohydrate-biased P:C ratio of 1:1.27 (44%:56%) self-selected by mated females of this species in an earlier study by Ponton et al. (2011). These selected P:C ratios reported from the past and present *T. molitor* studies are higher than those of cockroaches that accommodate nutritional endosymbionts (Jones and Raubenheimer, 2001; Raubenheimer and Jones, 2006; South et al., 2011), but are lower than those of omnivorous katydids that frequently consume animal prey (Simpson et al., 2006; Goeriz Pearson et al., 2011). Although relatively little is known about the nutrient balancing in omnivores, it appears that the self-selected P:C ratios of omnivorous insects vary among phylogenetically distinct groups.

Mainly driven by high protein demand for producing eggs, females are generally expected to be more protein-limited than males (Wheeler, 1996; Morehouse et al., 2010; Lee, 2010) and have an intake target that is often more biased toward protein than that of males. An alternative but not mutually exclusive possibility is that females may also require larger amounts of lipids than males because insect eggs consist of ca. 30–40% lipids (Ziegler and van Antwerpen, 2006). However, this chapter showed that there was no significant difference in the intake target between male and female *T. molitor* beetles.

Such a lack of sex difference in macronutrient intake may be attributed to the fact that the experimental beetles used in this chapter were not mated. Previous studies have shown that the egg production and other reproductive activities are stimulated by mating in many insects, largely mediated by male seminal fluid peptides transferred to the female reproductive tract during mating (Chen et al., 1988). Studies addressing the effects of mating on macronutrient preferences have been performed in greatest detail in *Drosophila melanogaster* (Ribeiro and Dickson, 2010; Lee et al., 2013). For example, sex-specific divergence in macronutrient intake is observed only when males are compared with mated females but not with virgin females (Lee et al., 2013), with mated females showing a considerably higher intake of macronutrients as well as preference for protein than males and virgin females. It has been reported that the fecundity of *T. molitor* females increases with mating frequencies and polyandry (Drnevich et al., 2001; Worden and Parker, 2001), raising the possibility that the intake for specific nutrients required for producing eggs can rise after mating in this species. A recent study from crickets indicated that the lack of difference in nutrient intake between male and female insects could be the result of intralocus sexual conflict (Maklakov et al., 2008).

When confined to nutritionally imbalanced foods in the no-choice experiment, beetles were prevented from achieving a state of nutritional balance (the intake target), thereby facing situations where they have to

compromise between the cost of over-ingesting the excessive nutrient in the food and that of under-ingesting the deficient one. The patterns of this ingestive trade-off are known to correlate with diet breadth or the degree of nutritional heterogeneity in insect herbivores (Raubenheimer and Simpson,1999, 2003; Simpson et al., 2002). For example, generalist grasshoppers (the gregarious phase of *Schistocera gregaria*) and caterpillars (*Spodoptera littoralis*, *Heliothis virescens*) tend to over-ingest the surplus nutrient to a greater extent than do their specialist counterparts (grasshoppers: the solitarious phase of *S. gregaria*, *Locusta migratoria*; caterpillars: *Spodoptera exempta*, *Heliothis subflexa*; Raubenheimer and Simpson, 2003; Lee et al., 2002, 2003, 2006; Simpson et al., 2002), forming a linear intake array (termed as, the 'equal distance rule' of compromise). A similar pattern of nutrient balancing between the excessive and deficit nutrients has been demonstrated from an omnivore and a generalist predator (Raubenheimer and Jones, 2006; Raubenheimer et al., 2007). By contrast, when restricted to imbalanced foods, the specialists are more prone to regulate their macronutrient intake in a way as to minimise the nutritional error relative to the intake target, exhibiting an arc-shaped intake array (the 'closest distance rule' of compromise). The nutritional heterogeneity hypothesis predicts that the tendency of the generalists to tolerate greater quantities of nutrient excesses is associated with their higher probability of encountering nutritionally

complementary foods, which will act as the nutritional antidotes for ameliorating the accrued nutritional imbalances (for further details, see Raubenheimer and Simpson, 1999, 2003; Lee et al., 2002, 2003, 2006; Simpson et al., 2002). *T. molitor* beetles are omnivorous scavengers that have adapted to high nutritional heterogeneity. Thus, they are expected to follow the nutrient balancing rule that is characteristic of the generalists. These results strongly agreed with the nutritional heterogeneity hypothesis, showing that the intake array of *T. molitor* beetles was apparently linear with negative slope, closely resembling that of generalist herbivores and omnivores. The slope of the intake array was close to 1 over the first 12 days post-eclosion, but became shallower as beetles on the high-protein diets ate more than those on the other diets during days 12–24, demonstrating the tendency of *T. molitor* beetles to prioritize the regulation of carbohydrate intake over that of protein intake. The slope of the intake array shallower than 1 may indicate that these omnivorous *T. molitor* beetles are highly capable of deaminating protein excesses and using the protein-derived carbon skeleton in energy metabolism to overcome carbohydrate limitation (Raubenheimer and Simpson, 2003; Thompson and Redak, 2000). Consistent with an earlier study from predatory beetles (Raubenheimer et al., 2007), the beetles in this experiment ingested more nutrients when given choice to self-select their preferred diet from two nutritionally complementary foods than when restricted to imbalanced single

foods. If nutrients were ingested in imbalanced quantities and ratios from one of the choice foods, beetles would be stimulated to eat the food nutritionally complementary to the most recently encountered food to redress the nutritional imbalances (Mayntz et al., 2005; Raubenheimer and Jones, 2006). Since the nutritional heterogeneity hypothesis predicts that the generalists have a propensity to over-eat when encountered with nutritionally imbalanced foods, continuously encountering the two nutritionally imbalanced but complementary foods would lead self-selecting beetles to eat more than those restricted to single foods. Previous studies from other insects have shown that males are more adept at laying down fats from ingested carbohydrates than females, reflecting their greater energy requirement to fuel their activities related to dispersal and courtship (Kolluru et al., 2004; Maklakov et al., 2008). However, the pattern was reversed for *T. molitor* beetles in this study, showing that females retained a higher level of body lipid reserves than males. Males in many species of beetles are expected to be metabolically more active than females, being predisposed to allocating more energy to activities like mate-searching and male-male fighting (Rogowitz and Chappell, 2000). Allocation of energy to these energetically costly premating activities is likely to divert energy away from storage in males, perhaps explaining why males were less efficient at retaining body lipid content than females. However, it remains to be elucidated whether males have higher resting and active metabolic rates than

females in *T. molitor*. The ability to withstand prolonged periods of acute food deprivation is determined primarily by the quantity of lipid reserves in insects (Rion and Kawecki, 2007; Lee and Jang, 2014). These data showed that females of *T. molitor* deposited more body fat and thus were more resistant to starvation than males. At low dietary P:C ratios, beetles may resist starvation well despite facing potential fitness costs associated with protein limitation. At high ratios, however, any benefits to be gained from eating sufficient protein is likely to be traded-off with the low probability of survival under food deprivation (Lee and Jang, 2014). This chapter provides the most comprehensive analysis of the nutritional regulatory responses of the two sexes of *T. molitor* beetles to a range of nutritional imbalances to date. These results agree with the idea that the heterogeneity of macronutrients encountered in the environment is a powerful factor shaping the behavioural and physiological mechanisms of nutrient regulation in insects (Raubenheimer and Simpson, 1999, 2003; Simpson et al., 2002). Given the importance of *T. molitor* beetle as a key model organism for studying both proximate and ultimate mechanisms of insect immunology (Siva-Jothy et al., 2005), demonstrating the detailed aspects of nutrition in this species will provide a solid platform for future studies addressing the interactions between nutrition and immune defence in the burgeoning field of nutritional immunology (Ponton et al., 2013).

CHAPTER 2.

Nutrient-specific food selection buffers the effect of nutritional imbalance in the mealworm beetle, *Tenebrio molitor*

Abstract

Ingesting nutritionally imbalanced food can cause a significant reduction in fitness in insects. Insects can avoid the negative consequences of nutritional imbalances by selectively foraging for nutritionally complementary foods. This chapter investigated the ability of the omnivorous beetle, *Tenebrio molitor* (Coleoptera: Tenebrionidae), to redress nutritional imbalances by selecting complementary foods. Beetles were fed one of three synthetic diets that varied in their protein: carbohydrate balance (p0:c42, p21:c21 or p42:c0) for 16 days and then allowed to select between two nutritionally imbalanced but complementary diets (p0:c42 vs. p42:c0) for 18 days. During the initial period, beetle survival was high on all three experimental diets, but their body composition was considerably skewed as a result of eating nutritionally imbalanced diets. Over the first 6 days of food choice (days 16–22), beetles previously fed a protein-rich, carbohydrate-deficient diet (p42:c0) preferred carbohydrate to protein, whereas those previously fed a carbohydrate-rich, protein-deficient diet (p0:c42) strongly preferred the protein-rich diet. When

the food choice period continued for longer than 6 days, the selection of diets by previously carbohydrate-deprived beetles (p42:c0) was similar to that of the control beetles previously fed an optimal food (p21:c21). However, beetles that were previously fed on the protein-deficient diet (p0:c42) selected protein and carbohydrate equally throughout the remaining period of food choice and the cumulative protein-carbohydrate intake of these protein-deprived beetles was similar to that of those fed the optimal diet (p21:c21). At the end of the experiment, the body composition of all beetles was similar, indicating that the effects of nutritional imbalance on body composition were buffered by the subsequent selection of complementary foods. This chapter results demonstrate that *T. molitor* beetles are capable of redressing nutritional imbalances and indicate that the way in which the nutritional balance of beetles is restored depends on the nutrient that is initially deficient in their food.

1. Introduction

Insects can maximize their Darwinian fitness when they acquire an optimal amount and blend of nutrients (Simpson et al., 2004; Lee et al., 2008; Jensen et al., 2012; Roeder and Behmer, 2014). Protein and carbohydrate are the two major macronutrients that are extensively used to investigate the effects of macronutrient balance on various aspects of physiology, behaviour and life-history performance in insects (Behmer, 2009; Simpson and Raubenheimer, 2012). When protein and carbohydrate are ingested in imbalanced quantities and ratios, insects suffer significant performance costs arising from ingesting not only too little of one nutrient (Mattson, 1980; White, 1993), but also too much of a nutrient that occurs in excess of their requirements (Simpson et al., 2004; Raubenheimer et al., 2005; Boersma and Elser, 2006; Zehnder and Hunter, 2009). For example, eating a diet containing excess protein relative to carbohydrate shortens lifespan in many insects (Lee et al., 2008, 2013; Fanson et al., 2012; Dussutour and Simpson, 2012).

The detrimental effects of ingesting nutritionally imbalanced food can be

ameliorated by insects using a variety of post-ingestive mechanisms (Zanotto et al., 1994, 1997; Raubenheimer and Bassil, 2007; Clissold et al., 2010), but probably the most effective way to correct nutritional imbalances is to forage for nutritionally complementary foods (Waldbauer and Friedman, 1991; Behmer, 2009; Simpson and Raubenheimer, 2012). Insects have a well-developed capacity to assess their current nutritional state and adjust their food preferences in order to counterbalance the nutritional imbalances accrued over a wide range of timescales (Simpson et al., 1988, 1990, 1991; Simmonds et al., 1992; Mayntz et al., 2005; Raubenheimer and Jones, 2006; Lee et al., 2012). The accuracy and efficiency with which insects restore their nutritional state are likely to depend on the extent and nature of the nutritional imbalance faced by insects, but little empirical work has been done to test this possibility in insects (but see Raubenheimer and Jones, 2006; Lee et al., 2012).

