

Convergence of carbohydrate-biased intake targets in caged worker honeybees fed different protein sources

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SUMMARY

The nutritional needs of bees are supplied by nectar carbohydrates and by protein and other nutrients in pollen but little is known of how bees achieve nutritional balance. Using newly emerged caged worker honeybees (*Apis mellifera scutellata*), we investigated whether bees maintain their intake target when confined to pairs of imbalanced complementary diets varying in protein to carbohydrate (P:C) ratio. Diets were formulated using three protein sources [casein, royal jelly or Feed-Bee® (a natural pollen substitute)] and sucrose. Within each protein type, honeybees switched between complementary diets and converged on the same P:C intake target. However, this target differed between protein types: P:C ratios were 1:12, 1:14 and 1:11 on casein, royal jelly and Feed-Bee® diets, respectively. Except for an early peak in protein consumption on royal jelly diets, these strongly convergent ratios remained constant over the 14 day experiment. This is probably due to the absence of brood, reflected in relatively stable values measured for haemolymph protein concentration and hypopharyngeal gland activation in bees on Feed-Bee® diets. Performance of caged workers was also assessed in terms of survival and ovarian activation. Survival was highest on casein diets and lowest on Feed-Bee® diets but ovarian activation was highest on royal jelly diets and lowest on casein diets. This may be due to additional components in Feed-Bee® and royal jelly (e.g. fatty acids), which are needed to activate the ovaries but also reduce survival. Nutrient intake of broodless workers is directly related to their own physiological requirements, and the strong carbohydrate bias may reflect the high metabolic rate of honeybees even under resting conditions.

Key words: nutrition, geometric framework, *Apis mellifera scutellata*, ovarian activation.

INTRODUCTION

The protein content of pollen is generally accepted as a measure of its nutritional value to bees, although pollen also contains lipids, carbohydrates, vitamins and minerals (Haydak, 1970). Adult honeybees (*Apis mellifera*) consume pollen for their own requirements but more importantly to feed larvae. Pollen is collected and transported by foragers, then consumed and digested by younger nurse bees, enabling them to produce jelly for feeding larvae, young workers and queens (Crailsheim, 1992). Nurse bees thus play a key role in digesting pollen and distributing the protein component as secretions from their hypopharyngeal glands (HPG). When the quality and quantity of pollen available to bees is limited, nutrition at both individual and colony levels can be severely affected (Brodtschneider and Crailsheim, 2010). Pollen quality, which can be expected to depend on amino acid composition (Cook et al., 2003; de Groot, 1953), is particularly important when honeybees are used in monoculture pollination and restricted to a single pollen type. Honeybees are frequently fed protein-rich pollen supplements or substitutes (usually based on soya flour) when pollen is scarce or of poor quality, or when colony strength must be increased to take advantage of a major nectar flow during commercial pollination.

However, the nutritional benefit of high protein levels in pollen warrants closer investigation. Although protein concentrations vary from 12 to 61% dry mass in bee pollens, there is no evidence that bees prefer to collect pollens of higher protein content (Pernal and Currie, 2001; Roulston et al., 2000). Excess protein has been shown to shorten the lifespan of honeybees (Standifer et al., 1960) and to reduce brood rearing (Herbert et al., 1977). We have previously found reduced survival and ovarian activation in caged workers of

Apis mellifera scutellata fed with the protein-rich pollen of *Aloe greatheadii* var. *davyana*, compared with those fed sunflower pollen (Human et al., 2007). Cohorts of caged adult workers given no-choice diets varying in protein to carbohydrate (P:C) ratio were subject to higher mortality as the protein level in the diet increased, regardless of whether the protein source was casein, aloe pollen or royal jelly (Pirk et al., 2010).

The optimal balance of nutrients required by honeybees can be investigated using a modelling approach to nutrition called the Geometric Framework (Raubenheimer and Simpson, 1993; Simpson and Raubenheimer, 1993; Simpson et al., 2004). This allows the calculation of an 'intake target', an animal's nutritional optimum when it is confronted by specific environmental variables at a particular stage in its life history. This optimum is often inferred experimentally from regulated points of intake when presenting animals with choices between complementary but unbalanced foods. The foods are represented as lines or 'nutritional rails' radiating out from the origin, with a slope equivalent to the ratio of the two nutrients examined (e.g. P:C ratio but could include any nutrient dimensions). When animals are provided with a single unbalanced diet and have no choice, they may over-ingest some nutrients in order to acquire enough of others even though both over- and under-ingestion of nutrients can have substantial fitness consequences (Raubenheimer and Simpson, 1999). Various nutritional challenges in insects have been examined using the Geometric Framework, mainly using solitary insect herbivores such as locusts and caterpillars as model organisms, with protein and carbohydrate being the two macronutrients most studied (Behmer, 2009).

