Regulation of protein and carbohydrate intake in caged honeybees *Apis mellifera scutellata*: assessment based on consumption and various performance measures

By

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Abstract

When provided with the opportunity to select their diet, most insect herbivores regulate their nutrient intake. However, in a nutritionally heterogeneous environment and with changing demands for growth, development and reproduction, obtaining the required amount and balance of nutrients is a challenge. This is especially true for social insects where the workers bring food into the colony to be shared by nestmates. The ability of insects to self-select their diet is an important trait related to fitness. In this study we investigated whether and how caged worker honeybees meet their nutritional requirements in response to the nutritional composition of the food they find. Using the 'geometric framework' we looked at the behavioural and physiological mechanisms used by caged worker honeybees in balancing their diet when provided with different pairs of complementary imbalanced foods.

First, we investigated whether caged worker honeybees maintain their intake target by providing them with pairs of complementary imbalanced foods with varying protein to

carbohydrate (P:C) ratios. Diets were formulated using different protein sources: casein, royal jelly and Feed-Bee®. Honeybees self-selected or balanced their diet by switching between the complementary foods in accordance with the composition of the food and the type of protein that they encountered. Honeybees selected average P:C ratios of 1:12, 1:14 and 1:11 on casein, royal jelly and Feed-Bee® diets respectively. The level of self-selection was confirmed using two performance measures: survival and ovarian activation. Both survival and ovarian activation differed depending on the type of protein source used.

Second, we investigated if honeybees regulated their growth target, which is the amount of nutrients incorporated into growth and storage tissue, by measuring physiological parameters in honeybees confined on imbalanced complementary food combinations having different P:C ratios. Feed-Bee® was used as a protein source. The physiological parameters measured were head fresh mass, hypopharyngeal gland (HPG) development, and protein concentration in the haemolymph. The bees fed on different diet combinations with different P:C ratios maintained each of the performance measures to the same level, which supports the ability of worker honeybees to self-select their diet. The measured physiological parameters were compared with other studies to asses the appropriateness Feed-Bee® diet as a protein source for the bees.

In the absence of brood the intake target is directly related to the physiological requirements of the worker bees. The behavior of these individual adult bees gives an insight in to the complex system; similar responses may be seen in nurse bees in the colony condition to obtain protein, carbohydrate and other nutrient requirements from stored pollen and nectar in the hive, either for their own nutritional requirements or for other colony members, especially larvae.

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Declaration

I declare that the thesis, which I hereby submit for the degree in Master of Science (Agric): Entomology at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at another university.

Solomon Zewdu Altaye

Signature…….

Date ……

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Chapter 1: General introduction

1.1. Honeybee nutrition

Bees depend on flowers to collect pollen and nectar from which they get their nutrient requirements. Nectar provides carbohydrate in the form of sugar, and pollen provides protein, lipids, carbohydrate, vitamins and minerals (Winston, 1987). These nutrients are required by honeybees for larval growth and metamorphosis, and for adult development and functioning (Winston, 1987). Pollen is the major natural source of protein in the honeybee diet and protein is essential for productivity of a colony (Kleinschmidt and Kondos, 1976). In particular, a substantial amount of protein is required for larval growth and development (Crailsheim *et al.*, 1992; Pernal and Currie, 2000). The larvae feed on royal jelly secreted by fully activated hypopharyngeal glands (HPG) of adult nurse bees (Snodgrass, 1925). Newly emerged bees also feed on royal jelly to develop their own HPG and begin to feed larvae as well as the queen, drones and other adult bees when they are around nine days old (Crailsheim, 1991). HPG activity depends on the age of worker bees (Huang & Otis, 1989) and its stage of development determines the tasks of worker bees (Free, 1961). In queenright colonies with open or unsealed brood, the size of the acinus reaches its peak in 8 day old bees (Suzuki, 1988; Crailsheim and Stolberg, 1989). The HPG reach their maximum weight from days 6-15 (Crailsheim and Stolberg, 1989; Knecht and Kaatz, 1990).

HPG development is influenced by the quality and quantity of pollen ingested by workers and the size of the HPG, as measured by acinus diameter, is related to its total protein content (Brouwers, 1983). The protein secreted by the glands is largely derived from the pollen consumed by the bees (Crailsheim *et al.*, 1992), thus the HPG provide a reliable measure of dietary protein assimilation in newly emerged workers (Crailsheim & Stolberg, 1989). When the quality and quantity of pollen is limited, it affects reproduction, brood rearing and longevity, thus ultimately the productivity of the colony (Kleinschmidt & Kondos, 1976; Human *et al.*, 2007). Moreover, when honeybees are confined to a single

pollen source or a pollen supplement or substitute with fixed proportions of protein and carbohydrate, which may not be balanced, this may affect the performance of the bees (Human *et al*., 2007; Pirk *et al*., 2009).

1.2. Effect of a single food with fixed nutrients: sunflower vs. aloe pollen study

The effect of considering only a single nutritional component like protein is illustrated by recent studies on the effect of two pollen sources, aloe (*Aloe greatheadii* var. *davyana*) and sunflower (*Helianthus annuus*) on ovarian activation of worker bees of *Apis mellifera scutellata* (Human *et al*., 2007). In this study, queenright workers kept on aloe fields performed better than those kept on sunflower fields. In contrast, workers kept in the laboratory and fed aloe pollen showed less ovarian activation and higher mortality than those fed sunflower pollen. The explanation given for this conflicting observation is that honeybees kept in the field survived well because of their access to other food sources to balance or dilute the effects of nutrients in their diet (Human *et al*., 2007). In a subsequent study, the impact of three different protein sources (casein, *Aloe greatheadii* var *davyana* pollen and royal jelly) as well as five different protein to carbohydrate (P:C) ratios (0:1, 1:3, 1:1, 3:1 and 1:0) on survival and ovarian activation was tested (Pirk *et al*., 2009). This study showed the importance of protein:carbohydrate balance and protein type for survival and ovarian activation.

For the above reasons, the approach of providing a single food with a fixed proportion of nutrients places too little emphasis on the deficient nutrient being the limiting one (Raubenheimer & Simpson, 1999); in other words, the balance of the macronutrients needs consideration. This is an important concept to be considered in diet preparation for feeding bees in winter, during which time there are nectar and pollen shortages (Johannsmeier, 2001). During winter, and also when honeybees are used in monoculture pollination, bees may not have access to other food sources to balance or dilute the effects of nutrients in

their diet. The best way of solving this problem is to follow an integrative nutritional approach which involves the simple 'geometrical framework'.

1.3. Geometric framework: an integrative approach to nutrition

The geometrical framework is an integrative approach that helps researchers to investigate how animals regulate their multiple and changing demands of nutrients in a heterogeneous environment (Simpson & Raubenheimer, 1993; Raubenheimer & Simpson, 1993; Raubenheimer & Simpson, 1999; Behmer, 2009). Using this approach we can measure the optimal balance and amounts of nutrients that need to be ingested and allocated to growth by an animal over a fixed period of time, i.e. the intake target and growth target respectively (Raubenheimer & Simpson, 1999). The intake target helps to empirically assess the influences on animals when their intake is constrained by circumstances such as nutritionally imbalanced food, the presence of plant toxins and unfavorable food distributions (Simpson & Raubenheimer, 2000; 2001). Different nutritional challenges have been thoroughly examined using solitary insects (Raubenheimer *et al.*, 2009, supplementary table 1) and also recently using a social insect, the ant *Rhytidoponera metallica* (Dussutour & Simpson, 2008a).

Regulating intake requires that the animal integrates information about the nutritional quality of its food and its current nutritional state, and meeting nutritional needs involves regulation of feeding and also post-ingestive utilization of food (Simpson *et al.*, 1995; Raubenheimer & Simpson, 2003). In addition to using the behavioural responses involved in nutritional homeostasis on feeding varying nutrient levels, the position of the intake target can also be estimated using performance criteria like survival, and developmental time and can also be calculated from the growth target (Simpson *et al.*, 1995), because an animal performs optimally when the proportion of nutrients in the food equals the self-selected intake target (Raubenheimer & Simpson, 1993; Behmer & Joern, 2008).

The quantity of nutrients in a food can be represented by a point in nutrient space, while the balance of nutrients is described as a linear trajectory that projects into the nutrient space at an angle describing the ratio of the nutrients: the lines representing the foods are then known as nutritional rails (Simpson *et al*., 1995; Raubenheimer & Simpson, 1999). For instance, when the ratio of nutrients in the food is the same as that required by an animal, it is a nutritionally balanced food (Fig. 1, Food B). The requirements of an animal can not be reached by feeding only on an imbalanced food (Fig. 1, Food A or C), but can be reached by switching between two complementary foods. Complementary foods allow the animal to zigzag its way through nutrient space (Raubenheimer & Simpson, 1999).

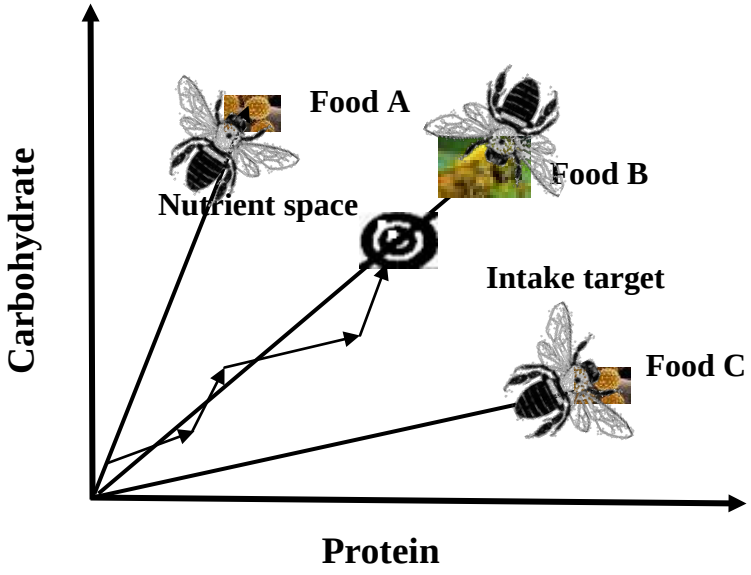

Figure 1. The quantity of nutrients in a food can be represented as a point in nutrient space at an angle describing the ratio of nutrients. If the ratio is the same as that required by the animal it is a nutritionally balanced food and the intake target can be reached by feeding on the food (Food B). The intake target cannot be reached by feeding on nutritionally imbalanced foods (Food A or C). The intake target can, however, be reached by switching between complementary foods (between Food A & C). Adapted from Raubenheimer & Simpson (1999).

1.4. Application of geometric framework to social insects

In social insects, the food entering the colony is brought by workers to be shared by the colony, which is an additional nutritional challenge to those faced by solitary insects (Dussutour & Simpson, 2008b). Thus foragers react to food encountered according to both individual need and internal demands for nutrients in the nest. Aphid-tending ants, *Lasius niger*, adjust their collective foraging response according to the nature of the food and the presence of brood in the colony (Portha *et al*., 2002). In this ant species, decisions at the individual level depend on the nature of the food (Portha *et al*., 2004); whereas at the collective level it is food type and food concentration that cause workers to recruit to the food source (Cassill & Tschinkel, 1999; Portha *et al*., 2002). Similarly ants were able to integrate foraging decisions based on food type and unrelated factors such as presence of brood (Portha *et al*., 2004); and also food quality (Beckers *et al*., 1993). Another ant species (*Solenopsis invicta*) also showed individual ability to assess food type and quality in order to ingest their intake target (Cassill & Tschinkel, 1999). Recently ants, (*Rhytidoponera metallica*) when given complementary imbalanced foods maintained their nutrient intake in response to the nutritional composition of foods (Dussutour & Simpson, 2008a).

To the best of our knowledge, investigation of how honeybees meet their nutritional requirements in response to varying nutritional composition of food at the individual level has not been carried out. At the colony level, honeybees modulate the intensity of nectar (Seeley, 1995) and pollen (Camazine *et al*., 1998; Dreller *et al*., 1999; Dreller & Tarpy, 2000) foraging according to the nutritional status of the colony. We have used the geometrical framework to investigate the behavioural and physiological mechanisms used by worker bees to achieve their intake and growth targets when provided with complementary imbalanced foods.

1.5. Supplemental feeding of honeybees

Searching for proper pollen substitutes has occupied the attention of many beekeepers for many years (Robinson & Nation, 1966). The aim of supplementary feeding using pollen substitutes or supplements is mainly to bridge the dearth periods by maintaining the colony strength for the coming nectar and pollen flow, as well as prior to the flowering of pollinated crops for pollination. The most widely used substitute consists of a mixture of soybean flour, dried brewers yeast and dry skim milk (Haydak, 1970). However, Saffari *et al.* (2006) stated that some pollen substitutes (e.g. dried egg yolk, meat scraps, milk products such as whey and wheast, and soy products) do not consider the issue of palatability, nutritional content, nutritional requirements of bees and biological effectiveness.

In designing pollen supplements and substitutes, the nutritional value to the bees should be considered, which means these diets must contain nutrients similar to the natural source, having proper texture and consistency so that they can be accepted by the animal, and digestibility, anti-nutritional factors, shelf life, availability and cost must also be taken in to account (Saffari *et al.*, 2006). Considering all those factors, Saffari *et al*. (2004) formulated a natural pollen substitute called Feed-Bee®, which performed to the same extent or more than pollen does (Saffari *et al*., 2004; De Jong *et al*., 2009). It has been shown to result in higher protein concentrations in haemolymph than pollen and acacia pod flour (De Jong *et al*., 2009). In another laboratory experiment, honeybees fed on Feed-Bee® consumed and weighed as much as other honeybees that consumed freshly collected pollen (Gregory, 2006). Because the nutritional composition of Feed-Bee® is known and less variable than natural pollen, correlation between the physiological parameters and diet can be investigated.

To date, honeybee feed formulation attempts have been concentrated on developing pollen substitutes or supplements depending on the pollen nutritional information whether the

materials are plant or animal in origin. When there is a scarcity of bee forage and little or no stores in the hive, bees may not have access to other diets; but feeding single pollen or pollen substitute or supplements only may not provide the proper balance and amount of nutrients required by bees (Human *et al*., 2007). That means that, in addition to focusing on other factors that are well explained by Saffari *et al.* (2004), honeybee feed formulation schemes should be based on the intake target of the bees as discussed earlier.

1.6. Aim of the study

The first aim (chapter 2) was to investigate whether and how honeybees meet their nutritional requirements in response to the composition of the food they encounter, by providing them with pairs of complementary imbalanced foods with varying protein to carbohydrate (P:C) ratios (casein, royal jelly and Feed-Bee®). We looked at the effects of these dietary P:C ratios and different protein sources on consumption and two performance measures, survival and ovarian activation. We expected the bees to compensate for an imbalanced diet by switching between two complementary foods provided, and also to change their choice of intake target with a change in protein source, as caterpillars showed an adaptive increase in protein intake when provided with low quality protein (Lee, 2007).