In this chapter, I investigate how protein:carbohydrate imbalances influence the pattern of complementary food selection in the mealworm beetle, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae). To accomplish this, I experimentally manipulated the nutritional state of *T. molitor* beetles by feeding them for 16 days on one of three foods that differed in terms of their protein and carbohydrate content and then determining the patterns in their selection of different nutritionally complementary diets over the next 18 days. During the pretreatment period, beetles were fed one of the following synthetic

diets with identical caloric contents: (1) protein-rich, carbohydrate-deficient diet, (2) equal protein: carbohydrate balanced diet or (3) carbohydrate-rich, protein-deficient diet. Recent work has shown that both male and female beetles of *T. molitor* are capable of regulating their protein and carbohydrate intake independently, resulting in an intake ratio of protein : carbohydrate close to 1 : 1 (Rho and Lee, 2014). This lead us to assume that a diet with an equal protein : carbohydrate (1 : 1) ratio is the optimal one for *T. molitor* beetles while the others are nutritionally imbalanced with one nutrient in excess and an insufficient quantity of the other. I hypothesized that beetles that were initially fed a diet devoid of a specific nutrient (protein or carbohydrate) would show a preference for a diet rich in the deficient nutrient. I also predicted that beetles would rely exclusively on controlling their food intake to compensate for protein-deficiency because nitrogen is only obtained by eating protein. When compensating for carbohydrate-deficiency, beetles are expected to be less reliant on their food intake because protein can be metabolized into carbohydrate by the process of gluconeogenesis (Thompson and Redak, 2000). To test these predictions, I examined the food preference patterns of beetles previously fed diets differing in the protein : carbohydrate balance. I also compared the body composition of beetles that were allowed to select between the diets with that of those that were fed only one of three diets in order to determine whether the nutritional balance of their bodies recovered as a result

of being able to select a particular diet.

2. Material and methods

2.1. Experimental foods

Dry, granular, chemically-defined synthetic diets were prepared based on the established protocol outlined by Simpson and Abisgold (1985). In total, I made three diets differing in protein (p) and digestible carbohydrate (c) content: 0% protein with 42% digestible carbohydrate (p0:c42), p21:c21 and p42:c0 (% dry mass). The total concentration of protein plus carbohydrate was fixed at 42% for all three diets. Since protein and carbohydrate are similar in caloric density (4 calories per gram), all three foods used in this study are near isocaloric. The protein component of all diets was a 3 : 1 : 1 mixture of casein, peptone and albumen, and sucrose was the digestible carbohydrate. All diets contained a fixed content of Wesson's salt (2.4%), cholesterol (0.5%), linoleic acid (0.5%), ascorbic acid (0.3%), a vitamin mix (0.2%) and indigestible cellulose (54%).

2.2. Experimental procedures

T. molitor were obtained from a stock culture maintained at Seoul National

University. During the larval stages, insects were kept in groups of 300-400 individuals per plastic container (40 × 18 × 8 cm^3 , L × W × H) where they had access to an ad libitum supply of wheat bran and water (water-filled plastic test tubes plugged with cotton wool) until pupation. Pupae were collected, sexed and allowed to complete their pupal development following the procedure described in Rho and Lee (2014).

On the first day of the experiment (day 0), a total of 120 newly emerged adults (60 males and 60 females) were weighed to the nearest 0.1 mg using a microbalance (Ohaus Co., Parsippany, NJ, USA), placed alone in experimental arenas (Petri dish diameter 90 mm) and then randomly divided into three experimental groups. Over the first 16 days post-eclosion (pretreatment period: days 0-16), beetles in each group were supplied with one of the three diets differing in protein : carbohydrate balance (p0:c42, p21:c21 and p42:c0). After experiencing this pretreatment, beetles were then provided with two dishes of nutritionally imbalanced but complementary diets (p0:c42 vs. p42:c0) for 18 days (choice period: days16-34). The upturned lids of 1.5 mL Eppendorf tubes (diameter 9 mm, depth 5 mm) were used as dishes for the dry, granular synthetic diet for the insects during the experiment. Before presenting them to insects, these dishes were filled with food, dried at 40°C for 24 h and weighed to the nearest 0.1 mg. Food and water (a water-filled 1.5 mL Eppendorf tube each capped with cotton wool) were replaced every other day. To minimize

any measurement error in food consumption, each dish was placed in a small Petri dish (diameter 40 mm) and any food spilled from the dish was put back in the dish before it was removed. Removed dishes containing uneaten food were dried to constant mass and weighed. Food consumption over each 2-day period was calculated as the difference between the dry mass of a dish initially given to insects and that of the same dish removed after two days of feeding. Food preference index was calculated for each beetle using the following formula:

$$\text{Preference index} = \frac{P - C}{P + C}$$

where P and C were the quantities of the p42:c0 and p0:c42 diets consumed, respectively. A positive value of this index thus indicates a preference for the p42:c0 diet and a negative value a preference for the p0:c42 diet. A value of zero indicates no preference. Protein and carbohydrate consumption were calculated as the product of food consumption and the concentration of each nutrient in the food. At the end of the experiment (day 34), beetles were frozen at -80°C and dried to constant weight at 50°C for 72 h. Dried carcasses were then weighed and lipid-extracted using three successive, 24 h chloroform washes (Simpson, 1982). Lipid-extracted carcasses were re-dried and then re-weighed, with total extractable lipid content being calculated as the difference

between the pre- and post-extraction dry mass. The proteineous body tissue of lean carcasses was digested and removed by soaking them in 0.35 M sodium hydroxide (NaOH) solution for three days at 33°C before being re-dried and re-weighed (Marden, 1987). Crude protein content was estimated by taking the difference between the dry mass of the sample before and after soaking them in sodium hydroxide. To measure the body composition of beetles at the end of the pretreatment period (day 16), I used another 120 newly eclosed adults (60 males and 60 females) that received the same pretreatment foods for 16 days, after which they were killed and subjected to body chemical composition analysis following the same protocol outline above. An earlier study indicated that there were no significant differences in dry body weight and body composition (protein and lipid content) between male and female beetles at adult emergence (day 0) (Rho and Lee, 2014). Throughout the whole experiment, all insects were kept in an incubator at 25°C under a 12L : 12D photoregime.

2.3. Data analysis

Both univariate and multivariate analysis of variance (ANOVA or MANOVA) were used to test the significance of the effects of pretreatment diet and sex on nutrient consumption and body composition. For the multivariate analysis, we used the Pillai's trace statistic because it is robust to

the violations of assumptions (Scheiner, 1993). Patterns of food preference in beetles that were offered two diets (p40:c0 vs. p0:c42) over three 6-day intervals were analyzed using a repeated measures ANOVA with sex and pretreatment diet as between-subject effects, and time (6-day intervals) and its interactions with sex and diet as within-subject effects. One-sample t-test was used to determine whether diet preference indices were significantly different from zero. Before the analyses, the data were checked for normality and heteroscedasticity using Kolmogorov-Smirnov and Bartlett's test, respectively. All data conformed to the assumptions of parametric tests. All statistical analyses were performed using SAS version 9.12 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Performance

All experimental beetles survived the experiment. However, 18 insects [four (males), four (one male and three females) and ten (four males and six females) individuals in the pretreatment groups p0:c42, p21:c21 and p42:c0, respectively] ate substantially less diet than the others and were not included in the analyses. There was no significant difference in the number of these beetles among the six sex × diet combinations (Analysis of deviance: $\chi 2 = 1.61$, df = 1,

$P = 0.204$). The dry body weight of beetles at the end of the experiment (day 34) was neither affected by experimental diet (ANOVA: $F_{2,96} = 0.08$, $P = 0.925$) nor by sex ($F_{1,96} = 0.14$, $P = 0.708$). Nor was there any interaction between diet and sex for body weight ($F_{2,96} = 1.19$, $P = 0.307$).

3.2. Food preference

Results from a repeated measures ANOVA indicated that the pattern in food preference was significantly affected by the P : C ratio of pretreatment diet, sex and time (three 6-day intervals; Table 1). A significant time × diet interaction indicated that the effect of the pretreatment diet on food preference changed significantly over time, but time × sex and time × sex × diet interactions were not significant (Table 1). When a repeated measures ANOVA with the pretreatment diet as the only between-subject effect was conducted separately for male and female beetles, time × diet interaction was significant for both male ($F_{4,104} = 9.35$, $P < 0.001$) and female beetles ($F_{4,88} = 6.07$, $P < 0.001$). Over the first 6 days of food choice (days 16-22), the pattern in diet preferences differed significantly among the three pretreatment groups (ANOVA, male: $F_{2,52} = 54.84$, $P < 0.001$; female: $F_{2,44} = 23.78$, $P < 0.001$; Fig.

1). During this period, both male and female beetles initially fed the carbohydrate-rich, protein-deficient diet (p0:c42) strongly preferred the protein-rich, carbohydrate-deficient diet (p42:c0) (positive signs; one-sample t tests, male: t_{19} = 7.80, P < 0.001; female: t_{15} = 2.29, P = 0.01), while the pattern was reversed for those initially fed the protein-rich, carbohydrate-deficient diet (p42:c0) (negative signs; male: t_{15}= -7.96, P < 0.001; female: t_{13}= -9.41, P < 0.001; Fig. 1). Beetle initially fed the equally balanced diet (p21:c21) preferred the carbohydrate-rich diet (p0:c42) to the protein-rich diet (p42:c0) (negative signs; male: t_{18}= -2.62, P = 0.017; female: t_{16}= -2.84, P = 0.012) over the first 6 days (days 16-22; Fig. 1), but the extent to which the former was preferred was less pronounced than for those that were initially fed the protein-rich, carbohydrate-deficient diet (p42:c0). Over the next two 6-day periods (days 22-28 and 28-34), the initial differences in the preferences among the three experimental groups were greatly reduced and eventually they became statistically indistinguishable from one another (days 28-34; ANOVA, male: $F_{2,52}$ = 3.16, P = 0.051; female: $F_{2,44}$ = 1.22, P = 0.305; Fig. 1). Preference indices recorded in these two 6-day periods were significantly negative for both male and female beetles initially fed the protein-rich, carbohydrate-deficient diet (p42:c0) and for female beetles initially fed the equally balanced diet (p21:c21), indicating a preference for the carbohydrate-rich, protein-deficient diet (p0:c42; all P values generated from one-sample t tests being

lower than 0.014). For both male and female beetles initially fed the carbohydrate-rich, protein-deficient diet (p0:c42), however, preference indices were not significantly different from zero (male: $t_{19} = 0.05$, $P = 0.959$; female: $t_{15} = -1.18$, $P = 0.258$), indicating that they did not show a preference for a specific food.

Table 1. Results of repeated measures ANOVA of the food preference indices of *T. molitor* beetles (male and female; sex) that were given a choice between two nutritionally complementary diets (p42:c0 vs. p0:c42) over three successive 6-day periods (days 16–22, 22–28 and 28–34; time) after initially being fed on one of three diets (p0:c42, p21:c21 or p42:c0; diet) for 16 days.