Most animals are required to eat a variety of foods to obtain optimal nutrition, and their decisions about the amount and balance of nutrients to be collected are based on their current nutritional state. Nutrient regulation becomes more complex in social insects, because foraging is restricted to a subset of individuals, whose requirements are likely to differ from other colony members such as growing larvae. Thus, foragers react to food they encounter according to both individual needs and internal demands for nutrients in the nest, and the food they collect is brought back to the colony for storage (Dussutour and Simpson, 2009). At the colony level honeybees modulate the intensity of foraging for nectar (Seeley, 1986) and pollen (Camazine et al., 1998; Dreller et al., 1999; Pernal and Currie, 2001) according to the nutritional status of the whole colony. However, how worker honeybees meet their individual nutritional requirements in response to the composition of food is unknown.

In the present study we apply the Geometric Framework to explicitly define the P:C intake targets of caged worker honeybees without the demands of larvae to feed. We provided newly emerged workers with complementary imbalanced foods of varying P:C ratio, containing three different protein sources: casein, royal jelly and Feed-Bee[®], a newly developed pollen substitute of plant origin (Saffari et al., 2006). We measured food consumption of honeybee workers over a 14 day period (i.e. before the transition from brood care to foraging), as well as their survival and ovarian activation. Depending on whether workers obtain their food directly or through trophallaxis, 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. In addition, we measured haemolymph protein concentration and HPG activation in bees maintained on the Feed-Bee[®] diets; like ovarian activation, these parameters reflect protein utilisation by the bees (Crailsheim and Stolberg, 1989; Cremonese et al., 1998; Pernal and Currie, 2000). We explored whether: (1) bees would compensate for an imbalanced diet by switching between complementary foods, and would change their regulated intake with a change in protein source, to reflect differences in amino acid balance; (2) the three protein sources would differ in their effects on worker survival and ovarian activation; and (3) P:C intake would vary with worker age. Most importantly, we expected that under broodless conditions, when workers do not have to activate their glands for feeding larvae, intake would reflect the physiological requirements of the workers and therefore be biased towards carbohydrates.

MATERIALS AND METHODS

Animals and cages

Five *Apis mellifera scutellata* Lepeletier colonies from the University of Pretoria apiary site were used to collect frames of capped worker brood, which were incubated at 34°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 cm × 8.5 cm × 7 cm) closed at both the front and back with movable glass slides and at the bottom with wire mesh to allow ventilation. Below the glass slide at the front of each cage, a plastic frame with round windows allowed insertion of three stoppered feeding tubes (plastic tubes with 2 cm × 1 cm apertures cut into them). Two feeding tubes contained the complementary imbalanced diets and the third contained water. Food and water were provided *ad libitum*. All of the cages were placed under standard conditions in an incubator at 34°C and 55–65% relative humidity (RH) for 14 days, and kept in the dark to simulate conditions within the colony.

Experimental protocol and diets

Based on a data from a preliminary experiment, we formulated four diets containing P:C ratios of 1:50, 1:25, 1:10 and 1:1. The diets (Table 1) were formulated using sucrose and three protein sources: casein (vitamin free, C 3400, Sigma-Aldrich[®], Schnellendorf, Germany), freeze-dried royal jelly (True Blue Health Products, Seven Hills, NSW, Australia) or Feed-Bee[®] (Bee Processing Enterprises Ltd, Toronto, Canada). The protein and carbohydrate contents of royal jelly (Johannsmeier, 2001) and Feed-Bee[®] (based on the manufacturer's figures) were taken into account during diet preparation. For all protein sources either casein or sucrose were added to achieve the desired P:C ratios (Table 1). These diets were paired in the following complementary food combinations for each protein source: 1:50 with 1:10, 1:50 with 1:1, 1:25 with 1:10, and 1:25 with 1:1. Each diet combination was fed to a different cage and replicated five times using different colonies, giving a total of 20 cages for each protein source.

Consumption

All diet components were dried at 45°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 dried to constant mass at 45°C over several days. Consumption was calculated as the difference in dry mass before and after feeding. 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. the intake target). Daily consumption (g bee⁻¹) was adjusted for declining bee numbers in each cage during the experiment.

Survival and ovarian activation

Survival was recorded by the daily removal and counting of dead bees from each cage, and on the last day surviving bees were frozen at -20°C and stored. For the determination of ovarian activation, 10 bees from each diet combination were selected for dissection. Fourteen days are sufficient for ovarian activation to take place in *A. mellifera scutellata* (Ruttner and Hesse, 1981). The stages of ovarian activation were visually scored following a standard method (Pirk et al., 2010) as: (1) inactive, (2) intermediate, and (3) active.