The second aim (chapter 3) was to investigate whether honeybees regulated their growth target when confined on different imbalanced complementary food combinations using Feed-Bee® as a protein source. We evaluated the performance of the bees based on the following physiological parameters: total protein concentration in the haemolymph, hypopharyngeal gland development and head fresh mass. Finally, based on the self-selected intake target and corresponding performances, we looked at the appropriateness of a Feed-Bee® diet as a protein source for the bees.

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Chapter 2:

Regulation of protein and carbohydrate intake in caged honeybees *Apis mellifera scutellata*: assessment based on consumption, survival and ovarian activation

Abstract

We investigated whether caged worker honeybees (*Apis mellifera scutellata*) maintain their intake target when challenged by the provision of one of four pairs of imbalanced complementary artificial foods varying in protein to carbohydrate (P:C) ratio (1:25 with 1:1, 1:25 with 1:10, 1:50 with 1:1, and 1:50 with 1:10). The foods were formulated using three protein sources (casein, royal jelly or Feed-Bee®, a natural pollen substitute) and sucrose, and the ratios were chosen based on an intake target of 1:17 P:C obtained by feeding pure sucrose and pure casein. Honeybees balanced their diet by switching between the complementary foods in accordance with the composition of the food and the type of protein that they encountered. Honeybees preferred average P:C ratios of 1:12, 1:14 and 1:11 on casein, royal jelly and Feed-Bee® diets respectively. The level of self-selection was confirmed using two performance measures: survival and ovarian activation, but here there were differences among the protein sources. In broodless caged worker honeybees the nutrient intake is directly related to their own physiological requirements, but requirements are likely to differ in the presence of brood.

2.1. Introduction

Honeybee colonies need a range of nutrients to satisfy their dietary requirements for larval growth and metamorphosis and for adult development and functioning (Winston, 1987). Nectar is the main source of carbohydrate in the form of sugar, and pollen provides protein, lipids, carbohydrate, vitamins and minerals (Haydak, 1970; Winston, 1987). Pollen is mainly consumed and digested by nurse bees, the age cohort of workers attending brood and the queen, enabling them to produce royal jelly, which is the main food source of queens, young workers and larvae (Crailsheim, 1991). When honeybees are maintained on pollens that are marginal in nutritive value, or when the quality and quantity of pollen is limited, reproduction, brood rearing, longevity, and eventually the productivity is affected (Kleinschmidt and Kondos, 1976; Human *et al*., 2007).

Sources of naturally occurring pollen are scarce during certain seasons (Johannsmeier, 2001). When bees are short of stored honey or pollen, they may be limited to a single food source. Moreover, supplements or substitutes with fixed proportions of protein and carbohydrate may not be balanced (Human *et al*., 2007). In addition, when honeybees are used in monoculture pollination, they may not have access to other food sources to balance or dilute the effects of other nutrients in the pollen they obtain. This has been illustrated by recent studies on the effect of two pollen sources, aloe (*Aloe greatheadii* var. *davyana*) and sunflower (*Helianthus annuus*) on ovarian activation of worker bees of *Apis mellifera scutellata* (Human *et al*., 2007). In this study, queenright workers kept on aloe fields performed better than those kept on sunflower fields. In contrast, workers kept in the laboratory and fed aloe pollen showed less ovarian activation and higher mortality than those fed sunflower pollen. The explanation given for this conflicting observation is that honeybees kept in the field survived well because of their access to other food sources to balance or dilute the effects of nutrients in their diet (Human *et al*., 2007). In a subsequent study, Pirk *et al*. (2009) showed the importance of protein:carbohydrate balance and protein type for survival and ovarian activation by providing bees with no-choice diets.

To investigate the optimal balance and amount of nutrients required by honeybees, we used the 'geometrical framework'. The geometrical framework measures the balance and amount of nutrients required to be ingested and allocated to growth by an animal over a fixed period of time: the intake and growth targets respectively (Raubenheimer and Simpson, 1997; Raubenheimer and Simpson, 1999; Simpson and Raubenheimer, 2000, 2001). The intake target helps us to empirically assess the influences on animals of constraints such as nutrient imbalances in food, plant toxins and unfavourable food distributions (Simpson and Raubenheimer, 2000, 2001). To investigate how an animal regulates its intake of nutrients, it must be challenged to alter its ingestive behaviour to maintain the intake target (Simpson *et al.*, 1995; Simpson and Raubenheimer, 2000). Different challenges have been thoroughly examined using solitary insects such as locusts, caterpillars, cockroaches and fruit flies, and also other animals as model organisms (Raubenheimer *et al.*, 2009, supplementary table 1).

How social insects maintain nutrient intake in response to the nutritional composition of foods they find is not well known (Kay, 2004; Dussutour and Simpson, 2008b). In social insects, workers bring food into the colony to be shared by nestmates, which is an additional challenge to those faced by solitary insects (Dussutour and Simpson, 2008b). Thus foragers react to food encountered according to both individual needs and internal demands for nutrients in the nest. Aphid-tending ants *Lasius niger* shape their collective foraging response according to the nature of the food and the presence of brood in the colony (Portha *et al,* 2002). Decisions at the individual level depend on the nature of the food (Portha *et al,* 2004); whereas at the collective level it is food type and food concentration that cause workers to recruit to the food source (Cassill and Tschinkel, 1999; Portha *et al*, 2002). Recently, Dussutour and Simpson (2008a) showed the ability of the ant *Rhytidoponera metallica* to compensate for changes in nutrient density by selecting among complementary foods. In case of honeybees, at the colony level they modulate the intensity of foraging for nectar (Seeley, 1995) and pollen (Fewell and Winston, 1992; Camazine *et al*., 1998; Dreller *et al.*, 1999) according to the nutritional status of the whole colony.

However, how caged worker honeybees meet their nutritional requirements in response to the composition of food has not been investigated.

We investigated whether honeybees maintain their intake target by providing them with pairs of complementary imbalanced foods with varying protein to carbohydrate (P:C) ratio using three different protein sources: casein, royal jelly and Feed-Bee®. Feed-Bee® is a natural pollen substitute of plant origin, with similar effects to pollen (Saffari *et al*., 2004; De Jong *et al.*, 2009). We measured the level of self-selection by measuring the feeding behaviour of honeybee workers and two performance measures: survival and ovarian activation. Depending on how workers obtain their food, there might be a strong relationship between rectal volume and ovarian activation (Schäfer *et* al., 2006): we therefore also measured the rectal volume of workers. Changing food choice with changes in protein quality is not common, but caterpillars showed an adaptive increase in protein intake when provided with low quality protein (Lee, 2007). We expected the bees to compensate for an imbalanced diet by switching between two complementary foods, and to change their intake target with a change in protein source.

2.2. Materials and Methods

2.2.1. Experimental animals and cages

Five *Apis mellifera scutellata* colonies from the University of Pretoria apiary site were used to collect frames of capped worker brood, which were incubated at 34^{o}C to obtain newly emerged adult worker bees of the same age. Groups of 100 newly emerged workers from each colony were confined in standard hoarding cages (11 x 8.5 x 7 cm) closed at both the front and back with movable glass slides (Fig. 1). The bottom of the cage was closed with wire mesh to allow ventilation. Below the glass slide at the front of each cage, a plastic frame was placed with round windows through which three stoppered feeding tubes (plastic tubes with standard windows 2 x 1 cm cut into them) were inserted into the cages. Two of the feeding tubes were used for providing complementary imbalanced foods and the third

for providing water. Food and water were provided ad *libitum*. All the cages were placed under standard conditions in an incubator at 34 ^{o}C and 55-65 % RH for 14 days, and kept in the dark to simulate conditions within the colony.

2.2.2. Experimental protocol and diets

In a first experiment, bees were allowed to feed on pure casein (vitamin free, C 3400, Sigma-Aldrich®, Germany) and sucrose provided separately with water in five replicates using different colonies for each. These colonies preferred an average intake target of 1:17 P:C ratio. Based on this first set of data, we formulated four diets, two from either side of this intake target, containing P:C ratios of 1:50, 1:25, 1:10, and 1:1 for the second experiment. The foods were formulated using three protein sources, casein, royal jelly or Feed-Bee®, and sucrose as digestible carbohydrate (Table 1). Royal jelly (Johannsmeier, 2001) and Feed-Bee® both contain a certain proportion of carbohydrate and this was accounted for during diet preparation. For all protein sources either casein or sucrose were added to make the desired ratios since casein had to be added to Feed-Bee® to reach the 1:1 ratio with Feed-Bee® being the major protein source (Table 1). These ratios were paired so as to give the following complementary food combinations (1:50 with 1:10, 1:50 with 1:1, 1:25 with 1:10 and 1:25 with 1:1) for each protein source. Each pair of complementary foods was fed to a different cage and replicated 5 times using different colonies, giving a total of 20 cages for each protein source.

All the foods were dried at 45 ^{o}C to remove moisture prior to weighing; water was then added to mix food into a homogeneous paste. Feeding tubes were replaced daily. After 24 h in the cages tubes were placed into the drying oven before reweighing and obtaining the final dry mass. Consumption was calculated as the difference in dry mass before and after feeding depending on the existing bees on each day.

In the same experiment we measured survival and ovarian activation. Each day dead bees were counted and removed from each cage, and on the last day honeybees that survived were frozen at -20^{0}C and stored. For ovarian activation determination, 10 bees from each food combination were selected for dissection from those frozen on day 14. Fourteen days are sufficient for ovarian activation to take place in *A. mellifera scutellata* (Ruttner and Hesse, 1981). The stages of activation were visually scored using a five point scale (Hess, 1942) (Fig. 2), and categorized as: 1) inactive ovary (stage 1), 2) intermediate active ovary (stage 2 and 3), and 3) active ovary (stage 4 and 5). To investigate if there was a correlation between ovarian activation and rectal volume, we scored the volume of the rectum as: 1) empty, 2) half filled and 3) full (Schäfer *et al.*, 2006).

2.2.3. Statistical analysis

The data passed the tests for normality and homogeneity of variance for each variable. To evaluate differences in cumulative consumption and average daily protein consumption between the different paired food combinations and protein sources, ANOVA with a post hoc Bonferroni test was performed, with the different pairs of foods and protein sources as the independent grouping variable and consumption as the dependent variable. Paired sample t-tests were used to test for differences in consumption between the pairs of foods presented. Kruskal-Wallis ANOVA with the post hoc test (multiple comparisons of mean ranks) was performed with each protein source or different food combination as grouping variable and ovarian activation, rectal volume, average daily survival, average daily consumption of total food and carbohydrate, and cumulative P:C ratio as dependent variables. Kaplan-Meier survival regression analyses were conducted for all pairs of food combinations in each protein source with the different protein sources as grouping variable. Multiple regression analysis was used for testing for interaction between factors. All the analyses were performed using STATISTICA software (StatSoft, Inc., Tulsa) version 7.

2.3. Results

2.3.1. Consumption

In the first the experiment, honeybees fed the pure casein and pure sucrose combination consumed significantly more sucrose than casein (T = 21.35, $P < 0.001$). There were no significant differences in daily consumption of casein ($F_{(13, 56)} = 1.87$, *n.s*) and sucrose ($F_{(13, 56)} = 1.58$, *n.s.*). In addition, there was no significant difference among the colonies for the cumulative protein ($F_{(4, 56)} = 0.74$, *n.s*) and carbohydrate ($F_{(4, 56)} = .74$, *n.s.*) consumption and honeybees preferred an average P:C of 1:17. When honeybees were fed the combination of pure casein and sucrose, the average mortality after the 14 days of the experiment was 6.8 %.

In the second experiment, colonies fed on casein, royal jelly or Feed-Bee® diets (hereafter referred to CA, RJ or FB respectively) with different P:C combinations (1:25 with 1:1, 1:25 with 1:10, 1:50 with 1:1, and 1:50 with 1:10) showed significant differences between the intake of the two macronutrients (CA: T = -50.71, N= 558, $P < 0.001$, RJ: T = -66.76, N = 558, $P < 0.001$, FB: T = -50.91, N= 558, $P < 0.001$), ingesting more of the carbohydrate. The average daily consumption of total food (including other nutrients in addition to protein and carbohydrate), protein and carbohydrate from the different protein sources is summarized in Table 2. There were significant differences among the three protein sources in the average consumption of total food ($H_{(2, n = 840)} = 138.41$; $P < 0.0001$), protein ($H_{(2, n = 840)} = 198.52$; $P < 0.0001$) and carbohydrate ($H_{(2, n = 840)} = 87.58$; $P < 0.0001$).

2.3.2. Reaching the intake target

When different colonies were fed with CA, RJ or FB diets, there were no significant differences either in the cumulative protein and cumulative carbohydrate consumption within each protein source (cumulative protein: CA ($F_{(4, 20)} = 0.044$, *n.s).;* RJ *($F_{(4, 20)}$* = 0.079, *n.s*).*;* FB ($F_{(4, 20)} = 0.36$, *n.s.*), and cumulative carbohydrate: CA ($F_{(4, 20)} =0.05$, *n.s*),

RJ ($F_{(4, 20)}$ = 0.067, *n.s.*) FB ($F_{(4, 20)}$ = 0.28, *n.s.*)) (Fig. 3A, B and C). The figures show that the different colonies showed no variation in their cumulative protein and carbohydrate intake within each protein source over 3 day intervals for 14 days (day 1-3, 4-6, 7-9, 10-12 and 13-14).

Cumulative protein consumption among the three protein sources showed significant differences ($F_{(2, 72)}$ =5.17, $P < 0.05$), whereas cumulative carbohydrate consumption did not ($F_{(2, 72)}$ =1.15, *n.s*). There was no colony effect on preferred P:C ratio within CA ($F=_{(4, 20)}$ = 0.039, *n.s.*), RJ ($F_{(4, 20)}$ = 0.17, *n.s.*) or FB ($F_{(4, 20)}$ =1.5209, *n.s.*) diets. However, there were significant differences (Kruskal-Wallis ANOVA: $H_{(2, n = 75)}$ = 42.59; $P < 0.0001$) in the preferred P:C ratio among CA, RJ and FB diets: 1:12, 1:14 and 1:11 respectively (Fig. 3A, B and C). Results from paired t-tests also showed that honeybees fed unequally from the two foods provided to them in all the treatments in each protein source except for the CA 1:25 and 1:1 combination (Table 3).