Source	df	Mean Square	*F*	*P*
Between subject effects				
Sex	1	0.78662	9.74	0.002
Diet	2	2.96271	36.69	<0.001
Sex × Diet	2	0.00652	0.08	0.923
Error	96	0.08076		
Within subject effects				
Time	2	0.44510	9.66	<0.001

Time × Sex	2	0.06897	1.50	0.227
Time × Diet	4	0.66104	14.35	<0.001
Time × Sex × Diet	4	0.03229	0.70	0.581
Error	192	0.04605		

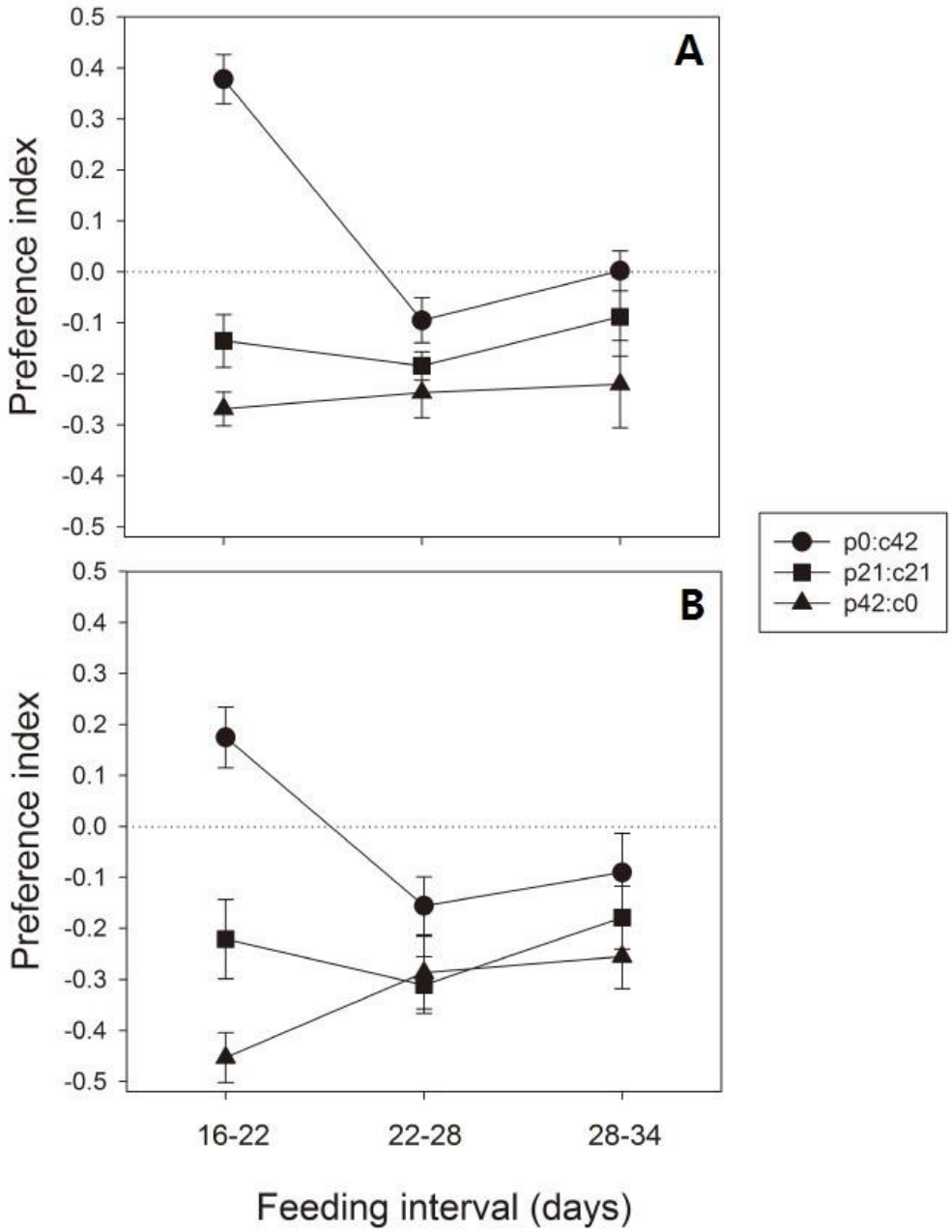

Figure 1. Trends recorded in the food preference indices of (A) male and (B) female *Tenebrio molitor* beetles allowed to select between two nutritionally complementary diets (p42:c0 vs. p0:c42) during three successive 6-day periods (days 16–22, 22–28 and 28–34) after initially being fed one of three diets (p0:c42, p21:c21 or p42:c0) for 16 days. Positive and negative indices indicate preferences for the p42:c0 and p0:c42 diet, respectively.

3.3. Macronutrient consumption

The cumulative patterns in protein-carbohydrate consumption are described separately for male and female beetles in Fig. 2 over the entire experimental period (days 0-34), including those for both the no-choice (days 0-16) and choice feeding (days 16-34) trials. During the no-choice period (days 0-16), males consumed significantly more nutrients than females on all three diets (ANOVA: $F_{1,96}$= 16.16, $P < 0.001$; Fig. 2). For both sexes, the total amount of macronutrients consumed over the no-choice period increased with increase in the P : C ratio of the no-choice diet ($F_{2,96} = 7.53$, $P < 0.001$; Fig. 2). There was no significant diet × sex interaction ($F_{2,96} = 0.05$, $P = 0.955$), indicating that both sexes followed a similar pattern of macronutrient consumption when each was provided with only one of the three diets. Results of a MANOVA and its post-hoc contrasts confirmed that the bivariate pattern of cumulative protein-carbohydrate intake over the no-choice period (days 0-16) differed significantly among the three experimental groups for both sexes (Table 2). When the trajectories of cumulative protein-carbohydrate intake moving through nutrient space over the food choice period (days 16-34) were

compared among the three groups (Fig. 2), the intake trajectory of beetles that were initially fed the protein-rich, carbohydrate-deficient diet (42:c0) almost paralleled that of those that were initially fed the equally balanced diet (p21:c21) in both sexes. However, this chapter also found that the intake trajectory of beetles initially fed the carbohydrate-rich, protein-deficient diet (p0:c42) had a much shallower slope than the trajectories of those fed the other two diets (p21:c21 and p42:c0 pretreatment groups) especially during the first 6 days of food choice (days 16- 22; Fig. 2). For both sexes, results of the MANOVA contrasts revealed that the cumulative protein-carbohydrate intake point of beetles initially fed the protein-deficient diet (p0:c42) and the equally balanced diet (p21:c21) converged and became statistically indistinguishable when measured at the end of the food choice experiment (day 34; Table 2; Fig. 2). Cumulative macronutrient intake of beetles initially fed on the protein-rich, carbohydrate-deficient diet (p42:c0) differed significantly from that of those initially fed on the other diets (Table 2; Fig. 2). While the cumulative protein intake of both male and female beetles on day 34 post-eclosion differed significantly among the three experimental groups (ANOVA, male: $F_{2,52}$ = 7.70, P = 0.001; female: $F_{2,44}$ = 7.44, P = 0.001), their final carbohydrate intake was statistically indistinguishable (male: $F_{2,52}$ = 2.44, P = 0.097; female: $F_{2,44}$ = 0.66, P = 0.521).

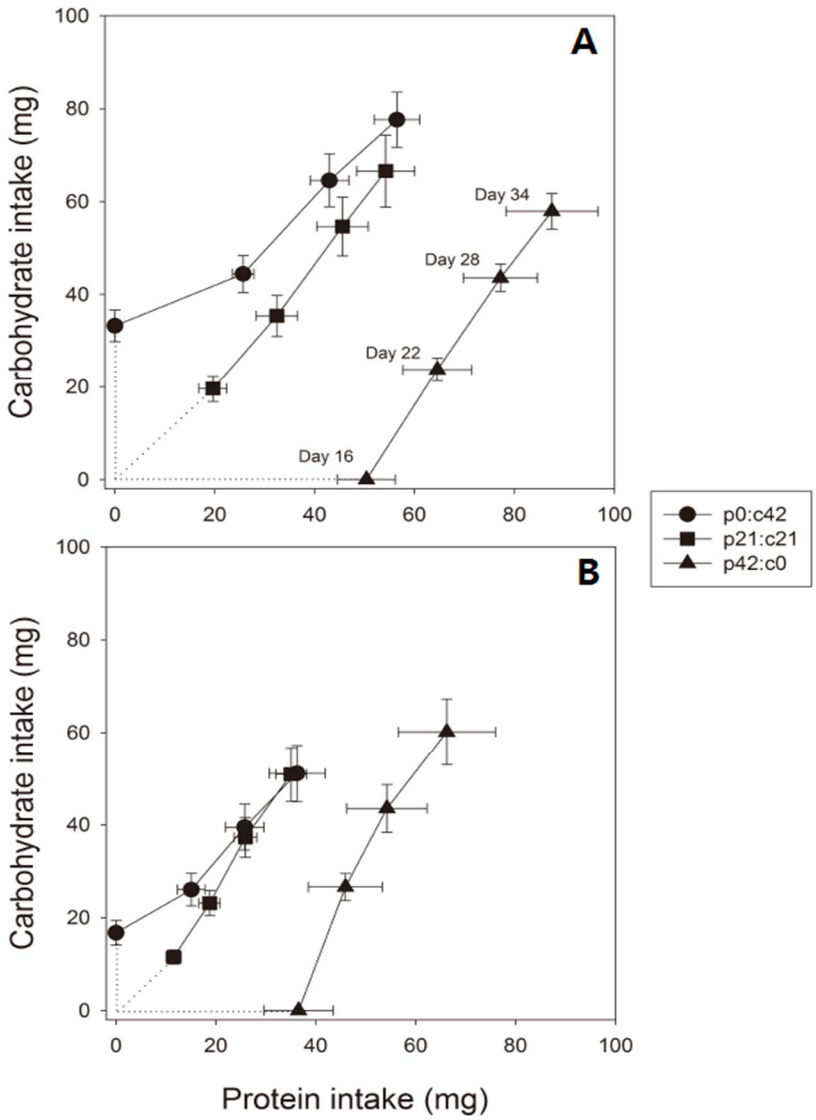

Figure 2. Means (+SE) of cumulative protein and carbohydrate eaten by (A)

male and (B) female *Tenebrio molitor* beetles allowed to select between two nutritionally complementary diets (p42:c0 vs. p0:c42) during three successive 6-day periods (days 16–22, 22–28 and 28–34) after initially being fed one of three diets (p0:c42, p21:c21 or p42:c0) for 16 days. Dotted and solid lines radiating from the origin represent the trajectories of cumulative protein-carbohydrate intake during the first 16 days of the no-choice feeding (days 0–16) and the three, 6-day periods (days 16–22, 22–28 and 28–34) of choice feeding, respectively. For each diet, four symbols starting from left to right indicate the cumulative nutrient intake on days 16, 22, 28 and 34, respectively.

Table 2. Results recorded for the MANOVAs of the protein-carbohydrate intake by (A) male and (B) female *T. molitor* beetles during the no-choice feeding period (days 0–16) and the entire experimental period (days 0–34). Results of multivariate contrasts between two diet groups (p0:c42 vs. p21:c21, p0:c42 vs. p42:c0 and p21:c21 vs. p42:c0) are reported below the MANOVA result for each sex.

Source	Days 0-16				Days 0-34			
	df	Pillai's trace	*F*	*P*	df	Pillai's trace	*F*	*P*
Male								
Diet	**4,104**	**0.83491**	**18.63**	**<0.001**	**4,104**	**0.58702**	**10.80**	**<0.001**
Contrasts								
p0:c42 vs. p21:c21	**2,51**	**0.46659**	**22.31**	**<0.001**	**2,51**	**0.05114**	**1.37**	
p0:c42 vs. p42:c0	**2,51**	**0.83409**	**128.19**	**<0.001**	**2,51**	**0.54272**	**30.26**	**<0.001**
p21:c21 vs. p42:c0	**2,51**	**0.64009**	**45.35**	**<0.001**	**2,51**	**0.44936**	**20.81**	**<0.001**
Female								
Diet	**4,88**	**0.70413**	**11.95**	**<0.001**	**4,88**	**0.32793**	**4.31**	**0.003**
Contrasts								
p0:c42 vs. p21:c21	**2,43**	**0.20051**	**5.39**	**0.008**	**2,43**	**0.00075**	**0.02**	**0.984**
p0:c42 vs. p42:c0	**2,43**	**0.69715**	**49.49**	**<0.001**	**2,43**	**0.26148**	**7.61**	**0.002**
p21:c21 vs. p42:c0	**2,43**	**0.52682**	**23.94**	**<0.001**	**2,43**	**0.28385**	**8.52**	**<0.001**

3.4. Body composition

The bivariate pattern of body protein and lipid content at the end of the initial no-choice period (day 16) differed significantly depending on the P : C ratio of the diet they fed on (MANOVA, male: Pillai's trace = 0.78971, $F_{4,102}$ = 16.64, $P < 0.001$; female: Pillai's trace = 0.75618, $F_{4,106} = 16.11$, $P < 0.001$; Fig. 3). Body lipid content of beetles on day 16 post-eclosion increased significantly with the carbohydrate content of the food they fed on (ANOVA, male: $F_{2,51} = 24.24$, $P < 0.001$; female: $F_{2,53} = 13.90$, $P < 0.001$). Body protein content on day 16 post-eclosion also varied significantly among the three pretreatment groups (male: $F_{2,51} = 5.62$, P = 0.006; female: $F_{2,53} = 8.84$, $P <$ 0.001). When the body composition was measured at the end of the food choice period (day 34), the bivariate pattern of body nutrient composition of the three experimental groups converged and became statistically indistinguishable from one another (MANOVA, male: Pillai's trace = 0.06691, $F_{4,104} = 0.90$, $P = 0.467$; female: Pillai's trace = 0.04491, $F_{4,88} = 0.51$, $P =$ 0.732; Fig. 3). The protein contents of the two sexes on day 34 post-eclosion were similar ($F_{1,96} = 0.24$, $P = 0.625$), but females had a significantly greater body lipid content than males (ANOVA: $F_{1,96} = 5.36$, $P = 0.023$), causing the bivariate body composition to be significantly different between the two sexes (MANOVA: Pillai's trace = 0.07182, $F_{2,95} = 3.68$, $P = 0.029$). MANOVA results indicated that there was neither a significant effect due to the P : C ratio

of the diet they were initially fed on (Pillai's trace = 0.02012, $F_{4,192}$ = 0.49, P = 0.745) nor a sex × diet interaction (Pillai's trace = 0.03888, $F_{4,192}$= 0.95, P = 0.4353) for body composition measured on day 34 post-ecolsion.