Table 1. Composition of diets

Protein source	P:C ratios			
	1:50	1:25	1:10	1:1
Casein (CA)	0.020 g CA 0.980 g sucrose	0.038 g CA 0.962 g sucrose	0.091 g CA 0.909 g sucrose	0.500 g CA 0.500 g sucrose
Royal jelly (RJ)	0.949 g sucrose 0.051 RJ	0.901 g sucrose 0.099 RJ	0.776 g sucrose 0.224 RJ	0.047 g sucrose 0.953 RJ
Feed-Bee [®] (FB)	0.947 g sucrose 0.053 FB	0.897 g sucrose 0.103 FB	0.763 g sucrose 0.237 FB	0.051 g casein 0.949 g FB

The mass of each constituent (g g⁻¹ diet) is given for each protein source and each protein to carbohydrate (P:C) ratio. Diet composition was calculated based on literature data on nutrient levels in royal jelly (Johannsmeier, 2001) and the manufacturer's information for Feed-Bee[®].

To investigate if there was a correlation between ovarian activation and rectal volume, we also scored the volume of the rectum as: (1) empty, (2) half full, and (3) full.

Haemolymph protein concentration and HPG activation

In a separate experiment using caged workers from three colonies maintained on Feed-Bee® diets and the same P:C diet combinations as above, haemolymph protein concentration and HPG activation were measured on day 0 (before bees were placed in the cages) and the days 3, 6, 9 and 14. 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 (Cremonese et al., 1998). For each diet combination we pooled the haemolymph of three individual bees from each cage (three replicates). The haemolymph of 36 newly emerged workers (day 0) was also collected. Protein concentration was determined spectrophotometrically at 595 nm using the Bradford assay and bovine serum albumin as standard (Cremonese et al., 1998).

HPG acinus area was measured as an indicator of gland activation, which should be due to diet alone within cohorts. Three bees were removed randomly from each cage on days 0, 3, 6, 9 and 14. The heads were dissected with a razor blade (cutting from the ocelli to the mandibles) under a stereoscopic microscope (Nikon SMZ800, Tokyo, Japan), and the HPG were removed. Each gland was mounted in a drop of distilled water on a glass slide and photographed using a Nikon transmission light microscope (Nikon Optiphot, Tokyo, Japan). Using these pictures, the areas (μm^2) of 10 randomly chosen acini per bee were measured by tracing the circumference, using Image Tool® analysis software (version 3, University of Texas, San Antonio, TX, USA). Diameters of the acini were measured at the same time.

Statistical analysis

Normality and homogeneity of variance were assessed for each variable; when the assumptions for parametric statistics were violated the corresponding non-parametric tests were performed. Analysis of variance (ANOVA) with Bonferroni tests were used to test the effects of diet combination, protein source, age and colony of origin on consumption, as well as age and diet combination on haemolymph protein concentration and HPG acinus area. Paired sample *t*-tests were used to test for differences in consumption between the pairs of foods presented. Kruskal–Wallis ANOVA with multiple comparisons of mean ranks were conducted to test if protein source and diet combination have an effect on ovarian activation, rectal volume and cumulative P:C ratio. Kaplan–Meier survival regression analyses were conducted for all diet combinations in each protein source with protein sources as grouping variable. Multiple regression analysis was used for testing for interaction between factors. All tests were conducted with STATISTICA software (StatSoft, Inc., Tulsa, OK, USA; version 7).

RESULTS

Consumption

Bees were fed 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). Honeybees fed unequally from the two foods provided to them in all of the treatments for each protein source except for the CA 1:25 and 1:1 combination (paired *t*-tests, $P=0.09$), and converged on the same P:C intake target within each protein source (Fig. 1). There were no significant differences between colonies in mean protein and carbohydrate consumption within each

protein source. There were significant differences among protein sources in mean daily consumption of protein, carbohydrate and total food on the different protein sources (Table 2).

Age did not significantly affect the P:C ratio in bees on CA and FB diets but there was a significant effect of age on P:C ratio for RJ [$F_{(13,266)}=8.4$, $P<0.0001$]; the *post-hoc* test showed that the workers consumed a higher ratio on day 2 compared with all of the other days except day 3 and 4 and on day 3 compared with all of the other days except days 2, 4 and 5.