2.3.3. Survival

Of the total 2000 honeybees fed with different pairs of complementary imbalanced foods within each protein source, 205 (10.25%) which fed on CA, 711 (35.55%), which received RJ and 754 (37.7%) of the FB treatment died before the experiment was completed. Significant interactions ($F_{(3,836)}$ =179.34, $P < 0.0001$; R^2 = 0.39) between survival and consumption of total food, protein and carbohydrate were found from the multiple regression analysis. Survival showed a significant positive relationship with average daily consumption of protein and carbohydrate. However survival showed a significant negative relationship with total consumption (including protein plus carbohydrate plus other nutrients).

As shown by Kaplan-Meier survival regression analysis, cumulative survival of bees sustained on the different food combinations within CA, RJ or FB diets showed significant variation (CA: X^2 = 11.03; df = 3; $P < 0.05$, RJ: X^2 = 30.15; df = 3; $P < 0.001$; FB: X^2 =

24.52; *df* = 3; *P* < 0.001) (Fig. 4A, B and C). Average cumulative survival significantly differed among protein sources (X^2 = 457.76; *df* = 2; *P* < 0.0001; Fig. 4D). Cumulative survival was lower in bees sustained on RJ than on CA diets (Cox's F-Test: $F_{(410,\ 1422)}$ = 3.95 *P* < 0.0001), lower on FB than on CA diets (Cox's F-Test: $F_{(410,\ 1508)}$ = 4.40 *P* < 0.0001, and lower on FB than on RJ diets (Cox's F-Test: $F_{(1422,\ 1508)}$ = 1.16 *P* < 0.01). Results also showed that average survival per day significantly differed within the different food combinations in each protein source: CA, RJ or FB (CA: $H_{(3,\ n\ =\ 280)}$ =27.39; *P* < 0.0001; RJ: $H_{(3,\ n=\ 280)}$ =11.17; *P* < 0.05; FB: $H_{(3,\ n\ =\ 279)}$ =17.84; *P* < 0.001). However, the average daily consumption and the different food combinations showed weak negative correlation with survival (Spearman rank correlation: *n* = 840; *R* = -0.11; *P* < 0.05).

2.3.4. Ovarian activation

A total of 600 bees from the different paired food combinations and protein sources were dissected. Ovarian activation of bees that were fed different food combinations within CA (X^2 = 1.74, *df* = 6 *n.s.*), RJ (X^2 = 5.57, *df* = 6 *n.s.*) and FB (X^2 = 10.52, *df* = 6, *n.s.*) did not differ significantly (Fig. 5A, B and C). However, there were significant differences in ovarian activation among the honeybees sustained on the different protein sources (*H* (2, *n* = 600) =131.53; *P* < 0.0001, Fig. 5D). Workers that consumed RJ had significantly more active ovaries than workers that consumed FB (*P* < 0.001) and CA (*P* < 0.0001) diets. Workers fed with FB diets showed more active ovaries than those fed CA (*P* < 0.01).

In general, for all three protein sources, ovarian activation and rectal volume of workers were negatively correlated (Spearman rank order correlation: *n* = 600; *R* = -0.223; *P* < 0.0001). In the bees fed on FB diet, ovarian activation was negatively correlated with rectal volume (Spearman rank order correlation: *n* = 200; *R* = -0.62; *P* < 0.05). The volume of the rectum varied significantly ($H_{(2,\ n=\ 600)}$ =130.1793 *P* < 0.0001) between bees sustained on the three protein sources (Fig. 6D). Honeybees sustained on RJ or FB diets had higher average rectal volume than those sustained on CA diets (*P* < 0.001). However, the rectal volume of bees fed RJ or FB diets did not differ. Bees sustained on the different food

combination of the same protein source (CA, RJ or FB) did not show significant variation in rectal volume (CA: $X^2 = 4.22$, *df* = 6, *n.s*; RJ: $X^2 = 2.17$, *df* = 6, *n.s*; FB: $X^2 = 10.08$, *df* = 6, *n.s*) (Fig. 6A, B and C).

2.4. Discussion

When caged worker honeybees were given complementary imbalanced food pairings, they behaviourally self-selected or balanced their diet and maintained their intake target by switching between the complementary foods in accordance with the composition of the food and the type of protein that they encountered. They regulated the intake of both protein and carbohydrate when fed different food combinations with different P:C ratios and preferred average P:C ratios of 1:12, 1:14 and 1:11 on casein, royal jelly and Feed-Bee® diets respectively. This indicates that worker bees were able to discriminate among different P:C ratios in the complementary foods provided.

Cumulative carbohydrate consumption on the three protein sources did not differ, but cumulative protein consumption did. This implies that colonies may change the amount of protein consumed to compensate for changes in protein quality. Indeed, this compensation for change in protein quality has been shown in caterpillar *Spodoptera littoralis*, (Lee, 2007). In connection with this, colonies also ingested different amounts of the total food and showed different intake targets on the different protein sources (Table 1). Generally the reason why colonies ingested more on the Feed-Bee® diet compared to the royal jelly diet may be a response to a lower quality diet (Slansky, 1993; Simpson *et al*., 2004; Lee, 2007).

In addition to protein quality, the complexity of the food has been shown to affect the intake target as well. When honeybees were restricted to a combination of pure casein and sucrose, they preferred an intake target of 1:17 P:C ratio, which was different from that obtained when fed casein diets with the different combinations, which is 1:12. In the pure food combination, the extreme nutrient content may have made it more difficult for the bees to reach the intake target than the later food pair combinations.

Survival showed a significant positive relationship with daily consumption of protein and carbohydrate. However it showed significant negative relationship with total consumption, which may be related to other nutrients found in the royal jelly and Feed-Bee® diets, unlike the casein diet, which only contains pure protein and carbohydrate. Lipid found in the royal jelly and Feed-Bee® diets may contain some fatty acids like oleic acid, which according to Manning *et al.* (2007) reduces the life span of honeybees when the concentrations is more than 2 %. The other reason is as honeybees do not defecate in the nest and can only defecate in flight (Michener, 1974; Barker, 1977), the accumulation of waste material in the rectum may depend on the amount of other nutrients found in each protein source, and may lead to premature death (Maurizio 1950; De Groot, 1953). These may be the reasons for the observed high mortality in those bees fed on royal jelly and Feed-Bee® diets compared to casein diets. This was also more pronounced on Feed-Bee® diets than royal jelly diets, which may again be due to ingestion of more food on Feed-Bee® diets.

Differences in survival between the different food combinations (1:25 with 1:1 and 1:50 with 1:1) and (1:25 with 1:10 and 1:50 with 1:10) may be due to ingestion of an excess of one of the high P:C ratio diets compared to the other complementary diet, as many animals have limited capacity to cope with nutrient excess (Raubenheimer 1992; Raubenheimer and Simpson, 1997). Excess protein has been shown to shorten the lifespan of honeybees (Standifer *et al.*, 1960; Pirk *et al.*, 2009) and ants (Dussutour and Simpson, 2009). However, bees were able to compensate or dilute the effects of other nutrients to the extent where at least 60% survived the 14 day experiment. In another study, caged honeybees fed on pure royal jelly alone showed 100 % mortality within three days (Lin and Winston, 1998). Similarly caged worker bees provided with no-choice diets in three different P:C ratios of 3:1, 1:1, and 1:3 using royal jelly or aloe pollen as protein source showed 100 % mortality within 5 and 8 days respectively (Pirk *et al.*, 2009).

Honeybees fed on the different food combinations within each protein source showed the same levels of ovarian activation. One of the reasons for this may be because honeybees fed

within each protein source maintained their intake target. However, ovarian activation varied significantly between protein sources. Ovarian activation is a nutrient limited process, depending on the availability and quality of food provided (Wheeler, 1996; Herbert *et. al*., 1977; Lin and Winston, 1998; Pernal and Currie, 2000; Hoover *et al.*, 2006). Colonies fed on royal jelly showed greater ovarian activation than those on casein and Feed-Bee® diets. Pirk *et al*. (2009) found higher ovarian activation on royal jelly diets compared to aloe pollen (*Aloe greatheadii var davyana*) and casein as protein sources in a no-choice experiment, which is due to quality of royal jelly to support development.

Due to social interaction among caged workers, they have two ways of obtaining protein for oogensis, either being fed by nestmates or feeding for themselves and those fed by other bees consume little and become reproductively active (Schäfer *et al.*, 2006). Supporting this, we found a strong significant relationship between rectal volume and ovarian activation in bees fed royal jelly and Feed-Bee®. The higher activation on Feed-Bee® diets compared to casein diets may be due to ingesting more protein from the Feed-Bee® diets than casein diets, which promotes the hypopharyngeal gland development for the rest of the workers to receive produced royal jelly by trophallaxis. The main reason for lower ovarian activation in those bees fed on casein compared to royal jelly and Feed-Bee® diets may be due to the lack of lipids in the casein diets. Lipids are important for maturation and ovarian activation (Dadd, 1985; Salomon *et al*., 2008). Interestingly, in two of the cages on Feed-Bee® diets, bees were observed to have active wax glands that enabled them to build a small comb on the foundation sheet (then they laid eggs in it on the last two days of the experiment): this indicates that the bees received all the necessary nutrients for developing their wax glands (see appendix 1).

The most important finding from the present study is that when honeybees were given complementary imbalanced food pairings, they behaviourally self-selected or balanced their diet and maintained their intake target by switching between the complementary foods in accordance with the complexity of the food and the type of protein that they encountered. Such kind of compensatory feeding to compensate for changes in the nutrient density has

been shown in other social insects like the ant, *R. metallica* (Dussutour and Simpson, 2008a). Similarly ants (*L. niger*) were able to integrate foraging decisions based on food type and unrelated factors such as the presence of brood (Portha *et al*., 2004) and also food quality (Beckers *et al*., 1993). Another ant species (*Solenopsis invicta*) showed individual ability to assess food type and quality to maintain their intake target (Cassill and Tschinkel, 1999). The position of the intake target can also be estimated using performance criteria like survival, development time and can be calculated from the growth target (Simpson *et al*., 1995), because an animal performs optimally when the proportion of nutrients in the food equals the self-selected intake target (Raubenheimer and Simpson, 1993; Behmer and Joern, 2008). Survival and ovarian activation varied between casein, royal jelly and Feed-Bee® as protein sources.

Why did honeybees on casein diets show high survival and low ovarian activation, and vice versa on royal jelly or Feed-Bee® diets? According to Maklakov *et al*. (2008) this may be because nutrient intake that improves survival results in poor reproductive performance and vice versa. For instance, in *Drosophila meligaster*, egg production was maximized at a cost to longevity: longevity is greatest on P:C ratio of 1:16, but the egg-laying is highest at a P:C ratio of 1:2 (Lee *et al.*, 2008). In addition, the absence of other nutrients in casein and their presence in royal jelly and Feed-Bee® diets may affect the preferred P:C ratio and the two performance measures used. Therefore in addition to the protein carbohydrate balance, the presence of other nutrients should also be considered in honeybee feed formulation; for this the recently developed pollen substitute, Feed-Bee®, is a good example.

Depending on the protein type the bees altered their protein to carbohydrate intake. In addition to protein quality that was discussed earlier, the variation in intake target between protein sources might be a strategy used to balance other nutrients like lipid and minerals found in royal jelly and Feed-Bee® diets compared to casein diets which only contain protein and carbohydrate. But when feeding on the different food combinations within each protein sources, the bees achieved the same intake targets and also showed similar levels of performance, which clearly indicate the ability of honeybees to self-select their diet.

Dietary self-selection behaviour helps insects to maintain optimal protein and carbohydrate intakes that are near to the optimal in terms of fitness (Waldbauer and Friedman, 1991; Simpson *et al*., 2004). The self-selected protein and carbohydrate intakes are optimal for adult worker bees, because in the absence of brood the nutrient intake is directly related to their own physiological requirements. The choice situation simulated in this experiment may reflect the behaviour of nurse bees where they have to choose protein, carbohydrate, and other nutrient requirements from stored pollen and nectar in a hive, either for their own nutritional requirements or for those of other colony members including larvae.

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2.7. Tables and Figures

Table 1. Preparation of diets. The mass (g) of each constituent per g food is given for each protein source and each P:C ratio. Diet composition was calculated based on literature data on nutrient levels in royal jelly (Johannsmeier, 2001) and Feed-Bee®.

Protein Source	P:C Ratios			
	1:50	**1:25**	**1:10**	**1:1**
Casein	0.0196 g CASEIN 0.9804 g SUCROSE	0.0384 g CASEIN 0.9615 g SUCROSE	0.0909 g CASEIN 0.9091 g SUCROSE	0.500 g CASEIN 0.500 g SUCROSE
Royal Jelly (RJ)	0.949 g SUCROSE 0.051 RJ	0.901 g SUCROSE 0.099 g RJ	0.776 g SUCROSE 0.224 g RJ	0.047 g SUCROSE 0.953 g RJ
FEED-BEE®(FB)	0.9468 g SUCROSE 0.0532 g FB	0.8967 g SUCROSE 0.1033 g FB	0.7631 g SUCROSE 0.2369 g FB	0.05123 g CASEIN: 0.9488 g FB

Table 2. Average consumption (in mg per bee per day) of total food, protein and carbohydrate. Experiment 1 included pure casein and sucrose. Experiment 2 included three protein sources (casein, royal jelly or Feed-Bee®) with P:C ratios of 1:25 with 1:1, 1:25 with 1:10, 1:50 with 1:1, and 1:50 with 1:10, fed to five different colonies. In the casein diets the total food consumed is the sum of the protein and carbohydrate consumed since it is a pure protein source, but in royal jelly and Feed-Bee® it is not, because they contain other nutrients in addition to protein and carbohydrate. Values are means ± s.e.m.

Experiment no	Protein source	Consumption in mg per bee per day			P:C ratio
		Total food	Protein	Carbohydrate	
Experiment 1	**Casein**	12.87 ± 0.54	0.55 ± 0.02	12.32 ± 0.54	01:17
Experiment 2	**Casein**	13.02 ± .23	1.01 ± 0.03	12 ± 0.21	01:12
	Royal jelly	14.83± 0.19	0.807 ± 0.02	13.4 ± 0.19	01:14
	FEED-BEE®	17.71 ± 0.29	1.32 ± 0.04	15.56 ± 0.28	01:11

Table 3. Paired sample t-tests showing difference in consumption between the two foods presented together for each combination of foods and each protein source. Significant values (in italics) indicate that honeybees did not feed randomly from the two foods presented to them.