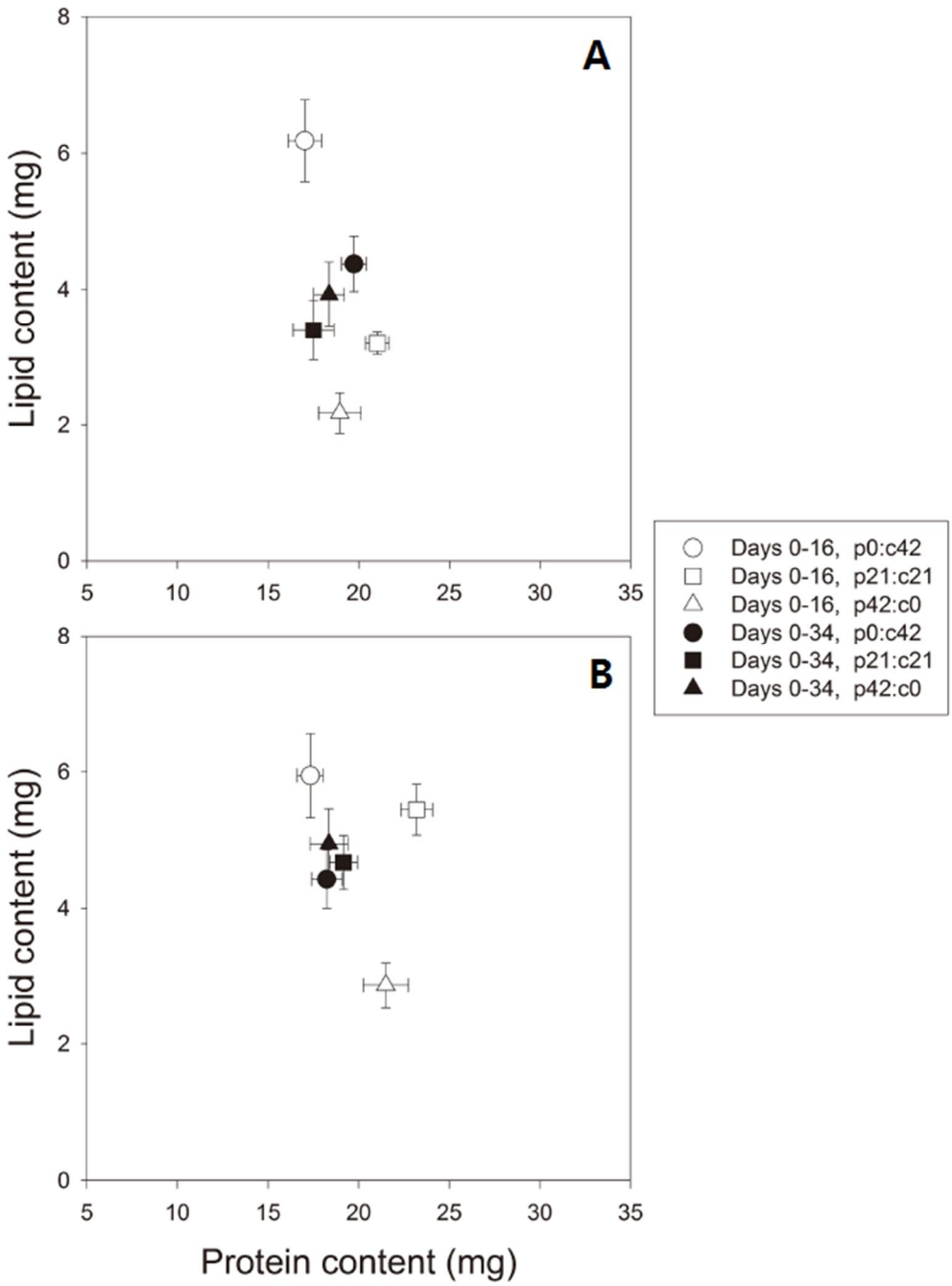

Figure 3. Bivariate means (±SE) of body lipid and protein content of (A) male and (B) female *Tenebrio molitor* beetles in the three pretreatment groups (p0:c42, p21:c21 or p42: c0) measured at the end of the no-choice (days 0–16; open symbols) and choice feeding periods (days 0–34; closed symbols).

4. Discussion

This chapter has previously shown that *T. molitor* beetles have the capacity to balance their intake of protein and carbohydrate by feeding on nutritionally complementary diets (Rho and Lee, 2014). In this chapter, I determined whether and how *T. molitor* beetles can recover from nutritional imbalances caused by ingesting diets that are deficient in one nutrient and contain an excess of another by compensatory food selection. To do so, I first subjected the experimental beetles to severe nutritional imbalances by feeding them diets that contained only protein or carbohydrate for 16 days during their early adulthood. Eating a food that is devoid of protein is likely to limit the ability of insects to repair existing proteins and to synthesize new ones while ingesting a food containing only protein is known to result in slow juvenile development, small body size and high mortality in many insects (Raubenheimer and Simpson, 2003; Simpson et al., 2004; Lee, 2010). However, all beetles in this chapter survived the initial treatment, which indicates that *T. molitor* beetles are capable of tolerating extreme nutritional imbalances for a prolonged period in their early adult life. Physiological processes enabling *T. molitor* beetles to withstand such extreme nutritional imbalances remain to be addressed, but may be linked to their ability to feed on a variety of nutritionally extreme plant- and animal-derived materials (Ramos-Elorduy et al., 2002). For example, it is likely that insects alleviate the extreme carbohydrate shortage resulting from

eating a protein-rich, carbohydrate-deficient food (p42:c0) by deaminating extra protein and using protein-derived carbon skeletons for energy (Thompson and Redak, 2000; Wilkinson et al., 2001; Raubenheimer and Simpson, 2003). Previous studies have shown that generalist-feeding insects, such as generalist herbivores and omnivores, tend to ingest a greater excess of protein than specialists because the former are more likely to utilize protein as an alternative source of carbohydrate than the latter (Lee et al., 2002, 2003; Simpson et al., 2002; Raubenheimer and Simpson, 2003). Similarly, these data indicated that *T. molitor* beetles fed a protein-rich diet (p42:c0) ate more than those fed a carbohydrate-rich diet (p0:c42) during the no-choice period, perhaps indicating that these omnivorous scavengers are also capable of using protein-derived carbon in energy metabolism (Rho and Lee, 2014).

These results indicate that *T. molitor* beetles are capable of redressing a long-term nutritional imbalance by adjusting their food preferences. When allowed to select between two nutritionally complementary diets (p42:c0 and p0:c42), beetles initially fed one of the two nutritionally imbalanced diets (p42:c0 or p0:c42) strongly preferred diets with the specific nutrient that was initially deficient in their diet. Male and female beetles that were fed on the optimal P : C diet (p21:c21) during the no-choice feeding period self-composed a slightly carbohydrate-biased ratio (P : C = 1 : 1.23 and 1 : 1.61 for males and females, respectively), which is close to the target intake ratio previously

reported for this species (Ponton et al., 2010). Restoring a balanced nutritional state by complementary food selection is recorded for predatory beetles, cockroaches, grasshoppers and caterpillars that have experienced severe nutritional imbalances over a wide range of timescales (Simpson et al., 1988, 1990, 1991; Simmonds et al., 1992; Mayntz et al., 2005; Raubenheimer and Jones, 2006; Lee et al., 2012), but there are not aware of any study on the ability of insects to redress the extreme nutritional imbalances accrued over 16 days. Specific foraging for deficient nutrients is likely to be controlled by blood-borne nutritional feedbacks that modulate the phagostimulatory responsiveness of peripheral taste receptors (Simpson et al., 1991; Simpson and Raubenheimer, 1993). For example, when an insect is deprived of carbohydrate but satiated with protein, sugar levels in the blood fall while the concentrations of circulating free amino acids rise, causing the gustatory receptors to be more sensitive to carbohydrate and less sensitive to protein. Another possible mechanism facilitating nutrient rebalancing is learning (Simpson and White, 1990).

An interesting aspect of these data is that the way in which beetles reinstated their nutritional balance by complementary food selection differed substantially depending on which specific nutrient was deficient in the pretreatment diet. Beetles initially fed on the synthetic diet containing only protein (p42:c0) strongly preferred the carbohydrate-rich diet during the first 6

days of food choice (days 16-22), but the extent to which this carbohydrate diet was preferred by them became less pronounced thereafter, with the pattern of food selection becoming closely similar to that of the control beetles that were initially fed the balanced diet (p21:c21) during the remainder of the food choice period (days 22-34). This indicates that self-selecting *T. molitor* beetles recovered from carbohydrate deficiency within 6 days by selecting the complementary diet. Similar compensatory feeding was recorded for beetles initially fed the protein-deficient diet (p0:c42). As expected, these protein-deprived beetles strongly preferred the protein-rich diet during the first 6 days of food selection and this preference decreased as the food choice continued beyond day 6. The cumulative protein-carbohydrate intake of the beetles initially fed on the protein-deficient diet (p0:c42) followed a trajectory with a shallower slope compared to that of those fed on the other initial diets and gradually converged to that of those initially fed on the balanced diet (p21:c21) during the course of the 18 days of choice feeding. By contrast, the cumulative macronutrient intake of the beetles that were initially deprived of carbohydrate (p42:c0) ran almost parallel to that of the control beetles (p21:c21). For carbohydrate-deprived beetles, the severe limitation in their source of energy experienced during the 16 days of the no-choice diet period is likely to be assuaged by using protein-derived carbon skeletons for energy metabolism (Raunbenheimer and Simpson, 2003), thereby reducing the amount of

carbohydrate that needs to be ingested for recouping the carbohydrate deficiency. In line with this prediction, I found that the behavioural compensation for carbohydrate-deficiency only lasted for the first 6 days of food choice. Since carbohydrate cannot substitute for protein, beetles initially fed on the protein-deficient diet (p0:c42) are expected to rely exclusively on the diet and hence may take longer to compensate for protein deficiency. The results presented in this study are largely consistent with the patterns of food preference previously recorded for *Spodoptera litura* caterpillars, which experienced extreme nutritional imbalances early in life (Lee et al., 2012).

The macronutrient balance of the pretreatment diet significantly influenced the body composition of beetles, but the composition of three pretreatment groups became statistically indistinguishable in nutrient space after 18 days of complementary food selection. This indicates that the effects of the nutritional imbalance on body composition were buffered by compensatory food mixing. A previous study on a predatory beetle (*Agonum dorsale*) indicates that the lipid content of self-selecting beetles increases rapidly from 14% to 46% over the first 48 h after emerging from winter diapause (Raubenheimer et al., 2007). In addition to complementary food selection, beetles might use a variety of post-ingestive regulatory mechanisms to cope with imbalanced nutrient intake, including the nutrientspecific modulation of the activity of digestive enzymes, physical restructuring of the alimentary canal and differential utilization of

absorbed nutrients (Zanotto et al., 1994, 1997; Clissold et al., 2010; Raubenheimer and Bassil, 2007). Despite having eaten significantly more protein than those initially fed on the balanced (p21:c21) and carbohydraterich, protein-deficient diets (p0:c42), beetles that were initially fed on the protein-rich, carbohydrate-deficient diet (p42:c0) had a similar level of body protein content to those initially fed on the other diets, raising the possibility that these post-ingestive mechanisms for balancing their nutrient intake might have played an important role in regulating body protein content.