Cumulative protein consumption showed significant differences among the three protein sources [$F_{(2,72)}=5.17$, $P<0.05$] whereas cumulative carbohydrate consumption did not [$F_{(2,72)}=1.15$, n.s.] (Fig. 1). 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 [$F_{(4,20)}=1.521$, n.s.] diets. The intake target differed between protein sources; on CA, RJ and FB diets the preferred P:C ratios (1:12, 1:14 and 1:11, respectively) were significantly different for all pair-wise

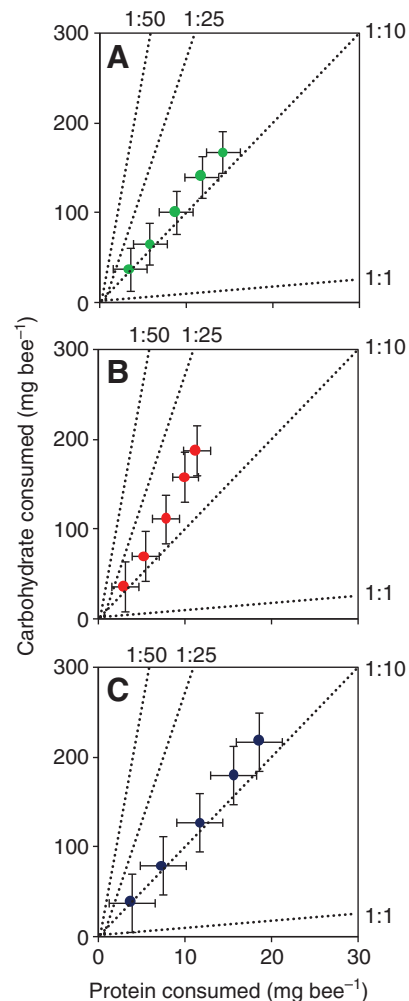


Fig. 1. Bivariate plots showing cumulative protein and carbohydrate consumption of five honeybee colonies fed complementary diets with different P:C ratios and different protein sources. Consumption is shown for 3-day intervals (days 1–3, 4–6, 7–9, 10–12 and 13–14). Bees were fed (A) casein, (B) royal jelly and (C) Feed-Bee® diets 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). Dotted lines radiating from the origin indicate the P:C ratios for the 1:50, 1:25, 1:10 and 1:1 diets. Within each protein type, bees converged on the same P:C intake target but this target differed between protein types.

Table 2. Daily consumption (mg per bee per day, means \pm s.e.m.) of total food, protein and carbohydrate

Protein source	Consumption in mg per bee per day			P:C ratio
	Total food	Protein	Carbohydrate	
Casein	13.02 \pm 0.23 ^a	1.01 \pm 0.03 ^a	12 \pm 0.21 ^a	1:12
Royal jelly	14.83 \pm 0.19 ^a	0.88 \pm 0.02 ^b	13.4 \pm 0.19 ^a	1:14
Feed-Bee [®]	17.71 \pm 0.29 ^b	1.32 \pm 0.04 ^a	15.56 \pm 0.28 ^b	1:11

Three protein sources (casein, royal jelly or Feed-Bee[®]), with protein to carbohydrate (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, were fed to five different colonies. In the casein diets the total food consumed is the sum of the protein and carbohydrate consumed, because 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. Different letters within columns indicate significant differences (ANOVA with *post-hoc* test), $P < 0.001$ for both tests.

comparisons [Kruskal–Wallis ANOVA: $H_{(2,75)}=42.59$; $P < 0.0001$, multiple comparison $P < 0.001$].

Survival

Of the total 2000 honeybees fed with different pairs of complementary imbalanced foods within each protein source, 205 (10.25%), which were fed on CA, 711 (35.55%), which received RJ and 754 (37.7%) on 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 multiple regression analysis. Survival showed a significant positive relationship with the average daily consumption of protein ($\beta=0.59$, $P < 0.0001$) and carbohydrate ($\beta=2.99$, $P < 0.0001$). However, survival showed a significant negative relationship with total consumption ($\beta=-3.75$, $P < 0.0001$).

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: $\chi^2=11.03$, d.f.=3, $P < 0.05$; RJ: $\chi^2=30.15$, d.f.=3, $P < 0.001$; FB: $\chi^2=24.52$, d.f.=3, $P < 0.001$) (Fig. 2A–C). Cumulative survival (all diet combinations) differed significantly among protein sources ($\chi^2=457.76$; d.f.=2; $P < 0.0001$; Fig. 2D).

Ovarian activation

A total of 600 bees from the different paired food combinations and protein sources was dissected. Ovarian activation of bees fed different diet combinations containing CA ($\chi^2=1.74$, d.f.=6, n.s.), RJ ($\chi^2=5.57$, d.f.=6, n.s.) and FB ($\chi^2=10.52$, d.f.=6, n.s.) did not differ significantly (Fig. 3A–C). However, there were significant differences in ovarian activation among protein sources [$H_{(2,600)}=131.53$; $P < 0.0001$, Fig. 3D]. 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 FB diets showed more active ovaries than those fed CA diets ($P < 0.01$).