Protein source	***Combination***	***t***	***df***	***P***
Casein	**1:25 with 1:10**	22.95	69	*.000*
	1:25 with 1:1	1.71	69	**.091**
	1:50 with 1:10	17.78	69	*.000*
	1:50 with 1:1	2.19	69	*.031*
Royal jelly	**1:25 with 1:10**	26.78	69	*.000*
	1:25 with 1:1	8.66	69	*.000*
	1:50 with 1:10	27.94	69	*.000*
	1:50 with 1:1	7.39	69	*.000*
Feed-Bee®	**1:25 with 1:10**	15.45	69	*.000*
	1:25 with 1:1	-3.17	69	*.002*
	1:50 with 1:10	26.19	69	*.000*
	1:50 with 1:1	-3.25	69	*.002*

Figure 1: Standard hoarding cages with feeding tubes (plastic tubes with standard windows 2 x 1 cm cut into them), through which bees can access food and water.

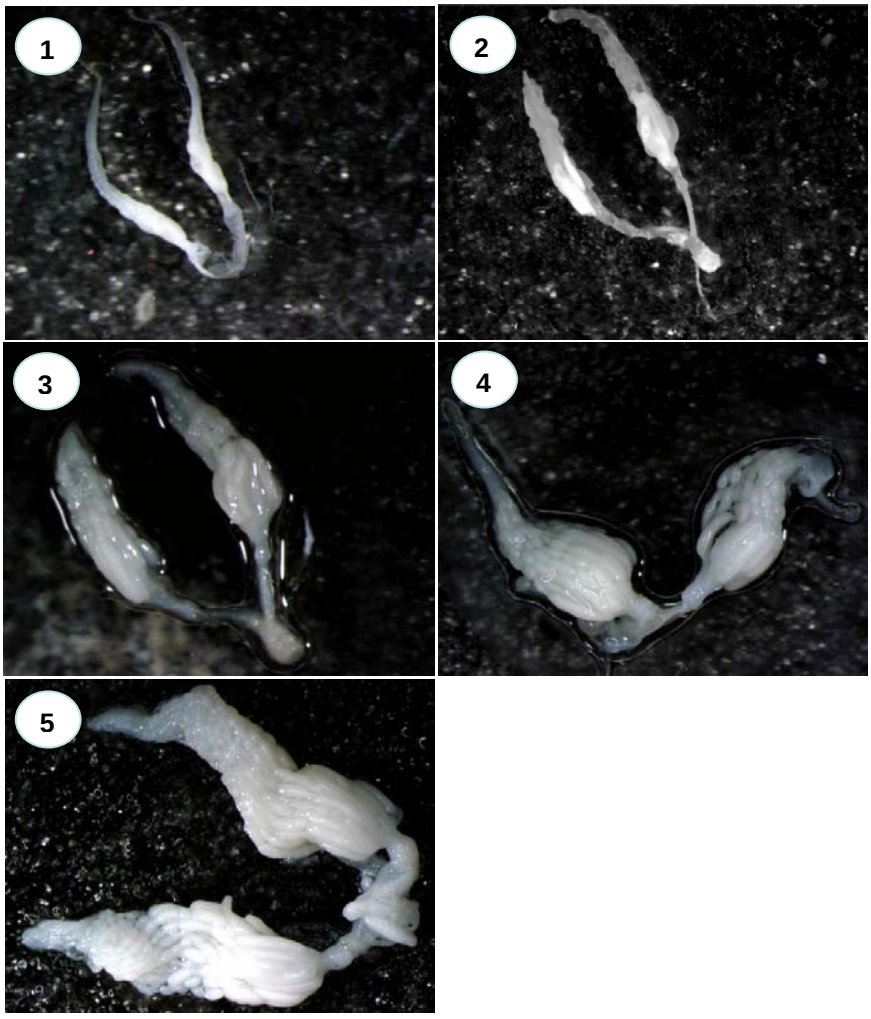

Figure 2: Different stages of ovarian activation: 1) inactive thread-like ovarioles without vitellus, 2) swollen ovarioles without vitellus, 3) swollen ovarioles with visible vitellus but without distinct oocytes, 4) the same as 3 but with distinct ovarioles and immature oocytes, 5) fully activated ovaries with distinct mature oocytes. The pictures were taken using Stereo microscope, Nikon SMZ800, Tokyo, Japan.

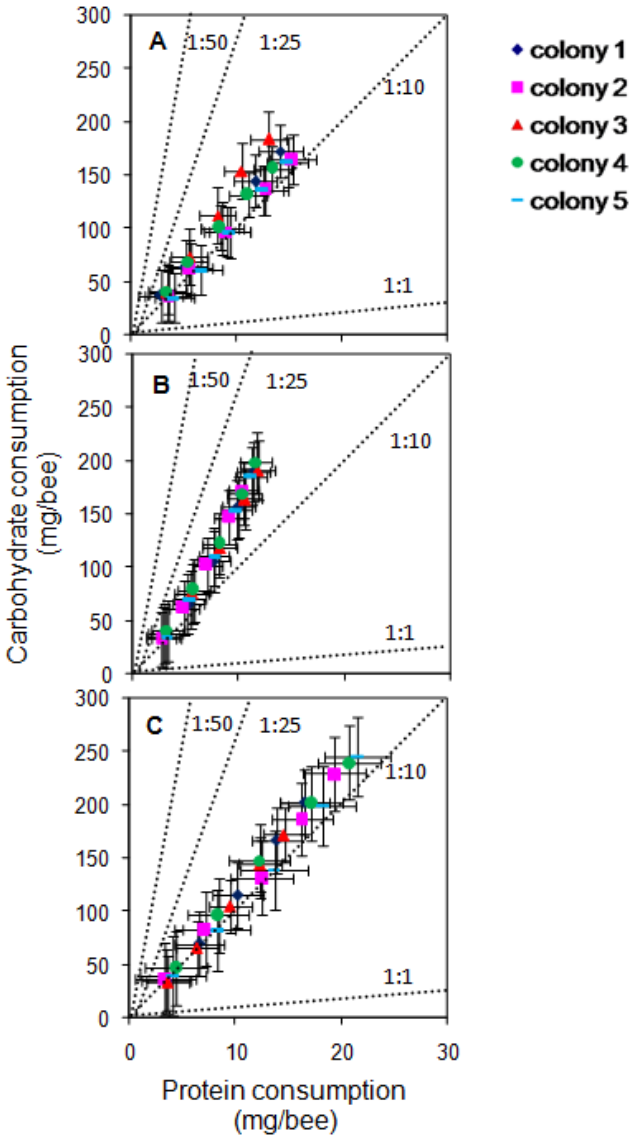

Figure 3. Bivariate plots showing average cumulative protein and carbohydrate consumption per bee (mean ± s.e.m) by different colonies of honeybees *(Apis mellifera scutellata)* over 3 day intervals for 14 days (day 1-3, 4-6, 7-9, 10-12 and 13-14) fed a) casein (CA), b) royal jelly (RJ) and c) Feed-Bee® (FB) diets with different pairs of food combinations (1:25 with 1:1, 1:25 with 1:10, 1:50 with 1:1, and 1:50 with 1:10). For each protein source, different colonies maintained their cumulative protein and carbohydrate intake to non statistical significant different points. There were significant differences in the preferred P:C ratio among CA, RJ and FB diets: 1:12, 1:14 and 1:11

respectively. Dotted lines radiating from the origin indicate the P:C ratio for the 1:50, 1:25, 1:10 and 1:1 diets.

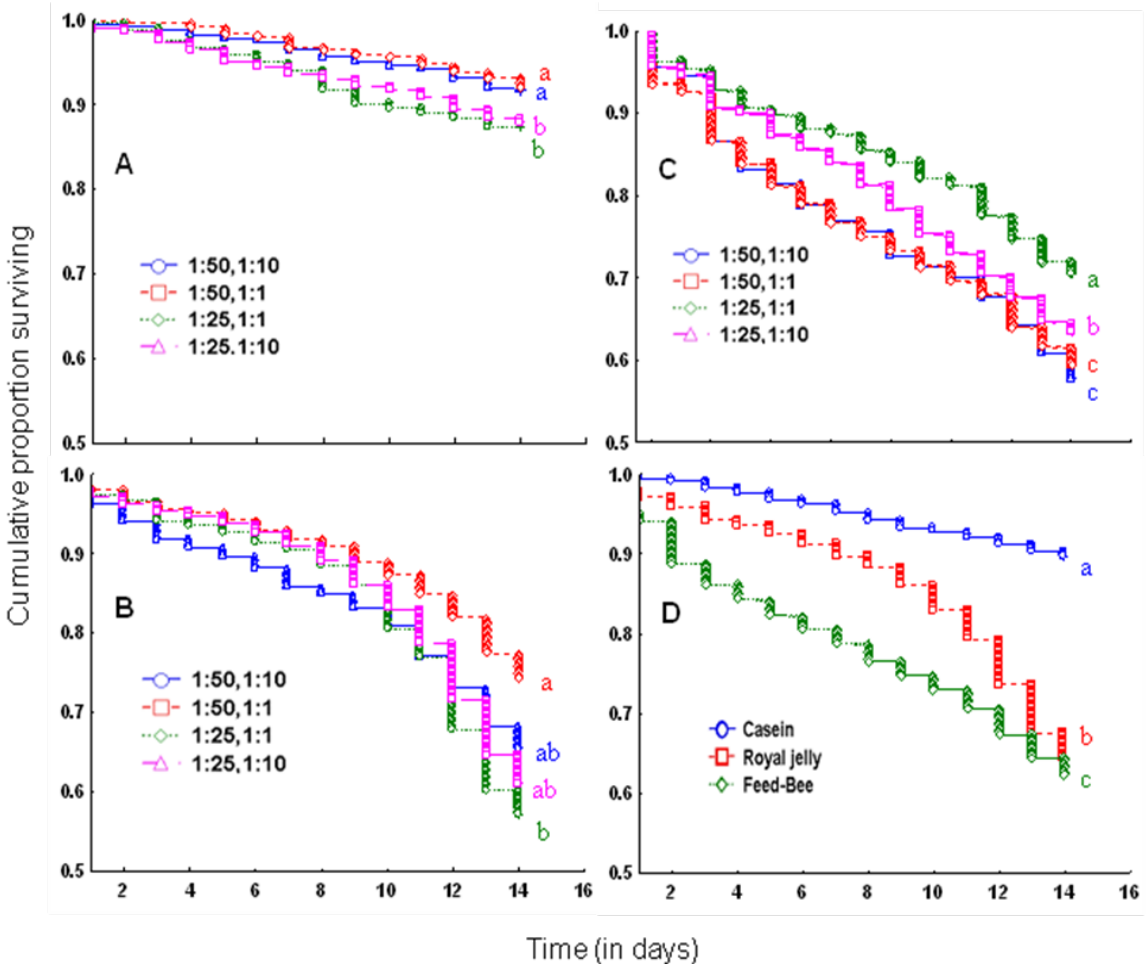

Figure 4. Cumulative proportion of bees surviving per day, sustained on the different pairs of food combinations: 1:25 with 1:1, 1:25 with 1:10, 1:50 with 1:1, and 1:50 with 1:10, using different protein source: a) casein, b) royal jelly and c) Feed-Bee®; and d) comparison among the three protein sources, using Kaplan-Meier survival regression analysis. Different letters denote significant differences at $p < 0.05$ using Cox's F test.

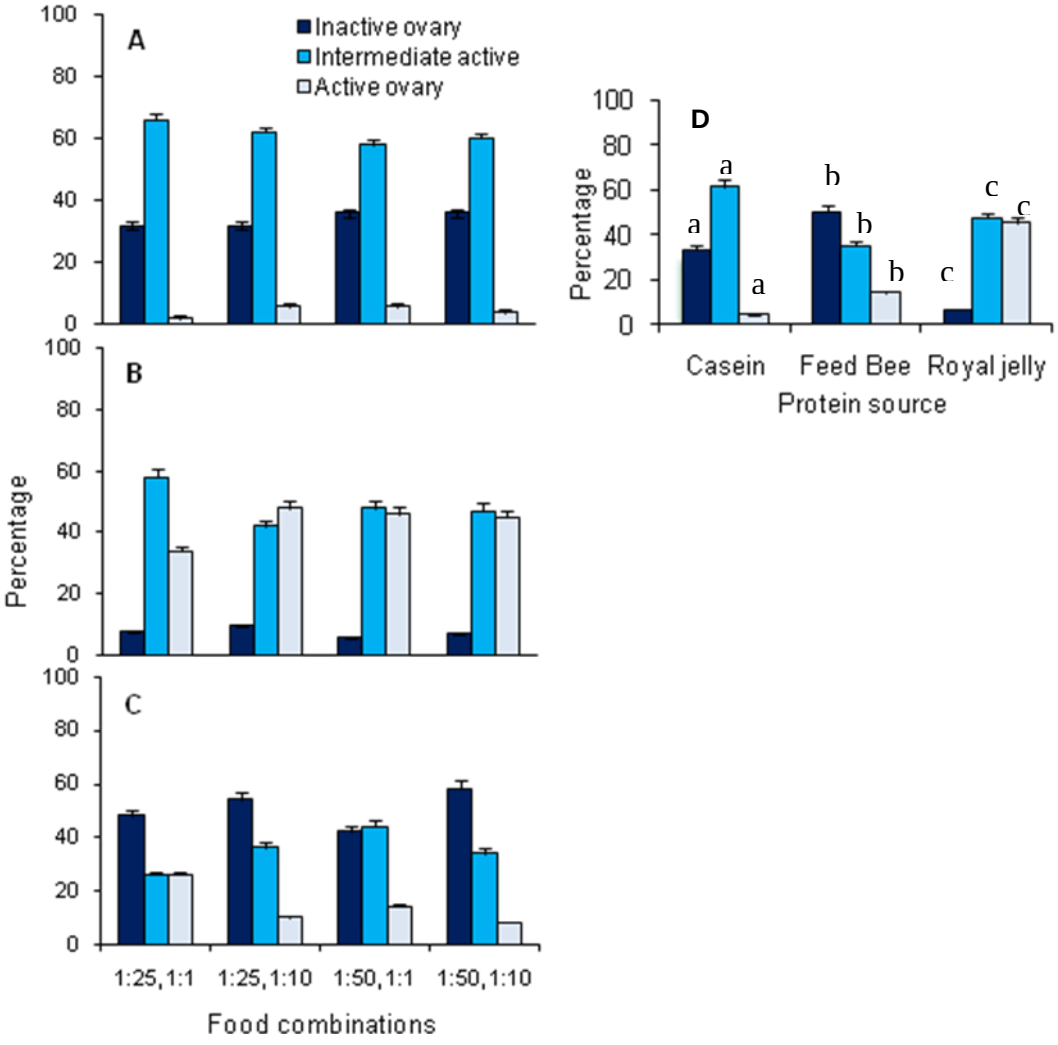

Figure 5. Bar graph showing percentage of mean ovarian activation of honeybees *(Apis mellifera scutellata)* sustained on three protein sources: a) casein, b) royal jelly and c) Feed-Bee® with different pairs of food combinations (1:25 with 1:1, 1:25 with 1:10, 1:50 with 1:1, and 1:50 with 1:10); and d) comparison among the three protein sources, different letters denote significant differences between groups among the protein sources. The level of activation was scored as: 1). inactive, 2). intermediate active and 3). active ovary.