In conclusion, these results indicate that the beetle *T. molitor* is very capable of redressing the nutritional imbalance accrued over a long timescale by plastically adjusting their food consumption. Beetles fully compensated for their protein-deficiency by food selection and their potential ability to use protein as a source of both nitrogen and energy might have enabled them to overcome the state of carbohydrate-deficiency not only behaviourally but also physiologically. These results have implications for the nutrient balancing and foraging behaviour of this omnivorous beetle.

CHAPTER 3.

Balanced intake of protein and carbohydrate maximizes lifetime reproductive success in the mealworm beetle, *Tenebrio molitor*

Abstract

Recent developments in insect gerontological and nutritional research have suggested that the dietary protein:carbohydrate (P:C) balance is a critical determinant of lifespan and reproduction in many insects. However, most studies investigating this important role of dietary P:C balance have been conducted using dipteran and orthopteran species. In this study, I used the mealworm beetles, *Tenebrio molitor L.* (Coleoptera: Tenebrionidae), to test the effects of dietary P:C balance on lifespan and reproduction. Regardless of their reproductive status, both male and female beetles had the shortest lifespan at the protein-biased ratio of P:C 5:1. Mean lifespan was the longest at P:C 1:1 for males and at both P:C 1:1 and 1:5 for females. Mating significantly curtailed the lifespan of both males and females, indicating the survival cost of mating. Age-specific egg laying was significantly higher at P:C 1:1 than at the two imbalanced P:C ratios (1:5 or 5:1) at any given age throughout their lives, resulting in the highest lifetime reproductive success at P:C 1:1. When given a choice, beetles actively regulated their intake of protein and carbohydrate to a slightly carbohydrate-biased ratio (P:C 1:1.54–1:1.64 for males and P:C 1:1.3–1:1.36 for females). The self-selected P:C ratio was significantly higher for females than males, reflecting a higher protein requirement for egg production.

Collectively, these results add to a growing body of evidence suggesting the key role played by dietary macronutrient balance in shaping lifespan and reproduction in insects.

1. Introduction

Food can have a profound impact on lifespan, with one prominent example being the life-extending effects of dietary restriction (DR) as reported from taxonomically disparate organisms from yeast to primates (Weindruch and Walford, 1988; Mair and Dillin, 2008; Colman et al., 2009; Fontana et al., 2010). The lifespan-extension by DR has been traditionally attributed to the restricted intake of calories, but there is a growing body of evidence suggesting that the balance of specific nutrients ingested is the most critical determinant of lifespan (Lee et al., 2008; Simpson and Raubenheimer, 2009; Piper et al., 2011; Tatar, 2011; Nakagawa et al., 2012; Simpson et al., 2015). For example, eating a food high in protein but low in carbohydrate has been shown to shorten lifespan in *Drosophila melanogaster* (Mair et al., 2005; Min and Tatar, 2006; Lee et al., 2008; Bruce et al., 2013; Lee, 2015; Jensen et al., 2015), tephritid flies (Fanson et al., 2009, 2012; Fanson and Taylor, 2012), crickets (Maklakov et al., 2008; Harrison et al., 2014), ants (Dussutour and Simpson, 2012), honeybees (Paoli et al., 2014), and even mice (Solon-Biet et al., 2014), indicating that the negative impact of high protein intake on lifespan is underpinned by a mechanism highly conversed across species (Tatar et al.,

2014; Mirzaei et al., 2014; Le Couteur et al., 2015).

Fecundity is another important fitness-related trait that is strongly influenced by the macronutrient balance of the food. In female insects, egg production rate has been shown to rise in response to a moderate increase in dietary protein:carbohydrate (P:C) ratio (Lee et al., 2008; Maklakov et al., 2008; Fanson et al., 2009; Lee, 2015; Jensen et al., 2015). More recent studies have also demonstrated that dietary P:C ratio has a major impact on traits linked to male fecundity (Reddiex et al., 2013; Sentinella et al., 2013; Jensen et al., 2015; Bunning et al., 2015; Rapkin et al., 2016; Morimoto and Wigby, 2016). High fecundity has been shown to be associated with reduced lifespan in a wide range of species in insects, a phenomenon known as the survival cost of reproduction (reviewed in Harshman and Zera, 2006; Flatt, 2011). Traditionally, a competitive allocation of resources between reproduction and somatic maintenance has been proposed as the prevailing explanation for this antagonism between these two traits, but empirical evidence substantiating this resource allocation trade-off still remains limited (O'Brien et al., 2008; Flatt, 2011). Recent evidence has suggested an alternative possibility that the negative relationship between lifespan and reproduction arises because the P:C ratio that maximizes reproduction differs from the one that maximizes lifespan (Lee et al., 2008). This divergence of nutritional optima for lifespan and reproduction has been demonstrated from *D. melanogaster* (Lee et al., 2008;

Lee, 2015; Jensen et al., 2015), tephritid flies (Fanson et al., 2009; Fanson and Taylor, 2012), and crickets (Maklakov et al., 2008; Harrison et al., 2014). However, it remains to be seen whether this divergent pattern is more widely distributed in insects other than dipterans and orthopterans.

Mealworm beetle, *Tenebrio molitor L.* (Coleoptera: Tenebrionidae), is a cosmopolitan pest scavenging on a variety of post-harvest grains. Since it is easy to rear in the laboratory, this species has become an important model organism to study behaviour, physiology, biochemistry, and immunology in insects. In recent years, there has been a growing interest in the commercial use of *T. molitor* larvae as a cost effective protein source for captive animals (Klasing et al., 2000; Ng et al., 2001; Ramos-Elorduy et al., 2002) and for humans (Aguilar-Miranda et al., 2002; Ghaly and Alkoaik, 2009; Siemianowska et al., 2013). Despite its emerging importance in both basic and applied entomological research, few studies have examined how long *T. molitor* lives and how many offspring it produces throughout its life (Morales-Ramos et al., 2012).

This chapter aims at determining the effects of dietary P:C ratio on lifespan and female fecundity (e.g., egg laying) in adult *T. molitor*. Since the consumption of high P:C food is commonly associated with shortened lifespan and elevated egg production rate in many female insects (e.g., Lee et al., 2008; Maklakov et al., 2008; Fanson et al., 2009), this chapter is particularly

interested in addressing whether such reduced lifespan on protein-rich foods is driven by a competitive resource allocation trade-off between lifespan and reproduction or by the detrimental effect of excessive protein intake. The nutritional costs of reproduction are assumed to be much higher in females than in males because eggs are more expensive to produce than sperms (Bonduriansky et al., 2008). Within females, mated females are expected to invest a substantially greater amount of resource in reproduction than unmated ones because mating stimulates egg production in many income-breeding insects, including *T. molitor* (Worden and Parker, 2001; Drnevich et al., 2001). Based on these assumptions, if shortened lifespan on high P:C food is primarily due to the reallocation of resources from somatic maintenance to reproduction, I predict that mated females will suffer a greater reduction in lifespan than males and unmated females upon consuming high P:C food. On the other hand, if the reduction of lifespan by eating a high P:C food is due to a mechanism completely independent of reproductive investment (e.g., protein toxicity), I predict that the extent to which lifespan is shortened by excessive protein intake will be similar across sex and mating status. To test these predictions, I employed a fully factorial experiment in which lifespan was measured from the mated and unmated beetles of both sexes feeding ad libitum on one of three synthetic foods differing in P:C ratio (P:C = 1:5, 1:1 or 5:1). From each mated female beetle, I quantified not only the total number of eggs laid throughout its

life (i.e., lifetime fecundity), but also the age-specific egg laying at each 7-day age interval from adult emergence to death. The latter was measured to investigate the effect of dietary P:C ratio on reproductive ageing in *T. molitor*, an aspect that has been rarely investigated in insects (but see Maklakov et al., 2009; Jensen et al., 2015).

Evolutionary theory predicts that animals forage optimally to maximize their fitness by balancing the intake of multiple nutrients (Simpson et al., 2004; Simpson and Raubenheimer, 2012) and this prediction was supported by a number of empirical studies demonstrating a close correspondence between the regulated macronutrient preference and the maximal expression of several fitness proxies in caterpillars (Simpson et al., 2004), *D. melanogaster* (Lee et al., 2008), and predatory beetles (Jensen et al., 2012). Having identified the nutritional optima for lifespan and fecundity for *T. molitor*, I then carried out an additional food choice assay to establish whether *T. molitor* had the capacity to balance the intake of protein and carbohydrate to maximize their Darwinian fitness. I offered the mated and unmated beetles of both sexes a choice between two nutritionally complementary foods and then analyzed the amount and proportion of protein and carbohydrate selected by them using a state-space modelling paradigm, the Geometric Framework for nutrition (Simpson and Raubenheimer, 2012). This experimental design allowed us to examine the effect of sex and mating status on macronutrient selection in *T. molitor*.

2. Materials and methods

2.1. Experimental insects

The beetles used in this study were obtained from a laboratory culture maintained at Seoul National University. During the pre-experimental period, larvae were reared in groups of 300–400 individuals in a clear plastic container ($40 \times 18 \times 8$ cm^3, L × W × H) where they had ad libitum access to wheat bran. A water-filled plastic tissue culture flask stoppered with cotton provided a water source. Newly molted pupae were collected from the culture, sexed, and placed in a 9 cm Petri dish until adult ecdysis. All insect rearing and experiments were carried out at 25 °C under a 12 h:12 h light:dark photoregime.

2.2. Synthetic foods

Following the protocol by Simpson and Abisgold (1985), we prepared three, synthetic foods that contained the same amount of calories but varied in the ratio of protein to digestible carbohydrate as follows: 7% protein with 35% digestible carbohydrate (p7:c35), p21:c21, and p35:c7 (% dry mass). Protein

consisted of a 3:1:1 mixture of casein, peptone, and albumen whilst digestible carbohydrate contained sucrose. Other constituents included 2.4% Wesson's salt, 0.5% cholesterol, 0.5% linoleic acid, 0.3% ascorbic acid, and 0.2% vitamin mix. The remainder of the food (ca. 54%) was filled with indigestible cellulose powder.

2.3. Experiment 1: lifespan and reproduction

To investigate the effects of dietary P:C balance, sex, and mating status on lifespan and fecundity, I employed a 2 × 2 × 3 factorial experimental design where sex (male and female), mating status (mated and unmated), and food treatment (p7:c35, p21:c21, and p35:c7) were fully crossed to yield a total of 12 treatments. Upon molting to adults (day 0), a total of 240 beetles (120 males and 120 females) were weighed to the nearest 0.1 mg using a microbalance (Ohaus Co., Parsippany, NJ, USA) and haphazardly allocated to one of three no-choice food treatments. Each insect was housed individually in a 9 cm Petri dish (feeding arena) containing a food dish [the upturned lids of 1.5 mL Eppendorf tube (9 mm diameter, 5 mm depth)] and a water-filled 1.5 mL Eppendorf tube capped with a cotton plug. Sufficient food was provided throughout the experimental period and beetles were moved to new feeding arenas every two days. When beetles were 8 days old (the age of sexual maturation; Happ, 1970), both males and females were split into two groups:

the mating and non-mating group. In the mating group, one male was randomly paired with one female from the same food treatment and each pair was placed in an empty 9 cm Petri dish for 24 h to allow mating to occur. The successful copulation of each pair was confirmed when male mounted and remained attached to female by its genitalia for 1–2 min (Font and Desfilis, 2003). All mating pairs successfully completed their copulation within 30 min. Although beetles in the mating group mated multiple times over 24 h, I did not count the total number of mating by each pair. In the non-mating group, two same-sex beetles from the same food treatment were paired and kept together in an empty 9 cm Petri dish for 24 h. In order to identify individuals during this pairing period, one individual in each pair was marked with a small droplet of white correction fluid (Worden and Parker, 2001). When this inter- or intra-sexual pairing period (24 h) was over, pairs were separated and each insect was put back to its own feeding arena and allowed to feed for 6 days. From day 8 until the end of the experiment when all beetles died, the pairing procedure was repeated once a week. Throughout their adult lives, beetles were paired with the same individuals. In pairs in which one individual died earlier than its partner, the surviving partner was newly paired with a same-aged individual that had been maintained on the same synthetic food. The number of eggs laid by each mated female was counted every two days until the end of the experiment. For each mated female, lifetime female fecundity was determined

as the total number of eggs produced throughout its adult life and egg production rate was computed as lifetime fecundity divided by the number of days between day 8 and the day the female died. The number of eggs summed over 6 days between two successive 24-h pairing rounds was used as the measure of age-specific egg laying (week based). Consistent with earlier studies (Worden and Parker, 2001; Drnevich et al., 2001), virgin females rarely laid eggs.