In the bees fed FB diets, but not the other diets, ovarian activation was negatively correlated with rectal volume (Spearman rank order correlation: $N=200$; $R=-0.62$; $P < 0.05$). Rectal volume varied significantly [$H_{(2,600)}=130.18$, $P < 0.0001$] between bees sustained on the three protein sources. Honeybees sustained on RJ or FB diets had higher mean rectal volumes than those on CA diets ($P < 0.001$). However, the rectal volumes of bees fed RJ or FB diets did not differ.

Haemolymph protein concentration

In the three colonies maintained on FB diets and the same P:C diet combinations, the intake target was again P:C 1:11 (data not shown). Protein concentration in the haemolymph of bees on FB diets showed significant variation [$F_{(4,55)}=5.3$, $P < 0.001$] between the different age groups (Fig. 4A). Bees fed the different FB diet combinations

showed similar protein concentrations in their haemolymph [$F_{(3,44)}=2.53$, $P=0.069$]. In addition, results from the two-way interaction between age groups and diet combinations showed no significant effect on protein concentration [two-way ANOVA: $F_{(9,32)}=0.73$, $P=0.68$]. The protein concentration in the haemolymph was positively correlated with the cumulative protein consumed ($R^2=0.12$, $P < 0.05$).

HPG acinus area

Worker bees on FB diets showed significant age effects on HPG acinus area [$F_{(4,1585)}=65.1$, $P < 0.0001$] (Fig. 4B). Diet combinations did not affect acinus area, with the sole exception of bees fed 1:25 with 1:10 and 1:25 with 1:1 diet combinations ($P < 0.0001$) on day 3. Results from the two-way interaction between age groups and diet combinations showed no significant effect on acinus area within the different age groups [two-way ANOVA: $F_{(9,32)}=1.87$, $P=0.094$]. There was a strong correlation between acinus diameters (data not shown) and acinus areas ($R^2=0.83$, $P < 0.0001$).

DISCUSSION

Intake targets of worker honeybees on different protein sources

To investigate how an animal regulates its intake of nutrients, it must be challenged to alter its ingestive behaviour to maintain the intake target. Caged worker honeybees actively regulated the intake of both protein and carbohydrate when given complementary imbalanced foods with different P:C ratios, preferring average P:C ratios of 1:12, 1:14 and 1:11 on CA, RJ and FB diets, respectively. The strong bias shown by these broodless caged workers towards dietary carbohydrate is confirmed by the similar intake targets on the three very different protein sources. Strong carbohydrate bias is also evident in the intake target of first-instar pea aphids, which perform best on a diet with an amino acid to sucrose ratio of 1:19 mg mg⁻¹ (Abisgold et al., 1994), and other insects with endosymbiotic bacteria that contribute to nitrogen metabolism (Raubenheimer and Simpson, 1997; Simpson and Raubenheimer, 1993). Recently it has been shown in both flies and grasshoppers that a strongly carbohydrate-biased diet maximises lifespan but not reproduction (Fanson et al., 2009; Lee et al., 2008; Maklakov et al., 2008).

It seems reasonable to assume that honeybee colonies will alter the amount of protein consumed to compensate for changes in protein quality. However, there is little evidence for this. Young caged workers do not increase pollen consumption to compensate for reduced protein content (Pernal and Currie, 2000). Bees prefer pollen of oilseed rape to that of field bean, and the former has more favourable proportions of essential amino acids (Cook et al., 2003), but it is not known whether bees can detect such differences. Compensation for change in protein quality is, however, seen in the

caterpillar *Spodoptera littoralis*, which increases protein intake when dietary CA is partly replaced with zein – a protein from maize with an imbalanced amino acid composition (Lee, 2007). Compared with CA, zein also reduced HPG activation and the longevity of honeybees (Standifer et al., 1960). However, CA is known to be

deficient in methionine – an amino acid that has recently been shown to influence life-history parameters of *Drosophila* (Grandison et al., 2009). Apart from amino acids, there are obviously differences in terms of the complexity and composition among the three protein sources that we fed to worker bees: in spite of this, there was still remarkable convergence in the intake targets.

Survival and ovarian activation on different protein sources

The performance measures of survival and ovarian activation differed between protein sources. Survival was highest on the CA diet combinations (90% after 14 days). This survival advantage was also apparent on no-choice diets when CA was compared with aloe pollen and RJ (Pirk et al., 2010). As honeybees do not defecate in the nest, the accumulation of waste material in the rectum, depending on other components present in each protein source, may lead to the premature death of caged workers (de Groot, 1953; Maurizio, 1950). This may contribute to the higher mortality in bees fed RJ and FB diets: rectal volume was lowest in bees on CA diets. When