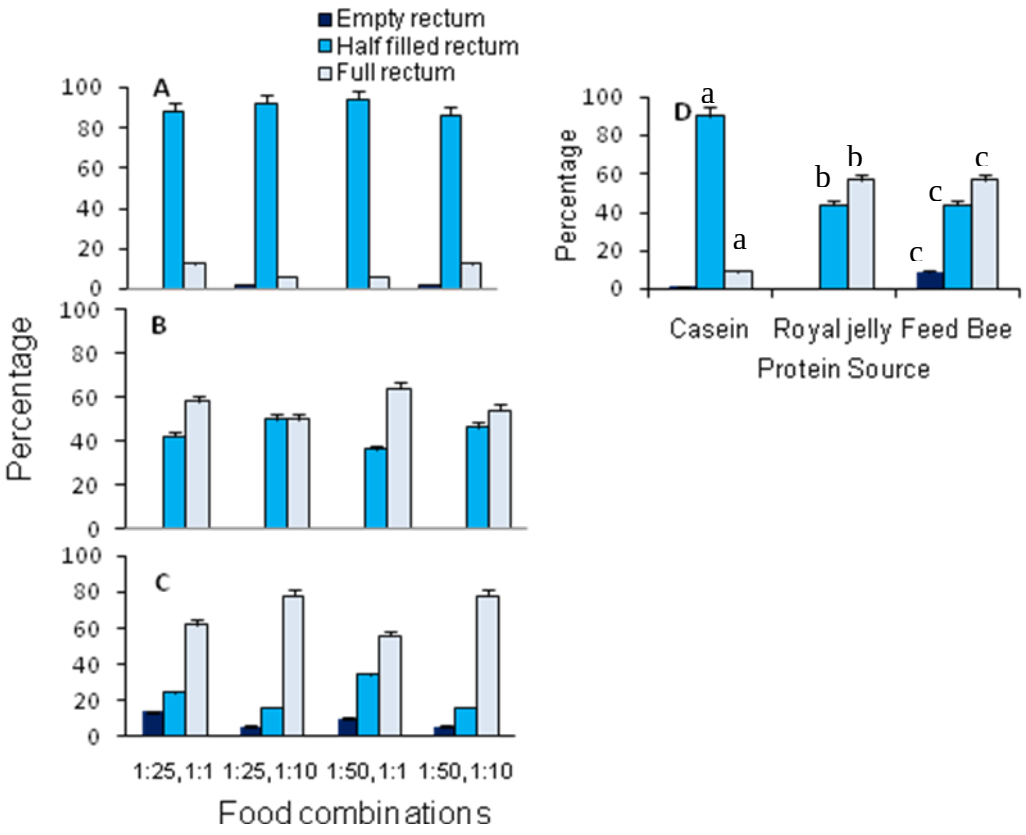

Figure 6. Bar graphs showing percentage of mean rectal volume of honeybees fed on three protein sources: a) casein, b) royal jelly and c) Feed-Bee® with different pairs of food combinations (1:25 with 1:1, 1:25 with 1:10, 1:50 with 1:1, and 1:50 with 1:10); and d) comparison among the three protein sources, different letters denote significant differences between groups among the protein sources. The volume pattern of rectum was scored as: 1). empty, 2). half full and 3). full.

Chapter 3:

Estimation of nutrient regulation based on haemolymph protein concentration and HPG development of caged honeybees (*Apis mellifera scutellata*) fed on Feed-Bee®

Abstract

In this study we measured honeybee physiological parameters to see whether newly emerged workers regulated their growth target by varying nutrient intake when confined in cages on different imbalanced complementary diets. The diets were formulated using the pollen substitute Feed-Bee® as a protein source. The parameters measured were hypopharyngeal gland (HPG) development, protein concentration in the haemolymph and head fresh mass. Protein concentration in the haemolymph ranged from 6.51 ± 0.59 to 12.3 ± 1.15 µg/µl (mean ± S.E.) between emergence (day 0) and day 14, the peak being on day 9. The acini diameter and area of HPG were maximal in 6 day old bees and reduced in 14 day old bees. Worker bees fed on the different diet combinations with different protein to carbohydrate (P:C) ratios maintained each of the performance measures at similar levels, which shows that honeybees maintained the required nutrients from each combination, which is supportive evidence for the ability of honeybees to self-select their diet.

3.1. Introduction

Pollen is the major natural source of protein in the honeybee diet and protein is essential for productivity of a colony (Kleinschmidt and Kondos, 1976). When there is a scarcity of natural pollen bees have to be provided with pollen substitutes or supplements. The aim of supplementary feeding using pollen substitutes or supplements is mainly to bridge the dearth periods by maintaining the colony strength for the coming nectar and pollen flow, as well as prior to the flowering of crops for pollination. Pollen supplements and substitutes should consider the nutritional value to honeybees. They should simulate the natural food source in nutrient content, texture and consistency, so that it can be easily accepted by honeybees and digestibility, anti-nutritional factors, shelf life, availability and cost should also be considered (Saffari *et al.*, 2006). Taking these factors into account, a newly developed pollen substitute, Feed-Bee®, performed the same as or more than pollen does (Saffari *et al.*, 2004; De Jong *et al.*, 2009). Feed-Bee® resulted in a higher protein concentration in honeybee haemolymph than pollen or acacia pod flour (De Jong *et al.*, 2009). In another laboratory experiment, honeybees fed on Feed-Bee® consumed and weighed as much as other honeybees that consumed freshly collected pollen (Gregory, 2006). Field tests with colonies showed that Feed-Bee® has been accepted and also enhanced brood rearing as well as pollen (Saffari *et al*., 2004).

In addition to focusing on the factors that are well explained by Saffari *et al.* (2004), it is shown that feeding single pollen or pollen substitute or supplements only may not provide the proper balance and amount of nutrients required by bees (Human *et al*., 2007). Thus honeybee feed formulation schemes should be based on the intake target of the bees as discussed earlier in chapter one. In our experiments we used Feed-Bee® diet as a protein source because it may enable the bees to perform optimally to reach their growth target, and moreover since Feed-Bee® is an industrially produced product, its nutritional composition is known (see appendix 2) and therefore we can easily correlate the intake target with the

honeybees traits such as hypopharyngeal gland (HPG) development, protein concentration in the haemolymph and fresh head mass.

Hypopharyngeal glands (HPG) are paired glands located in the head of honeybees that produce proteinaceous secretions (Huang & Otis, 1989; Hrassnigg & Crailsheim, 1998). HPG activity depends on worker bee age (Huang & Otis, 1989) and its stage of development determines worker bees task (Free, 1961). HPG development is influenced by the quality and quantity of pollen ingested by workers and the size of the hypopharyngeal gland, as measured by acini diameter, is related to its total protein content (Brouwers, 1983). The protein secreted by the glands is mainly derived from the food consumed by the bees (Crailsheim *et al*., 1992); thus HPG development is a reliable measure of dietary protein assimilation in newly emerged caged workers (Crailsheim & Stolberg, 1989). Generally, the condition of the HPG is used to describe the physiological status of worker honeybees (Hrassnigg & Crailsheim, 1998). HPG development gives a direct measure of fitness in bees, since it reflects any inherent differences in young workers or brood (Huang *et al.*, 1989).

In addition to HPG, haemolymph also has the potential to provide information about an insect's nutritional state, as haemolymph composition varies with quality of food and metabolic and growth demands of the insect (Simpson et al., 1995). Haemolymph plays a central role in pre-ingestive regulation as its composition changes with time since the last meal, amount and/or quality of food eaten, metabolic activity, and physiological needs of an insect (Simpson & Raubenheimer, 1993; Simpson *et al*., 1995; Behmer, 2009). When the haemolymph nutrient concentration decreases or becomes imbalanced, an insect starts to search for food along with determining the acceptability of the food (Behmer, 2009). The concentration of protein in the hemolymph is shown to be a rapid and reliable way of determining protein assimilation from a diet, and the quality of a protein diet is reflected by the protein content in the haemolymph (Cremonez et al., 1998).

Measuring these physiological parameters as indicators of adult bee performance enables us to estimate the position of the intake target (Simpson *et al*., 1995). An animal performs optimally when the proportions of nutrients in the food correspond to intake target (Raubenheimer and Simpson, 1993). Previously, in chapter 2 we investigated the behaviour of honeybees when provided with complementary imbalanced foods, and showed that they switched between two imbalanced but complementary foods, and achieved their intake target according to the composition of the food and the type of protein.

In this chapter we again fed caged worker bees imbalanced but complementary diet combinations having different protein:carbohydrate (P:C) ratios and measured performance based on the following physiological parameters: HPG development as measured by the acini diameter and area, protein concentration in the haemolymph and head fresh mass. Fresh mass of the head has been described as a simple and rapid method to describe glandular development (Hrassnigg & Crailsheim, 1998). We confined the bees to Feed-Bee®-based diets, in order to investigate the appropriateness of Feed-Bee® as a protein source.

3.2. Materials and Methods

3.2.1. Diet preparation and protocols

Three *Apis mellifera scutellata* colonies from the University of Pretoria apiary site were used to collect frames of capped worker brood, which were incubated at 34^{o} C to obtain newly emerged adult worker bees. Groups of 100 newly emerged workers from each colony were confined in standard hoarding cages (11 x 8.5 x 7 cm) closed at both the front and back with glass slides. The bottom of the cage was closed with wire mesh to allow ventilation. Below the glass slide at the front of each cage, a plastic frame was placed, with round windows through which three stoppered feeding tubes were inserted into the cages. Two of the feeding tubes were used for providing complementary imbalanced foods and the

third for providing water (Chapter 2, Fig 1.). All the cages were placed under standard conditions in an incubator at a temperature of 34 ^{o}C and 55-65 % RH for 14 days, and kept in the dark to simulate conditions within the colony.

The diets were prepared based on Feed-Bee®, with the protein and carbohydrate content considered in diet preparation (Chapter 2, Table 1). The same four different P:C ratios (1:50, 1:25, 1:10, and 1:1) were used as imbalanced foods as in the previous experiment (Chapter 2). The same complementary P:C ratios (1:50 with 1:10, 1:50 with 1:1, 1:25 with 1:10 and 1:25 with 1:1) were fed to four different cages, repeated three times using three different colonies, giving a total of 12 cages.

After drying until all the moisture was removed, dry masses were recorded before adding water to mix the food into a homogeneous paste. Feeding tubes were replaced daily after 24 h in the cages. They were removed and placed into the drying oven before reweighing and obtaining the final dry mass. Consumption was calculated from the change in dry mass. Based on the consumption data we determined the relative proportion of protein to carbohydrate ingested from each pair of complementary diets over 14 days (i.e. their preferred intake target). To confirm the level of self selection and evaluate the appropriateness of Feed-Bee® diet as a protein source we measured the following physiological parameters: haemolymph protein concentration, HPG development and head fresh mass, at days 0, 3, 6, 9 and 14.

3.2.2. Haemolymph protein concentration

3.2.2.1 Haemolymph extraction

Haemolymph was collected from a small incision at the level of the third dorsal tergite, using microcapillary tubes previously washed in a 0.1 % (w/v) phenylthiourea solution in water (Cremonez *et al.*, 1998). The haemolymph of 36 newly emerged worker bees less

than 24 h old (day 0) was collected, and then 100 bees were placed in each cage. After confinement and feeding on the different diets, the haemolymph of 3, 6, 9 and 14 day old workers was collected. The haemolymph of nine workers fed on each diet combination (three from each of the three replicates) was used for determination of protein concentration. To get adequate amount of haemolymph sample per cage, we pooled the haemolymph of three individual bees.

3.2.2.2 Protein determination

Protein concentration in the haemolymph was determined spectrophotometrically at 595 nm (Bradford, 1976), using bovine serum albumin (BSA) for the standard curve determinations at concentrations of 0-50 µg/ml. The protein reagent consisted of Coomassie Brilliant Blue G250 (100 mg) that was dissolved in 50 ml of 95 % ethanol. To this solution 100 ml of 85 % (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of 1 litre. The final concentrations in the reagent were thus 0.01 % (w/v) Coomassie Brilliant Blue G250, 4.7 % (w/v) ethanol, and 8.5 % (w/v) phosphoric acid (Bradford, 1976). 800 µl of the protein reagent was placed in a 10 ml test tube along with 200 µl of diluted haemolymph and then mixed. This solution was poured into a cuvette and the reading was taken after 5 min but before 1 hour (Cremonez *et al.*, 1998). Using the standard curve for 0-50 µg/ml BSA, we determined the protein concentration in the haemolymph after considering the dilution factors.

3.2.3. Head fresh mass, diameter and area of hypopharyngeal gland acini

Fresh mass of heads was measured, since it is a simple and rapid method to describe glandular development (Hrassnig & Crailsheim, 1998). Three bees were removed randomly from each cage on day 0, 3, 6, 9 and 14. The heads were weighed using analytical balance, (Ohaus), to the nearest 0.1 mg, and then heads were dissected for measuring the acini diameter and area of HPG. The heads were dissected with a razor blade (cutting from the

ocelli to the mandibles) under a stereoscopic microscope, and the HPG were removed. Each gland was mounted in a drop of distilled water (Malone *et al.,* 2004) on a glass slide and photographed using a Nikon transmission light microscope (Nikon Optiphot, Tokyo, Japan). Using these pictures, the areas (μm^2) and diameters (μm) of 10 acini per bee were measured using Image Tool® analysis software (version 3). The area was measured by tracing the circumference of each acinus and we also measured the acini diameters parallel to the axial duct (Fig. 1). Acini were chosen randomly by taking 10 acini from any side of the gland to avoid bias on the sizes of the acini.

3.2.5. Statistical analysis

To evaluate differences between the different diets, ANOVA with a post hoc Bonferroni test was performed, with diet as the independent grouping variable and haemolymph protein concentration, HPG acini diameter and area, and head fresh mass, as dependent variables. The relationship between haemolymph protein concentration and protein consumption, and between head fresh mass and HPG acini diameter and area, and between acini diameter and area was tested with correlation and regression analysis. Paired sample t-tests were used to test for differences in consumption between the diet pairs presented. Data for head fresh mass, acini diameter and area were pooled within each treatment after confining the bees to the different diet combinations. All variables were passed the tests for normality and homogeneity of variance. All tests were conducted with STATISTICA software (StatSoft, Inc., Tulsa, version 7).