2.4. Experiment 2: nutrient self-selection

To examine the effects of mating status and sex on macronutrient self-selection, I set up a 2 × 2 factorial experimental design, with sex (male and female) and mating status (mated and unmated) being the two main factors. The combinations of sex and mating status resulted in a total of 4 treatments. Freshly emerged adults (30 males and 30 females) were weighed to the nearest 0.1 mg and individually housed in their own feeding arenas containing two nutritionally complementary foods: one with five times more protein than carbohydrate (p35:c7) and the other with five times more carbohydrate than protein (p7:c35). Eight days after adult emergence, beetles were randomly divided into the mating and non-mating group and then paired with individuals of the same or opposite sex for 24 h following the protocol described above. When this first round of pairing was over, each insect was put back to its own

feeding arena and allowed to feed for 6 days (days 9–15). The second round of pairing took place on day 15, with each insect being paired with the same partner once again for 24 h. After the completion of the second pairing, insects were returned to their feeding arenas and continued to feed for another 6 days (days 16–22). During two 6-day feeding periods (days 9–15 and 16–22), each insect was provided with two pre-weighed food dishes that were replaced every two days. Old dishes that contained foods remaining uneaten were dried to constant mass at 40 °C for 48 h before being weighed to the nearest 0.1 mg. Food intake was determined as the mass difference between the food dish provided and that removed after two days of feeding, with the intake of protein and carbohydrate being calculated by multiplying food intake by the known concentrations (%) of these nutrients in the food. Throughout the experiment, beetles were provided with unlimited access to water as described above.

2.5. Data analysis

Twelve insects (10 and 2 from Experiment 1 and 2, respectively) that died within 10 days since adult emergence were discarded from the analyses because they failed to adapt to the experimental condition. Both univariate and multivariate analysis of variance (ANOVA or MANOVA using PROC GLM in SAS) were conducted to assess the significance of the main effects (sex, mating status, or food treatment) on nutrient intake, lifespan, and fecundity

(e.g., lifetime fecundity and egg production rate). When performing multivariate analysis, I adopted Pillai's trace statistic because it was considered the most robust to the violations of assumptions (Scheiner, 1993). Prior to these parametric analyses, the data were inspected for the conformity to the underlying assumptions of these parametric statistical tests (the normality and homoscedasticity of the data). The data strongly deviating from these assumptions were log-transformed (e.g., egg production rate). The longitudinal pattern of age-specific egg laying for individual beetles was analyzed using a repeated measures ANOVA using PROC MIXED, with age and food treatment being the fixed effects and female identity the random effect. I specified the Toeplitz covariance structure for the random effect because it generated the lowest Akaike's information criterion (AIC) compared to other structures (Little et al., 1996). Non-parametric LOWESS (locally weighted scatterplot smoothing using PROC LOWESS) was applied to illustrate the pattern of age-specific egg laying in mated females on each P:C food. All statistical analyses were performed using SAS version 9.12 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Experiment 1: lifespan and reproduction

The mean lifespan of experimental insects ranged between ca. 65.9 and 141.3 days depending on sex, food, and mating status. Lifespan was significantly affected by food (ANOVA: $F_{2,218} = 18.55$, $P < 0.001$) and mating status ($F_{1,218} = 12.24$, $P < 0.001$), but not by sex ($F_{1,218} = 0.51$, P = 0.474). The interactions between the main factors were not significant for lifespan (food × mating status: $F_{2,218} = 1.12$, $P = 0.327$; sex × food: $F_{2,218} = 2.17$, $P = 0.117$; sex × mating status: $F_{1,218} = 0.09$, $P = 0.763$; sex × food × mating status: $F_{2,218} = 0.53$, $P = 0.587$). Regardless of mating status, both male and female beetles had the shortest mean lifespan on protein-biased food (p35:c7; Fig. 1). Males lived significantly longer lives on equal-ratio food (p21:c21) than on carbohydrate-biased food (p7:c35) (Tukey test: $P = 0.037$), but there was no significant

difference in mean lifespan between female beetles on equal-ratio food (p21:c21) and those on carbohydrate-biased food (p7:c35) (P = 0.997). For both males and females, mating resulted in a shorter lifespan across all three food treatments, with mated beetles living ca. 15.8% shorter lives than unmated, virgins. Food treatment had a significant impact on both lifetime fecundity (ANOVA: $F_{2,46}$ = 16.51, P < 0.001) and egg production rate ($F_{2,46}$ = 13.09, P < 0.001). These two parameters representing female reproduction were the highest on equal-ratio food (p21:c21; Fig. 2). Post-hoc test revealed that there was no significant difference between beetles on two imbalanced foods (p7:c35 and p35:c7) with respect to their lifetime fecundity (Tukey test: P = 0.582) and egg production rate (P = 0.954). Partial correlation controlling for egg production rate revealed that lifetime fecundity was positively associated with lifespan within and across food treatments (Table 1). Positive correlation was also detecte d between lifetime fecundity and egg production rate when holding lifespan constant (Table 1).

As clearly illustrated in Fig. 3, the age-specific egg laying in mated females was significantly affected by food treatment (repeated measures ANOVA using a mixed model: $F_{2,46}$ = 13.19, P < 0.001) and age ($F_{17,559}$ = 6.20, P < 0.001). The age-specific egg laying was maintained high during early adulthood and fell gradually until the last egg laying before death. This pattern was consistent across food treatments, as indicated by non-significant food × age interaction

($F_{34,559}$ = 1.35, P = 0.093). Throughout their lives, mated females exhibited a constantly higher age-specific egg laying on equal-ratio food (p21:c21) than on two imbalanced foods (p7:c35 and p35:c7).

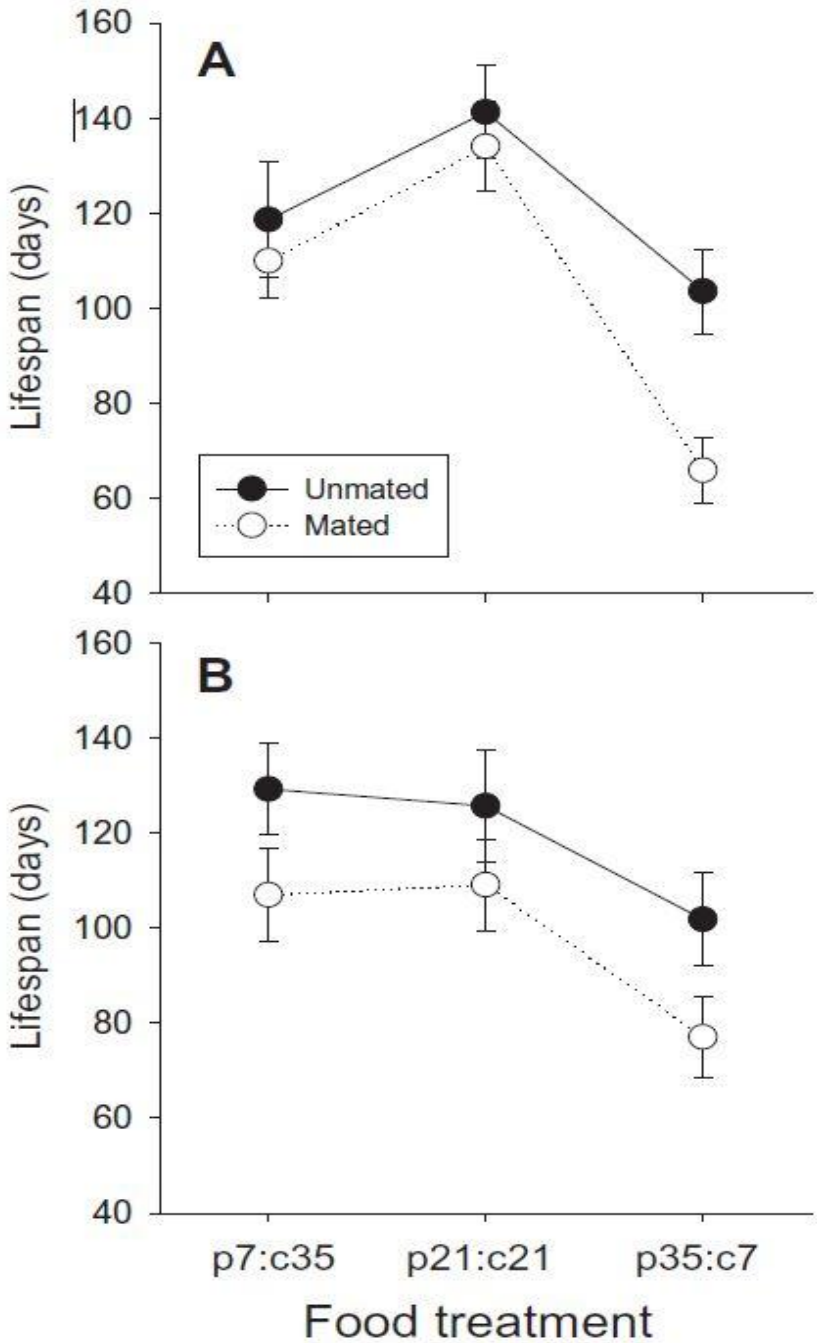

Figure 1. Mean (±SE) lifespan for (A) male and (B) female *Tenebrio molitor* fed on one of three synthetic foods differing in P:C ratio. For each sex, mated and unmated beetles are represented by open and filled circle, respectively.

Table 1. Partial correlations among lifespan, lifetime fecundity, and egg production rate for mated female *T. molitor* within and across three food treatments (all $P < 0.001$).

		Lifespan	Lifetime fecundity
Within treatment			
p7:c35	Lifetime fecundity	0.939	
	Egg production rate	−0.916	0.960
p21:c21	Lifetime fecundity	0.951	
	Egg production rate	−0.936	0.917
p35:c7	Lifetime fecundity	0.917	
	Egg production rate	−0.871	0.939
Across treatment			
	Lifetime fecundity	0.880	
	Egg production rate	−0.836	0.951