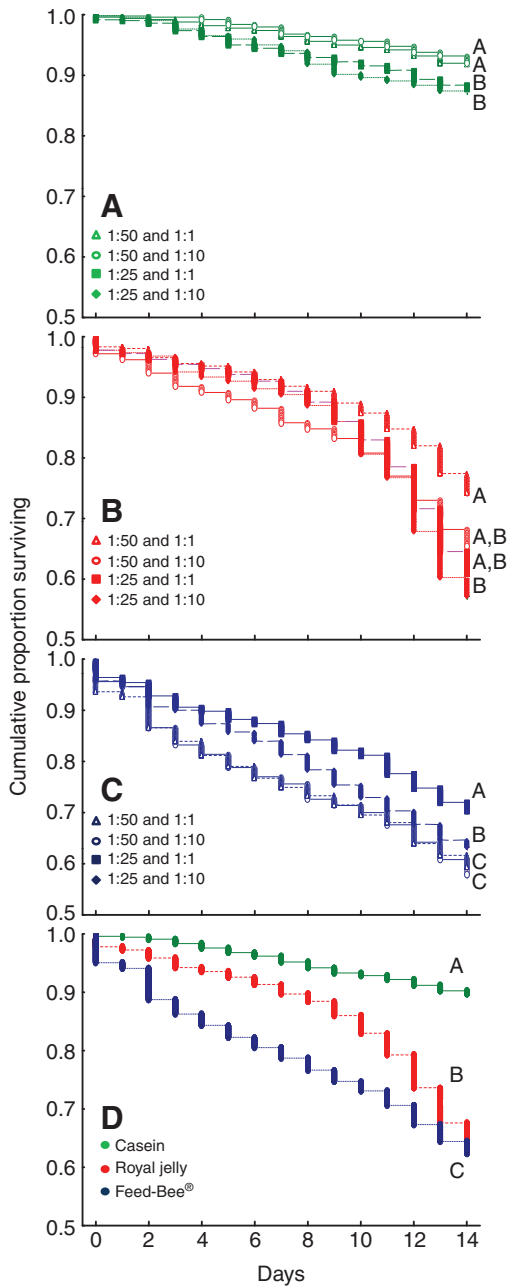


Fig. 2. Cumulative proportion of honeybees surviving on complementary diets with different P:C ratios and different protein sources. Bees were fed (A) casein (CA), (B) royal jelly (RJ) and (C) Feed-Bee® (FB) diets with different P:C combinations (1:25 with 1:1, 1:25 with 1:10, 1:50 with 1:1, and 1:50 with 1:10). (D) A comparison of the three protein sources. Data were analysed using Kaplan–Meier survival regression analysis, and different letters denote significant differences at $P < 0.05$ using Cox's F -test. 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: $F_{(1422,1508)}=1.16$, $P < 0.01$].

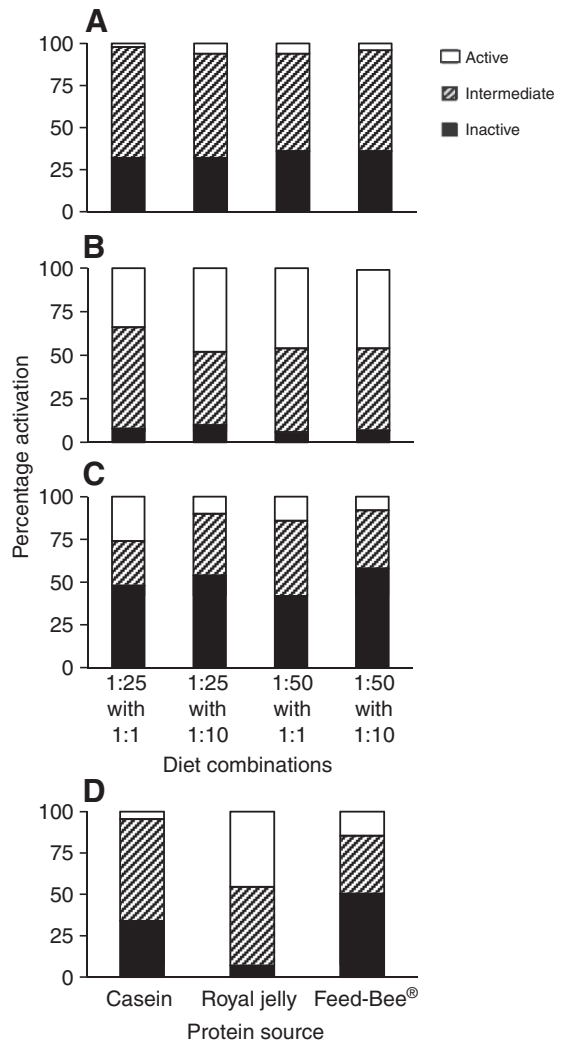


Fig. 3. Ovarian activation of honeybees fed complementary diets with different P:C ratios and different protein sources. Bees were fed (A) casein, (B) royal jelly and (C) Feed-Bee® diets with different P:C combinations (1:25 with 1:1, 1:25 with 1:10, 1:50 with 1:1, and 1:50 with 1:10). (D) A comparison of the three protein sources. The level of activation was scored as: (1) inactive, (2) intermediate active, and (3) active ovary.