3.3. Results

3.3.1. Nutrient intake

As previously observed in Chapter 2, the cumulative protein ($F_{(2, 12)} = 0.22$, $P = 0.80$) and cumulative carbohydrate ($F_{(2, 12)} = 0.32$, $P = 0.73$) consumed by the different colonies were not significantly different. Honeybees selected an average protein to carbohydrate ratio of

1:11 during the 14 days of the experiment. Results from paired t-tests also showed that honeybees fed unequally from the two foods provided to them in all the treatments (Fig. 2).

3.3.2. Haemolymph protein concentration

The mean protein concentration in the haemolymph showed significant variation ($F_{(4, 55)} = 5.3$, $P < 0.001$) between the different age groups, the peak being reached at day 9. The haemolymph protein concentration levels among 0, 3, 6 and 14 day old bees and between 9 and 14 day old bees showed no significant variation. Significant variation in mean protein concentration was shown between days 0 and 9 ($P < 0.01$), between days 3 and 9 ($P < 0.01$), and between days 6 and 9 ($P < 0.05$) (Fig. 3).

There was no significant effect of diet combination ($F_{(3, 44)} = 2.53$, $P = 0.069$) on the mean haemolymph protein concentration. Bees fed on the different diet combinations (1:50 with 1:1, 1:50 with 1:10, 1:25 with 1:1 and 1:25 with 1:10) showed similar protein concentrations in their haemolymph. In addition, results from the two-way interaction between the different age groups and diet combinations showed no significant effect on protein concentration in the haemolymph among bees fed on the different diet combinations within days 3, 6, 9 and 14 (two-way ANOVA: $F_{(9, 32)} = 0.73$, $P = 0.68$) (Fig. 4). The protein concentration in the haemolymph was significantly positively correlated with the cumulative protein consumed ($R^2 = 0.12$, $P < 0.05$).

3.3.3. Diameter and area of HPG acini, and head fresh mass

HPG acini diameters differed significantly between different age groups, including day 0 before bees were confined to the different food combinations ($F_{(4, 1585)} = 64.56$, $P < 0.0001$). Maximal development was observed in day 6. After pooling the data for each cage provided with a particular diet combination, again the different age groups (days 3, 6, 9 and 14) significantly affected the size of the acini diameter ($F_{(3, 44)} = 7.81$, $P < 0.001$). Acini

diameter significantly differed between days 3 and 14 ($P < 0.001$), between days 6 and 14 ($P < 0.01$), and between days 9 and 14 ($P < 0.05$), however it did not differ significantly among days 3, 6, and 9 (Fig. 5).

In addition to the acini diameter, we also measured the acini area of the HPG as a means of showing the level of the gland development. There were significant age effects on the acini area ($F_{(4, 1585)} = 65.1$, $P < 0.0001$). Maximal development was observed on day 6. Pairwise comparison among the different age groups showed that acini area among days 0, 3, 6 and 9 showed no significant variation, however, there was significant variation between days 3 and 6, and days 9 and 14 ($P < 0.0001$). After pooling the data for each cage provided with the different diet combinations, again the different age groups (day 3, 6, 9 and 14) were shown to significantly differ in acini diameter ($F_{(3, 44)} = 8.5$, $P < 0.001$). Acini diameter differed significantly between days 3 and 14 ($P < 0.05$), between days 6 and 14 ($P < 0.001$), and between days 9 and 14 ($P < 0.01$), however it did not differ significantly among days 3, 6 and 9 (Fig. 6).

There was no significant effect of diet combinations on the acini diameter ($F_{(3, 44)} = 2.52$, $P = 0.07$). The different diet combinations significantly affected the acini area ($F_{(3, 44)} = 2.91$ $P < 0.05$). However pair-wise comparison between the different diet combinations within the different age groups showed that except between those bees fed on 1:25 with 1:10 and 1:25 with 1:1 diet combinations ($P < 0.0001$) in the third day, the remaining did not lead to statistical difference in acini areas. Results from the two way interaction between the different age groups and different diet combinations showed no significant effect on the acini diameter or area within the different age groups (two-way ANOVA: $F_{(9, 32)} = 2.18$, $P = 0.051$; $F_{(9, 32)} = 1.87$, $P = 0.094$ respectively). There was a strong correlation between the acini diameter and acini area measurements ($R^2 = 0.83$, $P < 0.0001$).

There was a significant age effect on the head fresh mass of bees ($F_{(4, 55)} = 3.13$, $P < 0.05$). The mean head fresh mass was significantly higher ($P < 0.05$) in day 0 bees than those on day 14. However there were no significant differences in head fresh mass among days 0, 3,

6 and 9 (Fig. 7). Even though there was a decrease in head fresh mass from day 3 to day 14, this was not statistically significant ($F_{(3, 44)} = 1.47$, $P = 0.24$). The different diet combinations with different P:C ratios did not significantly ($F_{(3, 32)} = 1.11$, $P = 0.24$) affect the head fresh mass. Results from the two way interaction between the different age groups and different diet combinations showed no significant effect on head fresh mass (two-way ANOVA: $F_{(9, 32)} = 0.75$, $P = 0.66$). Head fresh mass showed a poor and insignificant correlation with the size of the acini diameter and area, $R^2 = 0.055$, ($P = 0.11$) and $R^2 = 0.027$, ($P = 0.27$) respectively.

3.4. Discussion

Protein concentrations in the haemolymph of worker honeybees fed on the different diet combinations were not statistically different from each other. Bees fed on the different diet combinations had to eat different proportions of the two foods, in order to maintain the amount of protein to be ingested against varying P:C ratios in the diet pairings. This may be due to using the haemolymph as a feedback mechanism in maintaining the food to be ingested, as shown in locusts, since haemolymph plays a central role in feeding behaviour and nutritional compensation (Simpson & Raubenheimer, 1993).

In honeybees the concentration of protein in the haemolymph is a good indication of the degree of protein assimilation from the diet (Cremonz *et al.*, 1998) and the benefit that a particular feed is giving to the bees (Szymas and Jedruszak, 2003). Caged bees fed on Feed-Bee® diets showed average protein concentrations in the haemolymph that ranged from 6.51 ± 0.59 to 12.3 ± 1.15 µg/µl (mean ± s.e.) between emergence (day 0) and day 14, the peak being on day 9. Our results agree with the protein concentrations obtained by feeding caged bees on pollen and other diets (Cremonz *et al.*, 1998, Table 2) and also with a study in which bees were fed Feed-Bee® itself, pollen and other diets (De Jong *et al.*, 2009, Table 1). For instance, De Jong (2009) found 9.42 µg/µl protein in the haemolymph of 6 day old workers fed on Feed-Bee®, which is statistically the same as obtained by feeding on Bee-Pro® (8.95 µg/µl) but different from obtained by feeding pollen (6.26 µg/µl), acacia pod floor (6 µg/µl), and sucrose (3.56 µg/µl). We found 7.75 ± 0.96 µg/µl

(mean ± S.E.) protein in the haemolymph of 6 day old workers. Our result is also close to the protein concentration in the haemolymph obtained from 5 day old feral and domesticated honeybees taken from colonies (Aase *et al*., 2005).

In addition, honeybees fed on the different diet combinations within the different age groups maintained the acini diameter and area of the HPG to the same level. The main reason for the bees fed on the different diet combinations having the same level of HPG development may be because all bees maintained their optimal amount and balance of required nutrients. HPG development as measured by acini diameter is influenced by the quantity and quality of pollen ingested (Brouwers, 1983). Acini diameter reflects the amount of protein the bees obtain from the diet they are provided (Babendreier *et al*., 2005).

We found that the acini diameter and area were high in the 6 day old bees and reduced in 14 day old bees. This is due to the effect of worker bee age on HPG activity (Huang & Otis, 1989), as the activity changes with the size of the gland (Babendreier *et al*, 2005). The acini diameter and area changed very little with age. This is due to absence of brood, as the development of the glands is highly dependent on the presence of brood to be fed (Suzuki, 1988, Huang & Otis, 1989) and also the presence of older adult bees to feed the young bees by trophallaxis (Naiem *et al*., 1999). In another study the acini diameter stayed unchanged throughout the experiment when bees were deprived of brood (Wegener *et al*., 2009).

In a queenright colony with open brood, the size of the acini diameter reached its peak on the eighth day (Suzuki, 1988; Cralisheim and Stolberg, 1989). When young bees live in the parent colony the acini of HPG develop rapidly from day 3 - 4, whereas singly isolated bees only maintain the size of the acini to that of the bees living to the parent colony during the first three days (Suzuki, 1988). In our study the Feed-Bee® diets initiated HPG development well enough; it is only due to the absence of brood that the development changed very little with age. We observed acini area to be a better measure of gland development than acini diameter, because, even when the gland increases in its area, the

diameter of the shortest axis parallel to the axial duct mostly remains the same (Fig. 1). Nevertheless we found a significant correlation between acini diameter and area, which indicated that both acini diameter and area measurements described the level of the HPG development closely. HPG development can also be assessed by measuring protein content of the glands (Pernal and Currie, 2000).

Worker bees fed on the different diet combinations with different P:C ratios showed no significant differences in head fresh mass. According to Hrassnigg & Crailsheim (1998), head fresh mass is a simple and rapid way to describe glandular development: they found 82 % correlation between HPG acini volume and head fresh mass. In contrast, Babendreier *et al.* (2005) found a low correlation of 35%. Our result agrees with the latter study: we found poor correlation with head mass: 25% for acini diameter and 16% for acini area. Therefore, as suggested by Babendreier *et al.* (2005), in caged bees measuring head fresh mass may not closely show the effects of the diet combinations on HPG activity.

The caged worker bees fed on the different diet combinations with different P:C ratio maintained each of the physiological parameters or performance measures discussed earlier to the same level. This indirectly indicates that the bees maintained the optimal amount and balance of the required nutrients, which also confirms the level of dietary self-selection. Dietary self-selection behaviour helps insects to maintain protein and carbohydrate intakes that are near to the optimal in terms of fitness (Waldbauer & Friedman, 1991; Simpson *et al*., 2004). The ability of the worker bees to discriminate among different protein sources was shown previously in Chapter 2 using different protein sources including Feed-Bee®. This study indirectly confirmed bees dietary self-selection behavior using the physiological parameters discussed earlier.

The protein concentration in the haemolymph was within the range found in other studies, which were done in colonies (queenright) (Aase *et al*., 2005) or caged bees fed on Feed-bee®, pollen or other pollen substitutes or supplements (Cremonz *et al*., 1998; De Jong *et al*., 2009). In laboratory experiments Feed-Bee® showed similar consumption and weight

to other honeybees that consumed freshly collected pollen (Gregory, 2006). Field tests with colonies showed that Feed-Bee® has been accepted and also enhanced brood rearing as well as pollen (Saffari *et al*., 2004). Compared with the other studies, in this study the performances of caged bees fed on Feed-Bee® diets with the different food pair combinations indirectly indicates the appropriateness of Feed-Bee® as a protein source for the bees. From this study it is evident that in no choice situations feeding Feed-Bee® alone might not provide optimum P:C ratio, as the bees preferred P:C ratio is quite different from the P:C ratio in the Feed-Bee®. Future studies should focus on what haemolymph protein concentration would be in the presence of brood and also how large HPG would get. There are also many more variables in colony studies that have to be tested in the presence of brood in either for further showing the appropriateness Feed-Bee® diet or improve it based on the information obtained.

3.5. References

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3.6. Figures

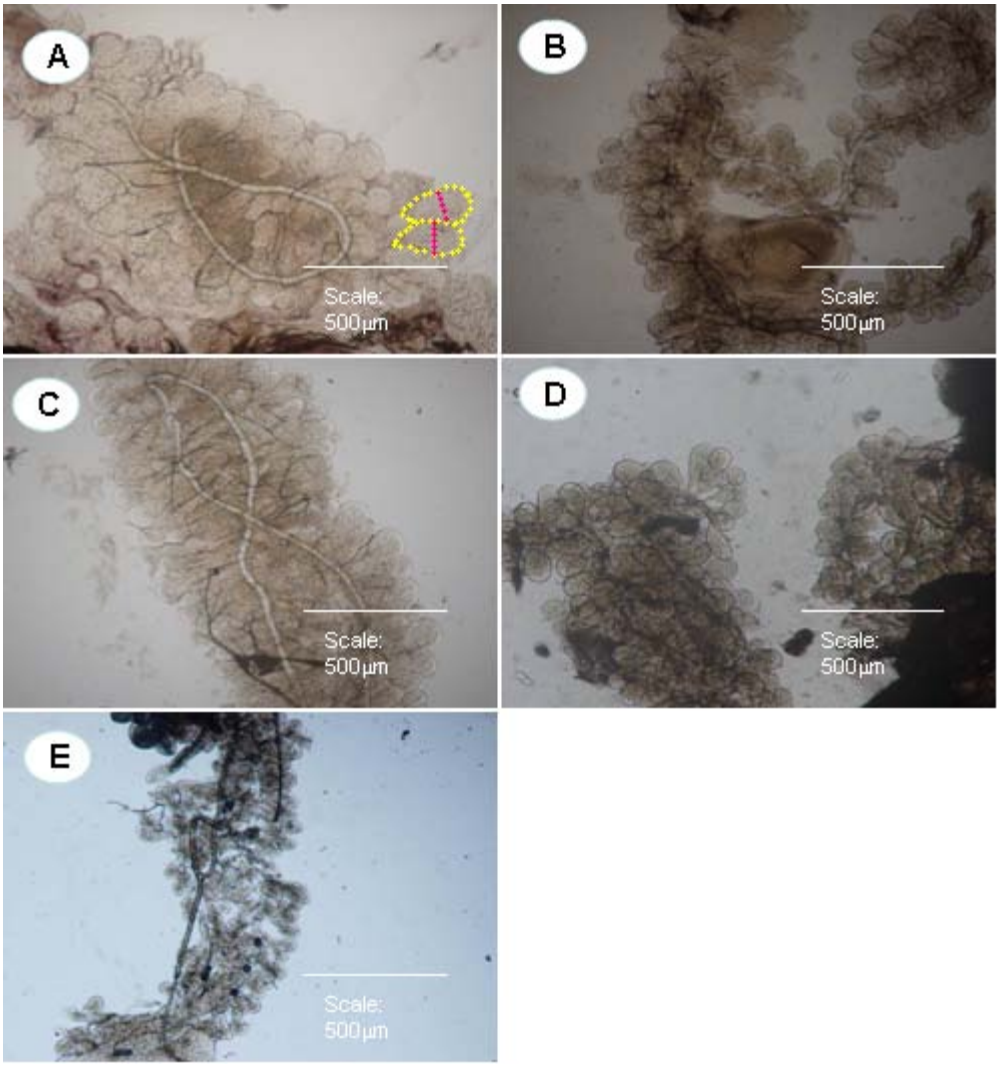

Figure 1. Pictures showing development of hypopharyngeal glands of honeybees *(Apis mellifera scutellata)* of different age groups: A) day 0, B) day 3, C) day 6, D) day 9 and E) day 14. Bees were fed Feed-Bee® diets with different paired P:C combinations. The area of the acini was measured by tracing the circumference of each acinus (Fig. A yellow dots) and we also measured the acini diameters parallel to the axial duct (Fig. A, pink dots). The pictures were taken using a Nikon transmitted light microscope, Nikon Optiphot, Tokyo, Japan.