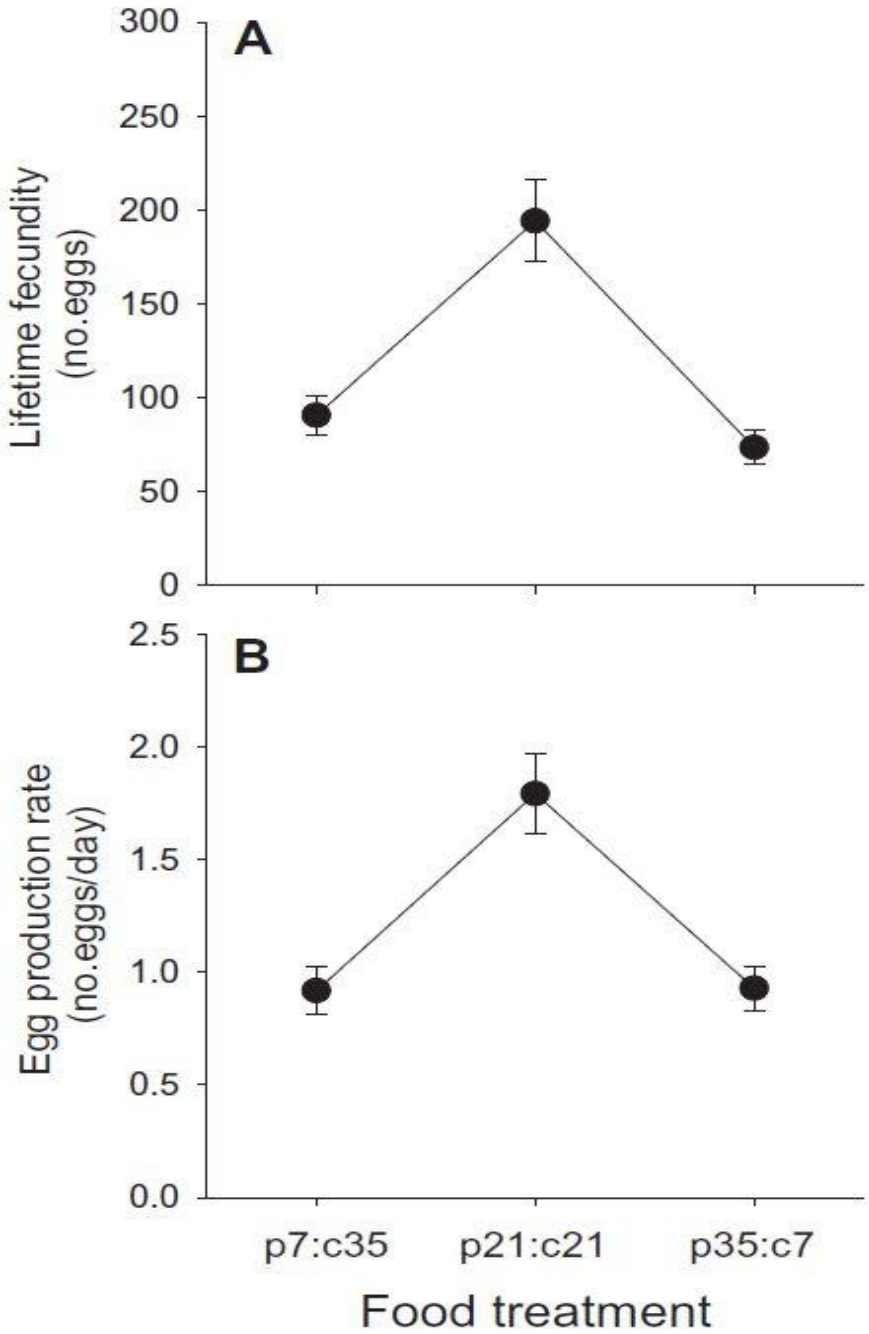

Figure 2. Mean (±SE) (A) lifetime fecundity and (B) egg production rate for mated female *Tenebrio molitor* fed on one of three synthetic foods differing in P:C ratio.

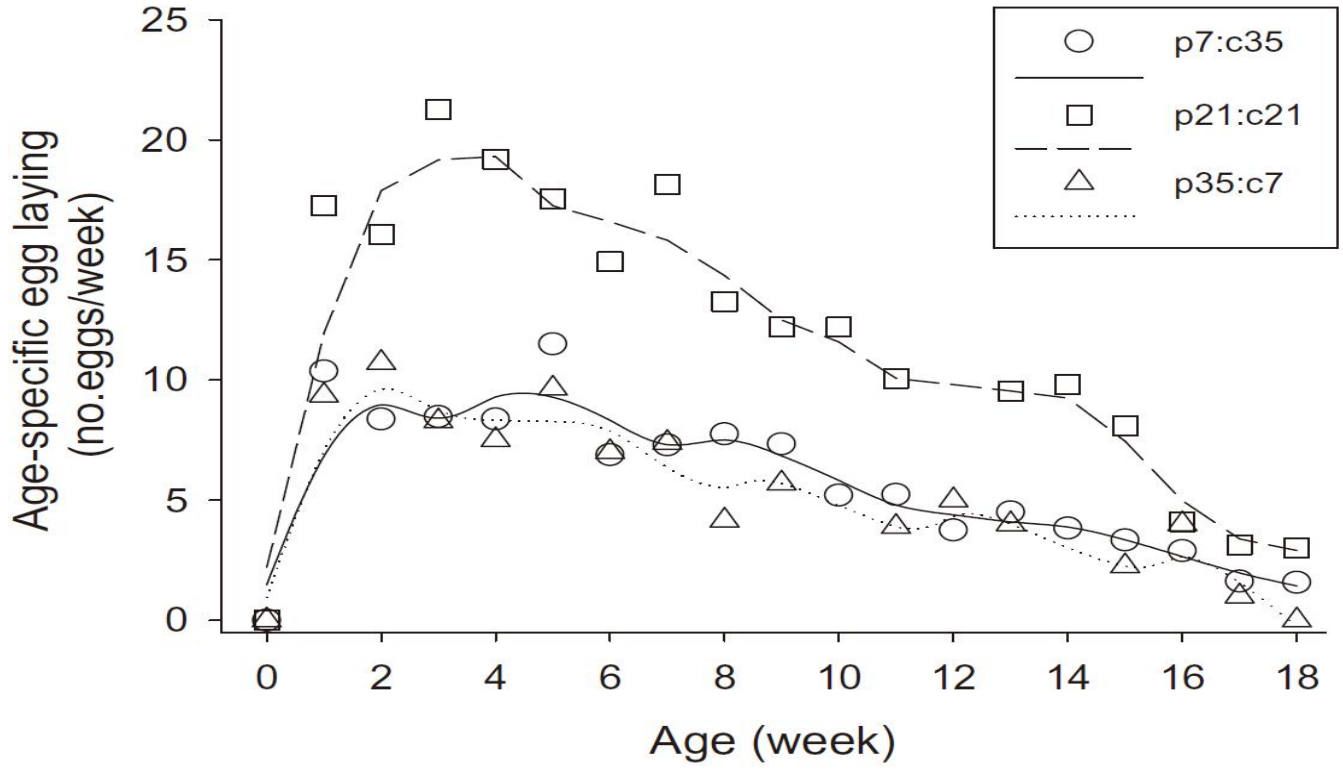

Figure 3. The pattern of age-specific egg laying by mated female *Tenebrio molitor* fed on one of three synthetic foods differing in P:C ratio from adult emergence until the 18th week of adulthood. For all three food treatments, smooth curves were fitted over the mean age-specific egg laying (open symbols) using LOWESS function with a smoothing parameter of 0.25.

3.2. Experiment 2: nutrient self-selection

Cumulative pattern of bivariate protein-carbohydrate intake over the two successive 6-day feeding periods (days 9–15 and 16–22) was significantly affected by sex (Fig. 4; MANOVA: Pillai's trace = 0.18292, $F_{2,53}$ = 5.93, P = 0.005), but neither by mating status (Pillai's trace = 0.07405, $F_{2,53}$ = 2.12, P = 0.130) nor by sex × mating status interaction (Pillai's trace = 0.00494; $F_{2,53}$ = 0.13, P = 0.877). Carbohydrate intake was similar between males and females (ANOVA: $F_{1,54}$ = 1.28, P = 0.262), but females consumed significantly more protein than did males ($F_{1,54}$ = 7.03, P = 0.011). The self-selected P:C ratio was thus significantly higher in females than in males ($F_{1,54}$ = 8.44, P = 0.005), with the mean ratio being 1:1.54 for unmated males, 1:1.30 for unmated females, 1:1.64 for mated males, and 1:1.36 for mated females (Fig. 4). These self-composed P:C ratios were all significantly different from 1:1 (one-sample t-test: all $P < 0.01$).

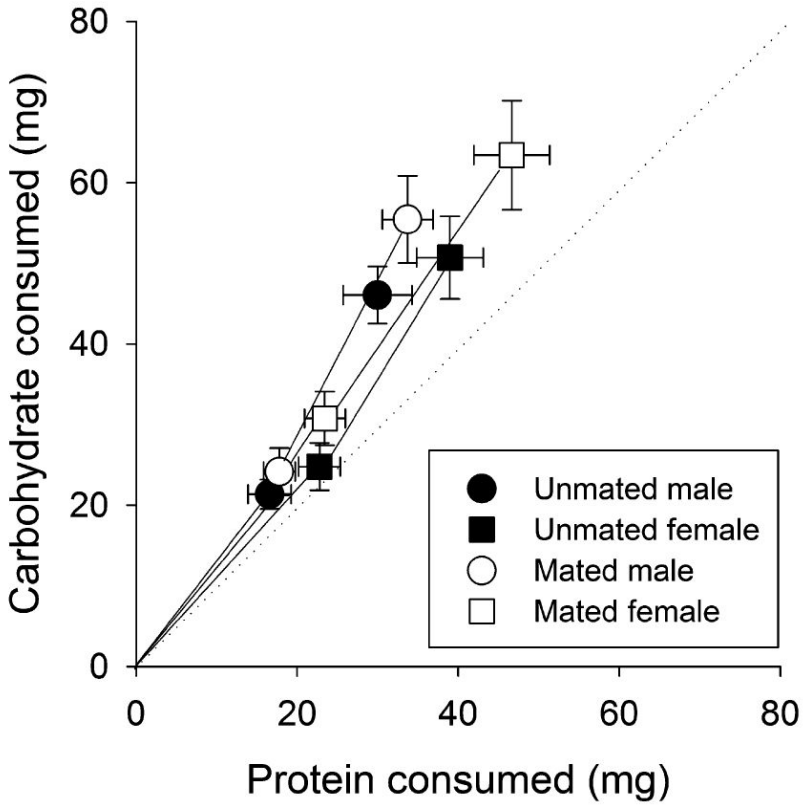

Figure 4. Bivariate mean (±SE) amount of protein-carbohydrate consumed by male and female *Tenebrio molitor* given a food choice between two nutritionally unbalanced P:C foods (p7:c35 and p35:c7) over the two 6-day feeding periods (days 9–15 and 16– 22). For each sex, mated and unmated beetles are represented by open and filled circle, respectively. Solid lines represent the trajectories of the protein-carbohydrate consumed over the experimental period (days 9–15 and 16–22). Dotted line indicates the P:C ratio of 1:1.

4. Discussion

The most important finding of this chapter is that the lifespan of *T. molitor* was critically influenced by dietary P:C ratio, lending strong support to the emerging notion that the macronutrient balance of the food is the key determinant of lifespan and ageing in insects. In agreement with the patterns previously observed from dipteran and orthopteran insects, the consumption of a high P:C food resulted in a shorter lifespan for *T. molitor*. However, it is important to note that there was a qualitative difference in the way in which lifespan responded to dietary P:C ratio between *T. molitor* and other insects and between male and female *T. molitor*. For example, the lifespan of most insects investigated to date peaked at the lowest P:C ratio and fell gradually with increasing P:C ratio (see references cited in Le Couteur et al., 2015), but this was not the case for *T. molitor*. In male *T. molitor*, the mean lifespan peaked at the intermediate P:C ratio of 1:1 and fell as the ratio deviated from 1:1. However, in female *T. molitor*, the mean lifespan increased as the P:C ratio declined from 5:1 to 1:1 and reached a plateau thereafter, demonstrating that females exhibited the longest lifespan at both P:C 1:5 and 1:1. These results indicate that the sensitivity to which the underlying mechanism of lifespan is controlled by dietary P:C ratio may not be uniform across species and sex.

By comparing the lifespan responses of tephitid flies that varied in reproductive status (mated, virgin, and sterilized females, and virgin males) to foods differing in protein and carbohydrate content, Fanson et al. (2012) found that the extent to which lifespan was shortened on high P:C food was similar across sex and reproductive status, providing empirical evidence that reduced lifespan on high P:C food is not due to a competitive resource allocation trade-off between lifespan and reproduction, but to the detrimental effect of excessive protein intake perse. These results from *T. molitor* were largely congruent with this so-called 'lethal protein hypothesis' (Fanson et al., 2012) in two respects. First, in a manner similar to what was demonstrated by Fanson et al. (2012), I found that the magnitude of the lifespan-shortening effect of high P:C food was similar between reproductively active, mated females and non-egg-producing, virgin females and also between males and females, reinforcing the argument that the reduction in lifespan on high P:C food occurred through a mechanisms independent of reproductive investment. Second, instead of demonstrating an antagonistic relationship, lifespan and egg production were shown to decrease concomitantly with increasing dietary P:C ratio, indicating that excessive protein intake had a negative impact on both lifespan and reproduction. The mechanisms by which excessive protein consumption leads to shorter lifespan remains poorly understood, but may include increased mitochondrial generation of reactive oxygen species (ROS),

increased production of toxic nitrogenous waste products, and increased activity of mTOR (target of rapamycin) (Kapahi et al., 2004; Sanz et al., 2004; Tatar et al., 2014; Mirzaei et al., 2014).