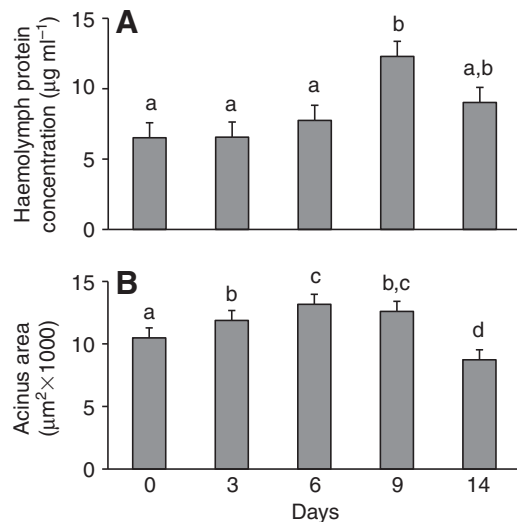


Fig. 4. Physiological parameters of worker honeybees fed Feed-Bee® diets with different P:C combinations (1:25 with 1:1, 1:25 with 1:10, 1:50 with 1:1, and 1:50 with 1:10). (A) Haemolymph protein concentration ($\mu\text{g ml}^{-1}$, means \pm s.e.m.). $N=12$ (haemolymph of three bees per cage was pooled). (B) Acinus area of hypopharyngeal glands (HPG) (μm^2 , mean \pm s.e.m.; $N=10$ acini per bee, 9 bees per diet combination per day). Different letters denote significant differences between days (ANOVA with *post-hoc* test).

protein sources and diet combinations were compared, differences in survival tended to be due to the time of mortality rather than the survival at 14 days. At least 60% of worker bees survived the 14 day experiment on RJ and FB diets, compared with only 10% survival of bees given a no-choice RJ diet of 1:3 P:C (Pirk et al., 2010). In another study, caged honeybees fed on pure RJ alone showed 100% mortality within three days (Lin and Winston, 1998).

When worker bees are caged in queenless groups their ovaries are activated, and dietary protein is used for ovarian activation rather than brood rearing (Crozier and Pamilo, 1996; Pernal and Currie, 2000). This effect is more pronounced in African honeybees (Ruttner and Hesse, 1981). Ovarian activation varied significantly between protein sources. RJ diets supported the highest ovarian activation, as we have shown previously (Pirk et al., 2010) in a no-choice experiment comparing RJ with aloe pollen and casein. Because of social interactions among caged workers, some obtain protein for oogenesis through being fed by nestmates instead of feeding for themselves; these individuals become reproductively active but consume little of the food provided (Schäfer et al., 2006). In support of this, we found a significant inverse relationship between rectal volume and ovarian activation in bees fed Feed-Bee® diets. Lower ovarian activation in bees fed on CA compared to RJ and FB diets is not surprising, as the casein-based combinations were incomplete diets lacking other nutrients such as lipids (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 (eggs were laid in it on the last two days of the experiment): this indicates that the bees received all the necessary nutrients for normal development of their glands.

It is becoming increasingly apparent that different nutrient intakes favour different life-history traits. The nutritional trade-off between longevity and reproduction is striking in *Drosophila melanogaster*; longevity is greatest on a carbohydrate-biased P:C ratio of 1:16,

similar to that of broodless worker honeybees, but oviposition requires a higher protein intake and is maximal at a very different P:C ratio of 1:2 (Lee et al., 2008). When flies are offered complementary yeast and sugar solutions, they converge on an intake target of 1:4 P:C, which represents a compromise between the optima for longevity and reproduction (Lee et al., 2008). Similar divergence in optimal P:C ratios has been observed in Queensland fruit flies *Bactrocera tryoni* (Fanson et al., 2009). Optimal P:C ratios for reproduction differ between male and female crickets (*Teleogryllus commodus*), and both are higher than the low P:C ratio that is ideal for extended lifespan (Maklakov et al., 2008). Mortality of worker ants also increases when they are restricted to higher P:C ratio diets (Cook et al., 2009; Dussutour and Simpson, 2008b), and larvae of *Rhytidoponera metallica* die if too much protein is collected by the workers (Dussutour and Simpson, 2009).