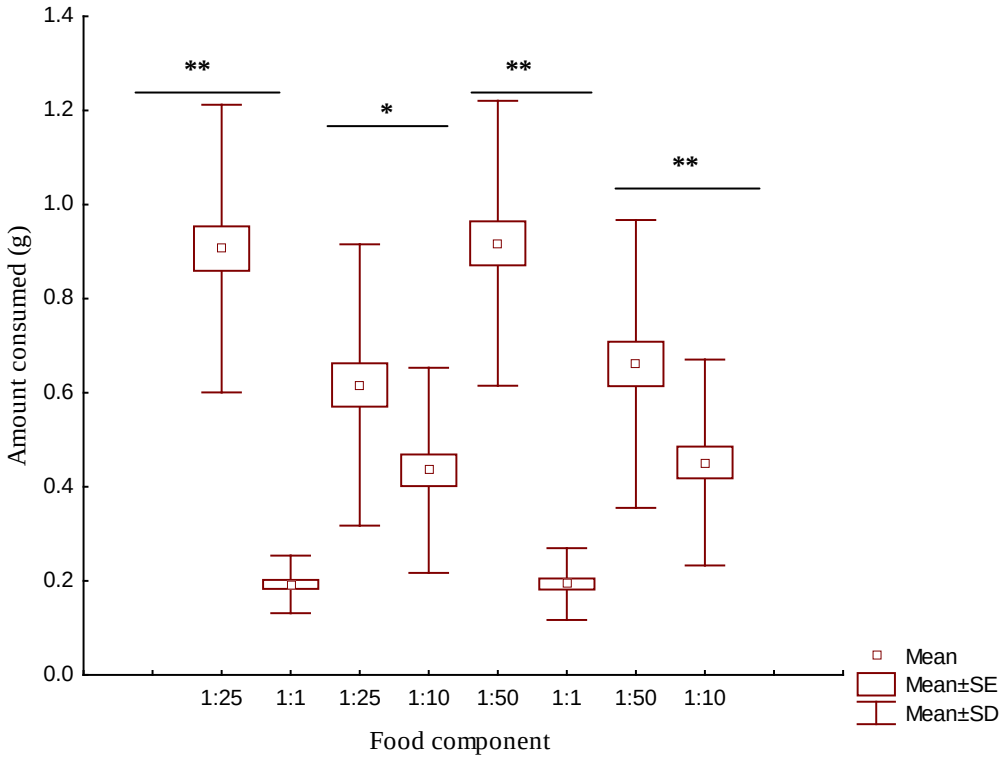

Figure 2. Box and whisker plot showing mean amounts of food consumed from each P:C ratio in each food pair (1:25 with 1:1, 1:25 with 1:10, 1:50 with 1:1, and 1:50 and 1:10) over 14 days of the experiment. Asterisks above the line denote significant differences in consumption between the two diet pairs (**$p < 0.001$ and *$p < 0.05$).

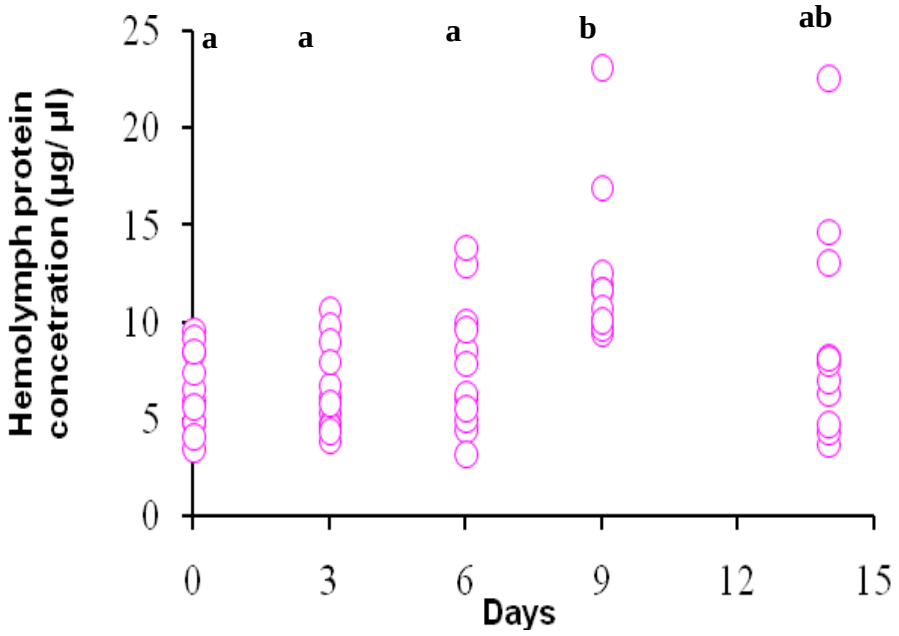

Figure 3. Scatter plot showing protein concentration (µg/µl) in the haemolymph of honeybee workers of day 0, 3, 6, 9 and 14 day bees fed on different paired food combinations (1:25 with 1:1, 1:25 with 1:10, 1:50 with 1:1, and 1:50 with 1:10). Different letters denote significant differences in protein concentration in the haemolymph ($p < 0.05$). Haemolymph of three bees was pooled together per combination, i.e. per cage, when replicated 3 times it is 36 bees per day.

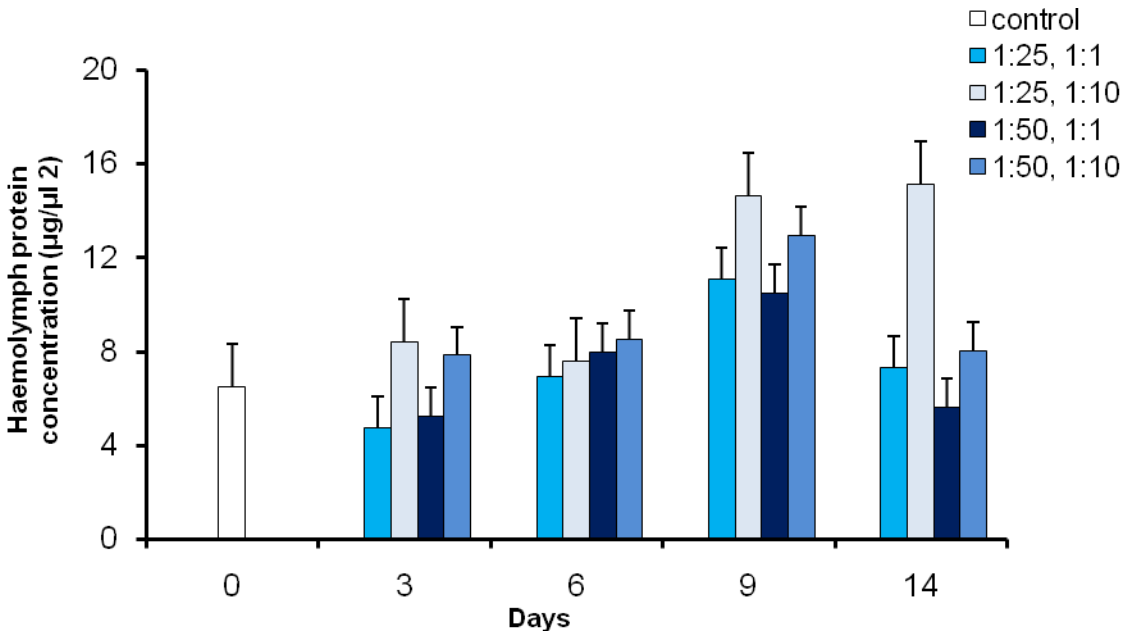

Figure 4. Bar graph showing the total protein concentration in the haemolymph (µg/µl) of worker honeybees of 0, 3, 6, 9 and 14 days fed on Feed-Bee® diets with different pairs of food combinations (1:25 with 1:1, 1:25 with 1:10, 1:50 with 1:1, and 1:50 with 1:10). Haemolymph of three bees was pooled together per combination, i.e. per cage, when replicated 3 times it is 36 bees per day. Protein concentrations in the haemolymph of worker honeybees fed on the different diet combinations within each day were not statistically different from each other.

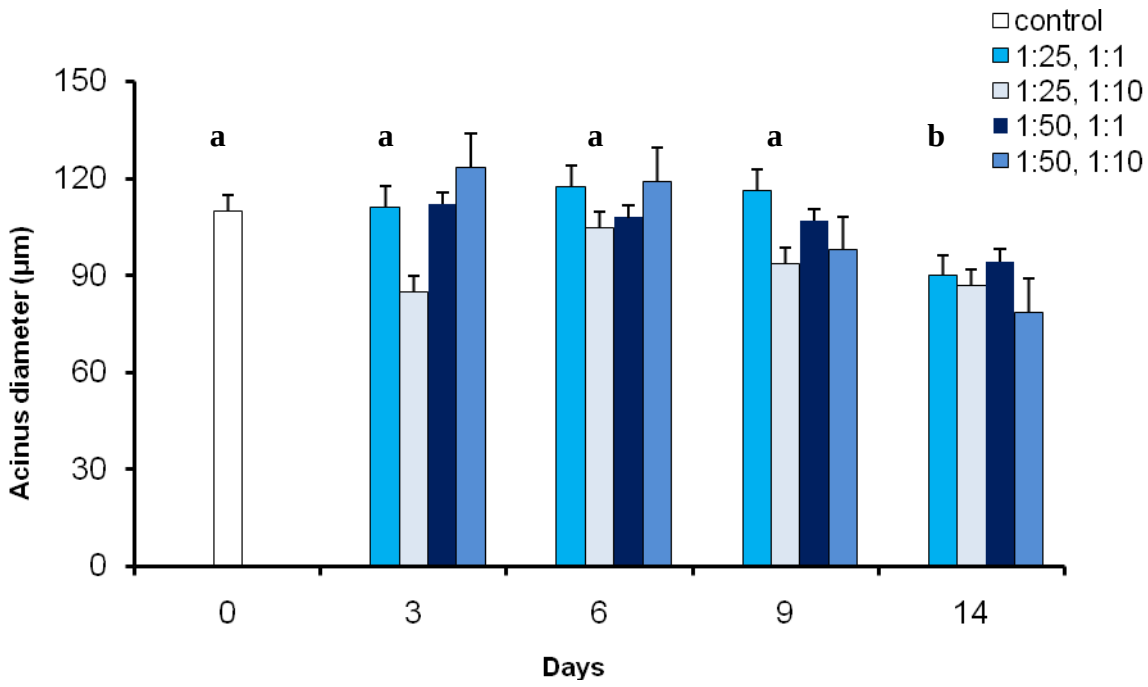

Figure 5. Bar graph showing the acinus diameter (µm) of hypopharyngeal glands of 0, 3, 6, 9 and 14 day old bees fed on Feed-Bee® diets with different pairs of food combinations (1:25 with 1:1, 1:25 with 1:10, 1:50 with 1:1, and 1:50 with 1:10). Different letters denote significant differences in the size of acinus diameter among the different age groups. Sample size, n = 10 acini per bee, 9 bees per diet combination per day.

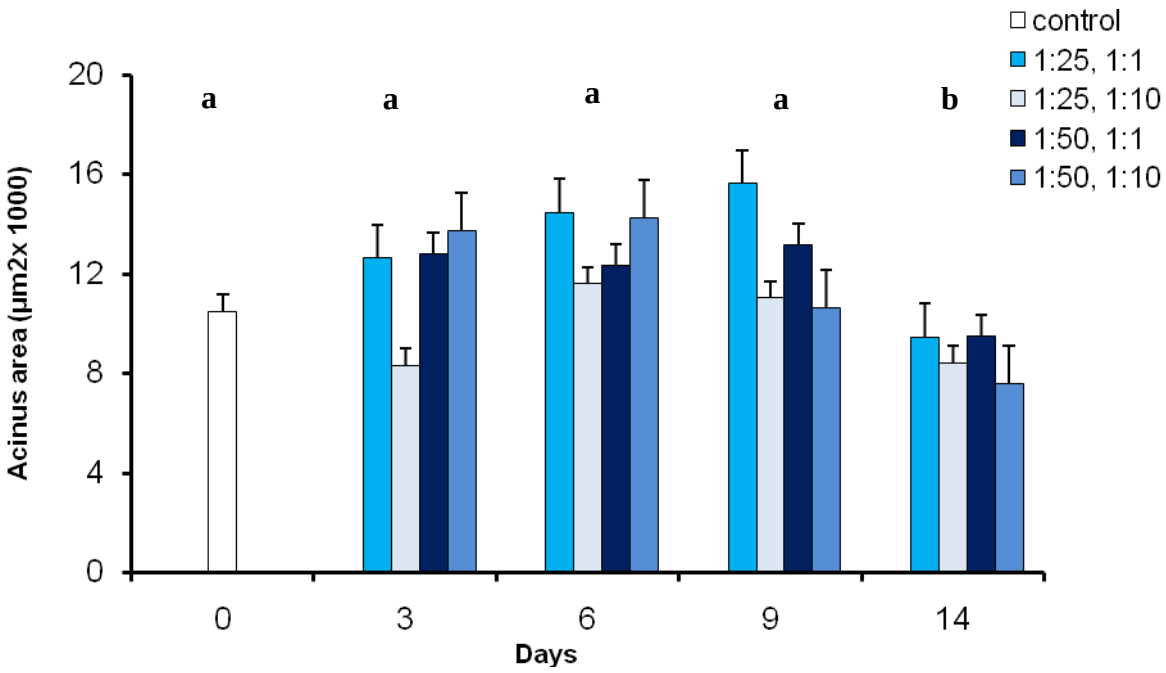

Figure 6. Bar graph showing the acinus area (µm^2) of HPG of honeybees of 0, 3, 6, 9 and 14 day old bees fed on Feed-Bee® diets with different pairs of food combinations (1:25 with 1:1, 1:25 with 1:10, 1:50 with 1:1, and 1:50 with 1:10). Different letters indicate significant differences in the average size of acinus area among the different age groups. Sample size n = 10 acini per bee, 9 bees per diet combination per day.