These data showed that mating stimulated egg production but curtailed lifespan in both male and female *T. molitor*. There are two possible mechanisms explaining the mating-induced reduction in lifespan in females. First, although the 'lethal protein hypothesis' seems to be a reasonable explanation for the observed association between high protein intake and reduced lifespan in *T. molitor*, there still remains the possibility that increased resource investment in egg production after mating has reduced the amount of resources that might be destined for somatic maintenance and repair (Kirkwood, 1977; Harshman and Zera, 2006; Flatt, 2011). Second, as previously reported for *D. melanogaster*, it is possible that the shortened lifespan of mated females is caused directly by the toxic effect of male seminal fluid products (e.g., sex peptides) transferred from male accessory glands to females during mating (Chapman et al., 1995; Wigby and Chapman, 2005). Potential mechanisms underlying the lifespan-shortening effect of mating in males remain far more elusive, but it is possible that elevated metabolic costs associated with courtship and increased sperm production could have been traded off against somatic maintenance and repair in males (Partridge and Farquhar, 1981; Van Voorhies, 1992).

Although it was not explicitly examined in this study, the observed mating-induced decrease in lifespan could be attributed to post-mating increase in juvenile hormone (JH) level in the haemolymph of both male and female *T. molitor* (Rolff and Siva-Jothy, 2002). Besides its roles in regulating post-embryonic development, reproduction, polyphenism, and other physiological functions, there is increasing evidence that JH is a key regulatory endocrine of lifespan in insects (Flatt et al., 2005). For example, increased JH level was associated with reduced lifespan in monarch butterflies (Herman and Tatar, 2001). Recent study also reported that reduced JH level extended the lifespan of female *D. melanogaster* (Yamamoto et al., 2013). In addition to its direct effect on lifespan, post-mating increase in JH secretion might have indirectly caused mated male and female *T. molitor* to die earlier through suppressing their immune function and thus making them more vulnerable to infection (Rolff and Siva-Jothy, 2002; Rantala et al., 2003). This hypothetical role of JH in mediating the link between mating, lifespan, and immunity in *T. molitor* needs to be verified in future studies.

Reproductive ageing is defined as the gradual deterioration of age-specific reproductive output with advancing age (Maklakov et al., 2009; Jensen et al., 2015). These beetles followed this general pattern of reproductive senescence, laying more eggs early in adulthood and less as they got older. Females consistently laid more eggs on equal-ratio food (p21:c21) than on protein- and

carbohydrate-biased foods (p7:c35 and p35:c7) at any given age throughout their lives. This pattern of age-specific egg laying resulted in the highest lifetime fecundity at dietary P:C ratio of 1:1. Results from partial correlation analyses indicated that beetles achieved the highest lifetime fecundity on equal-ratio food not only because they lived the longest but also because they had the highest egg production rate on this food.

In most insects investigated to date, lifespan and reproduction were maximized at different P:C ratios, with the peaking P:C ratio for reproduction being higher than that for lifespan (Lee et al., 2008; Maklakov et al., 2008; Fanson et al., 2009; Jensen et al., 2015; Lee, 2015). In contrast to this general pattern, I did not find any strong patterns of divergence in nutritional optima between lifespan and reproduction in *T. molitor*. However, it should be noted that we used only three dietary P:C ratios in this study, thus leaving open the possibility that the P:C optima for two traits could be actually divergent. In order to address this unresolved issue of the potential divergence in nutritional optima, I need to employ a more extensive experimental design that includes more dietary P:C ratios as it had been done in some recent studies using the Geometric Framework (see Lee et al., 2008; Maklakov et al., 2008; Fanson et al., 2009; Harrison et al., 2014; Jensen et al., 2015; Lee, 2015).

The fact that lifetime reproductive success was maximized at the P:C ratio of 1:1 led us to predict that *T. molitor* would exhibit a strong behavioral

regulation to reach to this ratio by selectively foraging between nutritionally complementary foods. Consistent with this prediction, this data showed that female *T. molitor* regulated their protein and carbohydrate intake to a ratio slightly lower than 1:1 (1:1.30 and 1:1.36 for unmated and mated females, respectively). It was evident that *T. molitor* actively regulated their food selection because these self-composed P:C ratios deviated significantly from 1:1, a P:C ratio expected if beetles ingested two foods at random (Rho and Lee, 2014, 2015). When the regulated protein-carbohydrate intake was compared between males and females, I found that females consumed more protein than did males, reflecting extra protein requirement for egg production (Wheeler, 1996; Lee, 2010; Lee et al., 2013). In conclusion, this chapter provides strong support to the idea that dietary P:C ratio is a critical determinant of both lifespan and fecundity in *T. molitor*, substantiating the results previously obtained from other insects. Among three P:C ratios tested, both lifespan and lifetime reproductive success were the highest at P:C 1:1, indicating that the nutritional optima of these two important fitness components did not show a strong pattern of divergence in *T. molitor*. When given a choice, mated female beetles actively regulated their protein and carbohydrate intake to a slightly carbohydrate-biased ratio of P:C 1:1.36. In this chapter, I proposed that *T. molitor* can be an excellent model organism for studying lifespan and ageing in insects. Given the significant role played by *T. molitor* in the field of sexual

selection and ecological immunology in insects, it is hoped that the experimental system I established in this study will provide a useful platform upon which I can continue to develop testable hypotheses regarding how nutrition mediates the complex interactions between sex, immunity, and ageing (Simpson and Raubenheimer, 2012).

CONCLUSION

This thesis is composed of three experimental chapters, with each describing the mechanistic, functional, and adaptive aspects of nutritional regulatory responses of *T. molitor.* As far as I am aware, this is the first empirical demonstration of the nutrient balancing and dietary intervention on lifespan in this important model insect. There are four main conclusions arising from this thesis.

First, this study has established that *T. molitor* beetles have well-developed capacity to balance the intake of protein and carbohydrate by selectively mixing nutritionally complementary foods. When offered a choice, *T. molitor* beetles actively regulated their protein and carbohydrate intake to a 1:1 ratio. When prevented from achieving their regulated target intake, these omnivorous beetles exhibited a nutrient balancing rule which enables them to eat and tolerate greater quantities of nutrient excesses in the diets. This strategy has been previously reported from other omnivores and generalist herbivores and is

thought to be associated with their high likelihood of encountering nutritionally complementary foods from nutritionally diverse environments.

Second, it is clearly demonstrated that *T. molitor* beetles can recover from the negative consequences of ingesting nutritionally imbalanced diets by plastically adjusting their diet selection. However, it is noteworthy that the adjustments in diet preference enabled the beetles to fully compensate for the deficiency of protein in their previous meal, but not that of carbohydrate. A potential explanation for this limited capability of *T. molitor* beetles to behaviourally compensate for carbohydrate shortage is that carbohydrate shortage can be physiologically compensated by converting ingested protein to carbohydrate via gluconeogenesis (Thompson & Redak, 2000; Wilkinson et al., 2001), thus precluding beetles from foraging for extra carbohydrates. The biochemical foundation for this interesting possibility warrants further investigation.

Third, the current study confirms that the balance between two macronutrients, protein and carbohydrate, in the food is the key determinant of lifespan and reproduction in *T. molitor* beetles. This result is to large extent consistent with those previously reported from other insects, but there are also some major differences in the way in which both lifespan and reproduction respond to macronutrients. Whilst most insects examined to date showed diverging nutritional optima for lifespan and reproduction (e.g., Lee et al., 2008; Maklakov et al., 2008; Fanson et al., 2009; Jensen et al., 2015), both lifespan

and lifetime egg production of *T. molitor* beetles were maximized at the same P:C ratio of 1:1. However, a major limitation of this study is that only three P:C diets were used to examine the relationship between diet and fitness in *T .molitor* beetles. This approach is rather too simplistic because it does not allow us to investigate both the separate and interactive effects of individual nutrients. Hence, a more extensive experimental design which includes a comprehensive mixture and concentration of protein and carbohydrate content in the diet should be employed in future researches in a manner similar to that used for exploring the dietary intervention on lifespan and reproduction in *D. melanogaster* (e.g., Lee et al., 2008).

Last, this study provides experimental evidence that the regulated mixture of protein and carbohydrate selected by *T. molitor* closely corresponds to the P:C ratio that maximizes their Darwinian fitness. This finding is congruent with the prediction based on an evolutionary hypothesis that the optimal foraging of insects has evolved to maximize their fitness through achieving an optimal balance of multiple nutrients (Simpson et al., 2004). An emerging question to be addressed in future research is whether this regulated target intake defended by *T. molitor* beetles can be altered by environmentally-mediated changes in nutritional requirement, such as immune assault and warming.

The conclusions derived from this thesis offer a unique insight into understanding the adaptive significance of the nutritional balancing and

foraging behaviour in this omnivorous beetle and also highlight that *T. molitor* can be an excellent model organism for studying nutrition, lifespan, and ageing. It is my firm belief that the experimental protocol I have developed in my thesis will serve as a useful platform for future investigations on the nutritional biology of this species of emerging scientific and commercial importance.

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KOREAN ABSTRACT

갈색거저리의 영양섭식행동조절에 관한 연구

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초록

갈색거저리(*Tenebrio molitor*)는 생리학, 행동학, 면역학 등과 같은 다양한 곤충학 관련 연구에 널리 사용되고 있는 중요한 모델생물이며, 최근 동물사료와 식용곤충으로써 그 산업적 중요성이 부각되고 있는 유용곤충이다. 이러한 중요성에도 불구하고, 갈색거저리의 영양요구, 영양조절기작, 그리고 영양섭취에 따른 갈색거저리의 적응도 발현과 같은 기초적인 영향학적 지식에 대한 연구는 아직도 부적한 것이 현실이다.

본 연구는 우선 갈색거저리 성충이 단백질과 탄수화물 섭식을 과연 어떻게 행동적 · 생리적으로 조절하는지를 규명하기 위해 통제된 실험실 조건에서 갈색거저리의 영양섭식조절행동과 식후영양이용효율을 측정하였다(제1장). 갈색거저리 수컷과 암컷 성충들에게 단백질과 탄수화물 조성이 비율이 다른 2가지 먹이를 자유로이 선택하게끔 허용했을 때, 이들은 단백질과 탄수화물을 1 대 1의 비율로 일

관적으로 선택하는 것으로 확인되었다. 갈색거저리에게 단백질과 탄수화물의 조성비율이 불균형한 음식물만을 제공하였을 경우, 이들은 주어진 음식물에 과하게 함유된 영양분의 섭식을 극대화하는 영양균형전략을 보였는데, 이는 잡식곤충이나 광식성 초식곤충과 같이 자연환경에 매우 광범위한 영양환경에 적응한 곤충들에게서 일반적으로 보이는 섭식전략으로 알려져 있다.

이렇게 갈색거저리의 최적영양요구와 영양균형전략을 규명한 후, 본 연구는 과연 갈색거저리가 자신의 영양선호도를 능동적으로 변화시킴으로써 장기간 노출되었던 영양불균형상태를 극복할 수 있는지를 확인하는 실험을 추가적으로 수행하였다(제2장). 이를 위해 갈색거저리를 단백질과 탄수화물이 각각 결여된 음식에 18일 동안 노출시킨 후, 이들에게 단백질과 탄수화물을 동시에 제공하여 자신이 선호하는 영양분을 선택하도록 허용하였다. 연구 결과, 18일 동안 단백질이 결여된 음식을 섭취한 개체들은 유의하게 단백질을 탄수화물에 비해 선호하는 것으로 나타났으며, 6일 만에 정상적인 수준의 영양조건으로 회복하는 것을 확인할 수 있었다.

본 연구는 또한 갈색거저리가 섭취한 음식물의 단백질과 탄수화물 간의 균형이 갈색거저리의 진화적 적응도를 결정하는 중요 생활사 형질인 수명과 산란에 미치는 영향을 규명하기 위한 실험을 진행하였다(제3장). 갈색거저리의 수명, 산란율, 그리고 평생 동안 낳은 알의 수 모두 단백질 대 탄수화물이 1 대 1일 때 가장 높았다. 이와 같은 연구결과는 곤충은 행동적으로 영양섭식을 조절하는 과정을 통해 자신의 진화적 적응도를 극대화시킨다고 예측하는 진화적 가설을 지지하는 실험적 증거를 제시하고 있다.

검색어 : 갈색거저리, 먹이선택행동, 생활사 특성, 식후영양이용조절, 영양조절, 적응도

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