Implications of studying caged workers without brood

Intake targets can be expected to change with development. During the first 15 days of imaginal development in female locusts, protein and carbohydrate intakes are relatively constant for the first few days but protein intake then decreases as tissue growth declines (Chyb and Simpson, 1990; Raubenheimer and Simpson, 1999). In worker honeybees, there was no effect of age on P:C intake, except for a higher P:C ratio on days 2–3 in bees fed RJ diets. This may be because the HPG develop rapidly in the first few days of adult life under colony conditions, even in the absence of brood (Crailsheim and Stolberg, 1989). The generally constant P:C ratios selected by caged worker bees can be explained by the absence of brood. This is also why haemolymph protein levels and HPG acinus areas changed very little with age in workers on the FB diets. The haemolymph protein concentrations that we recorded are lower than those of caged bees fed pollen and other diets (Cremonez et al., 1998) but similar to those in a study in which bees were fed FB itself, pollen and other diets (de Jong et al., 2009). Activation of the HPG is maximal after about eight days in colony conditions but is reduced in the absence of brood and in caged bees (Crailsheim and Stolberg, 1989; Huang and Otis, 1989; Lass and Crailsheim, 1996); an additional influence on HPG activation which was also lacking in our cages is the presence of older adult bees to feed the young bees by trophallaxis (Naiem et al., 1999). There are thus multiple reasons why HPG activation was reduced in our bees. Incidentally, although acinus area will be a more reliable measure if growth on different axes varies, we also measured diameters of the same HPG acini, and our mean values of 110 μm from days 0 to 9 (data not shown) are very similar to those in the caged bees fed pollen by Crailsheim and Stolberg (Crailsheim and Stolberg, 1989).

Nutritional regulation in social insects is complicated by the different requirements of the different life stages, and the limitation of foraging to a fraction of the colony's members. Ant colonies, like those of bees, require carbohydrates as an energy source for adults, and protein for larval growth (Dussutour and Simpson, 2008a). Artificial colonies of *R. metallica*, a primitive omnivorous ant, regulate both sugar and protein intake more precisely as larval numbers increase (Dussutour and Simpson, 2008b; Dussutour and Simpson, 2009). Development of a synthetic diet has shown that colony performance of this species, measured in terms of both worker mortality and the number of larvae raised, is optimal on a 1:2 P:C ratio (Dussutour and Simpson, 2008b). Ants manipulate the nutritional content of collected food so that the proportion of protein is higher in stored food, indicating selective extraction of carbohydrate (Cook et al., 2009; Dussutour and Simpson, 2009). When the amounts of protein and carbohydrate collected by *R. metallica* are corrected for

this rejection of excess protein, the P:C intake targets are 1:1.5 in colonies with larvae and 1:2 in those without. Why is the intake target of caged worker honeybees without brood so different? The extreme carbohydrate bias can not be attributed to endothermy because our bees were in an incubator but the metabolic rates of caged worker bees will be much higher than those of *R. metallica* colonies maintained at 25°C (Dussutour and Simpson, 2009). The resting metabolic rate of honeybees increases steeply with ambient temperature, and is 47nl of oxygen at 35°C (Kovac et al., 2007), which requires the metabolism of 4.83 mg of sucrose per bee per day. This is 40% of the daily sucrose consumption that we measured on CA-based diets (Table 2). Activity, which was excluded in the measurements of Kovac and colleagues (Kovac et al., 2007), will increase the individual requirement for carbohydrate, especially after the transition to foraging. The intake target may also be influenced by the fact that bees specialise in mass storage of nectar carbohydrates. It would be interesting to find out whether abdominal lipid stores increased in caged workers during the 14 day experiment; under colony conditions, a decline in stored lipid precedes the onset of foraging (Toth and Robinson, 2005). In addition, the strong carbohydrate bias is representative of natural conditions during a broodless period such as winter.

Honeybees manipulate the nutritional content of collected food extensively by converting nectar into honey and pollen into bee bread, in addition to the manufacture of easily digestible jelly from pollen by nurse bees. Queen larvae and laying queens are likely to have a high demand for protein, as reflected in the near 1:1 P:C ratio of RJ (Johannsmeier, 2001), but worker larvae are fed nectar and pollen as well as jelly so the P:C ratio of their diet may differ. In samples collected for analysis immediately after discharge by worker bumblebees *Bombus terrestris*, Pereboom measured a low P:C ratio of 1:15 in the larval food (Pereboom, 2000). The nutritional rails approach should be extended to examine the intake targets of worker honeybees in the presence of larvae, when we might expect a shift towards protein (at least until the larvae pupate), and also the nutritional optima of artificially reared larvae. There are many factors, such as low temperature, toxins and diseases that could potentially cause the intake targets of adult and larval bees to shift. The Geometric Framework is a powerful tool in research aimed at identifying how foragers select pollen and nectar resources to feed not only themselves but the entire colony.

LIST OF ABBREVIATIONS

CA	casein
FB	Feed-Bee®
HPG	hypopharyngeal glands
P:C ratio	protein to carbohydrate ratio
RJ	royal jelly

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