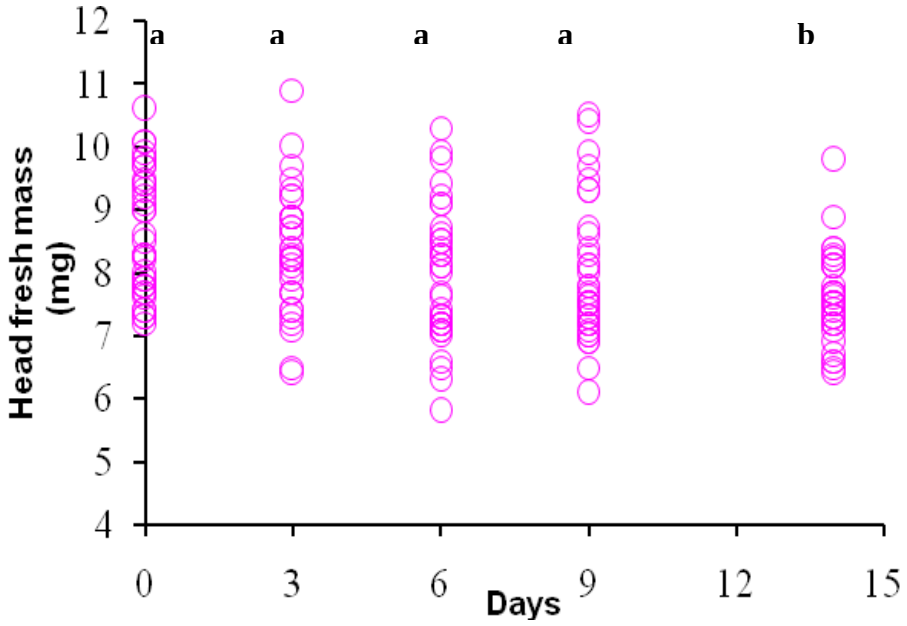

Figure 7. Scatter plot showing head fresh mass of honeybees of days 0, 3, 6, 9 and 14 (n =36) fed on Feed-Bee® diets with different pairs of food combinations (1:25 with 1:1, 1:25 with 1:10, 1:50 with 1:1, and 1:50 with 1:10). Different letters denote significant difference in head fresh mass ($p < 0.05$). Sample size, n = 3 bees per cage, 9 bees per diet combination and 36 per day.

Chapter 4: General discussion

When honeybees are limited to a single pollen or a supplement or substitute with fixed proportions of protein and carbohydrate that may not be balanced, and the bees do not have access to other diet sources to provide a balance of nutrients (Human *et al*., 2007), this affects reproduction, brood rearing, longevity, and eventually the productivity of the colony (Kleinschmidt & Kondos, 1976; Human *et al*., 2007). During winter when food resources are scarce or when honeybees are used in monoculture pollination, bees may likewise have no dietary choices. Furthermore the aim of supplementary feeding using pollen, pollen substitutes or supplements is mainly to bridge the dearth periods by maintaining the colony strength for the coming nectar and pollen flow, as well as prior to the flowering of crops requiring pollination. This kind of situation has also been investigated in caged worker bees by Pirk *et al*. (2009) using no-choice diets with different P:C ratios and protein sources. That means to solve this problem, honeybee feed formulation schemes should be based on the intake target of the bees to make them more effective. This intake target, which is the amount and balance of nutrients that has to be ingested by an animal, can be determined using the geometrical framework (Simpson *et al*., 1995; Raubenheimer & Simpson, 1999). To investigate how an animal regulates its intake of nutrients, it must be challenged to alter its ingestive behaviour to maintain the intake target (Simpson *et al*., 1995; Simpson & Raubenheimer, 2000).

We investigated first (in Chapter 2) the behaviour of caged honeybees when provided with complementary imbalanced foods: they switched between two imbalanced but complementary diets and maintained their intake target according to composition of the food and the type of protein that they encountered. The position of the intake target can also be estimated using performance criteria like survival and developmental time (Simpson *et al*., 1995). Honeybees fed on the different diet combinations with different P:C ratios regulated survival and ovarian activation to the same level, confirming the position of the intake target.

Differences among the different protein sources may be related to the amount of other nutrients found in royal jelly and Feed-Bee® diets, unlike the casein diet, which only contains pure protein and carbohydrate. Depending on the amount of the other nutrients found in each protein source, the accumulation of waste material in the rectum may differ, and the lack of defecation in caged bees may lead to premature death (Maurizio 1950; De Groot, 1953), because honeybees can only defecate in flight (Michener, 1974; Barker, 1977). This may be one of the reasons for the observed high mortality in those bees fed on royal jelly and Feed-Bee® diets compared to casein diets. Similarly honeybees provided with no-choice diets showed high mortality in those bees fed on aloe pollen and royal jelly compared to casein in three different P:C ratios of 3:1, 1:1, and 1:3 (Pirk *et al*., 2009). Therefore in such studies when bees are confined in cages and unable to excrete, survival should not be used as a performance measure to predict the optimal P:C ratio for the reason discussed above.

Lipids are important in honeybee nutrition and their presence may have increased ovarian activation in those bees fed royal jelly or Feed-Bee® diets. Moreover, in two of the cages on Feed-Bee® diets, bees were shown to build a little comb on the foundation sheet, which indicates that the bees received all the necessary nutrients to develop their wax glands (see appendix 1). In addition to the protein:carbohydrate balance, the presence of other nutrients should be considered in honeybee feed formulation, and for this the recently developed pollen substitute Feed-Bee® is a good example.

In Chapter 3 we measured additional physiological parameters in honeybees confined on imbalanced complementary food combinations having different P:C ratios. Results showed that the protein concentration in the haemolymph was within the range found in other studies, which were done in colonies (queenright) (Aase *et al*., 2005) or caged bees fed on Feed-bee®, pollen or other pollen substitutes or supplements (Cremonz *et al*., 1998; De Jong *et al*., 2009). HPG development as measured by acini area or diameter changed very little with age because of absence of brood (Suzuki, 1988, Huang & Otis, 1989). We also

observed the acini area to be a better measure of gland development than the acini diameter, because, even when the acini increase in area, the diameter of the shortest axis parallel to the axial duct mostly remains the same due to growth on the longest axis. Thus, when the development of the HPG increases with age especially in the presence of brood, measuring the acini area may be a better measure of gland development than the diameter. The head fresh mass also showed no difference with age which indicates the activity of the HPG was not changing, again associated with the absence of brood (Suzuki, 1988, Huang & Otis, 1989).

When honeybees were given complementary imbalanced food pairings, they behaviourally self-selected or balanced their diet and maintained their intake target by switching between the complementary foods provided. This kind of compensatory feeding to compensate for changes in nutrient density has been shown in other social insects like the ant, *R. metallica* (Dussutour and Simpson, 2008a). The caged worker bees fed on the different diet combinations with different P:C ratio on the Feed-Bee® diet maintained each of the physiological parameters or performance measures discussed earlier to the same level (chapter 3). This indirectly indicates that the bees maintained the optimal amount and balance of the required nutrients, which also confirms the level of dietary self-selection. This dietary self selection behavior is an important trait in worker bee; it enables the workers to explore a wide variety of nutritionally different sources. It might also be this behaviour which is used by nurse bees to obtain protein and carbohydrate and also other nutrient requirements from stored pollen and nectar in the hive, either for their own nutritional requirements or for other colony members including larvae. The intake target and other many more variables should also be tested at colony level in the presence of larvae; but the tendency of the bees to store foods in the combs needs to be considered before planning such a study.

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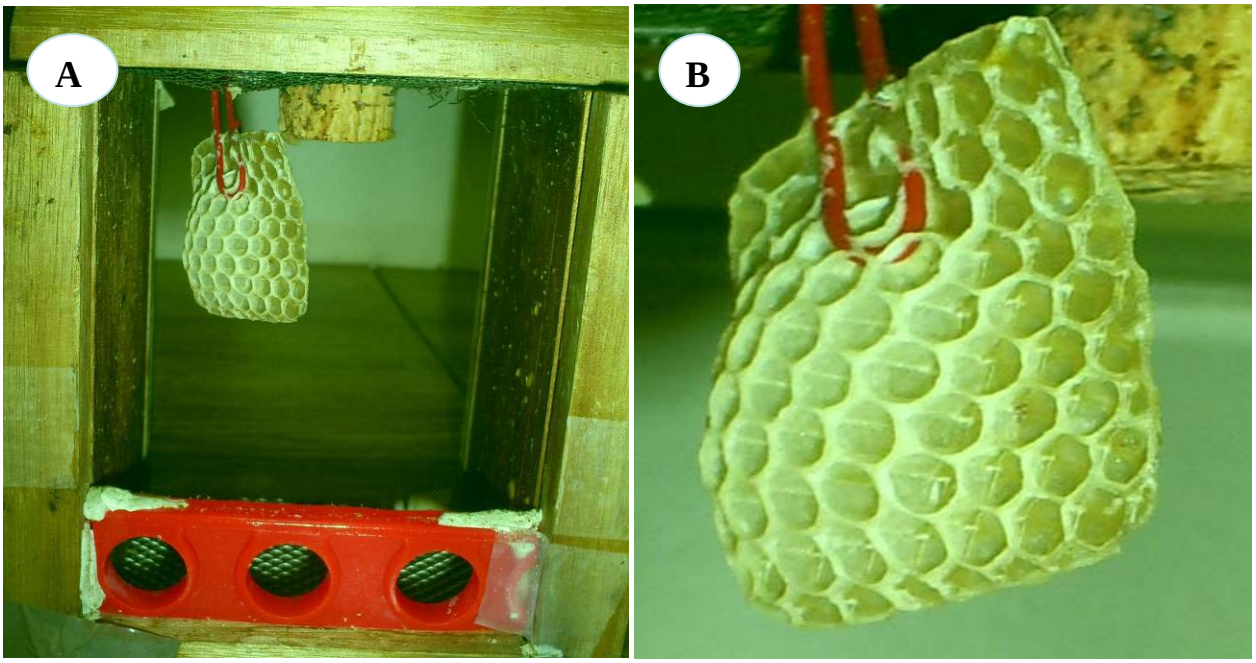
Appendices

Appendix 1*:*

Honeybee comb constructed by caged worker bees fed on Feed-Bee® diets,



In two of the cages on Feed-Bee® diets, bees were observed to have active wax glands that enabled them to build a small comb on the foundation sheet (a). Then they laid eggs in it on the last two days of the experiment (b): this indicates that the bees received all the necessary nutrients for developing their wax glands

Appendix 2:

Feed-Bee® Composition

According to the manufacturers information Feed-Bee® is made from 100% processed plant materials, which is free from pollen, hive products, animal products/by-product, soy products/by-products, genetically modified organism, chemicals, antibiotics/medicines, artificial colours, and flavours or preservatives.

Nutrition Facts:

Protein…….....36.4%

Fat…….....3.9%

Carbohydrates (dietary fibre etc.)….....41.8%

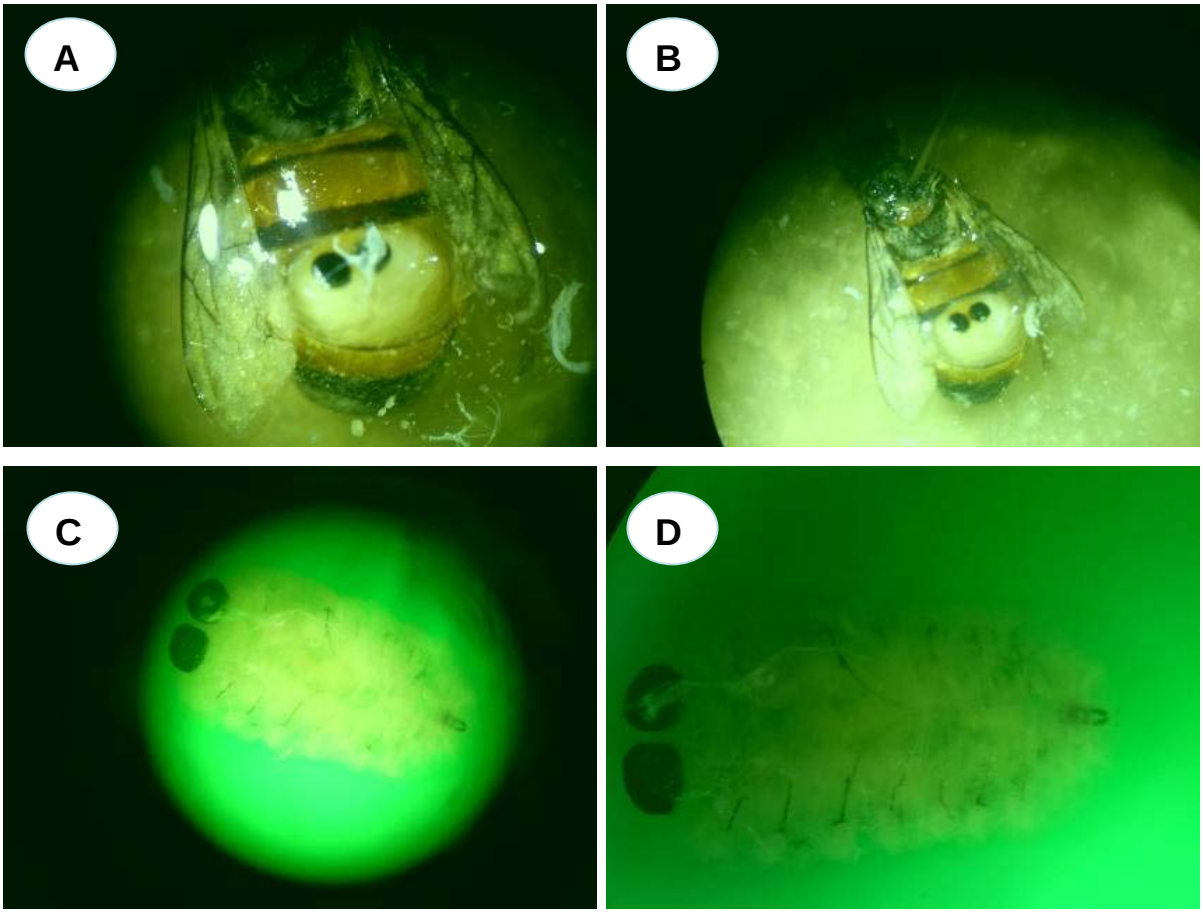
Sugar (natural occurring, not extracted)……....10.0%

Minerals (natural occurring, not synthetic)…….....3.1%

Source: http://www.feedbee.com

Appendix 3:

Parasite found in the thorax of caged honeybees



A parasite found in the thorax of honeybees between the fourth and the sixth tergite is shown in (a) and (b). The parasite is closely 0.5 cm long and has two black dots in the in front side as shown in (c) and (d).