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The Effect of High and Low Fat Pollens on Honeybee Longevity

by Rob Manning

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Foreword

Australia's major honey producing species are eucalypts. By European standards, the pollen of these plants are low in lipid and therefore may have affected the longevity of European honeybees.

Additions of two common fatty acids found in pollen, identified in RIRDC project DAW-100A found that longevity was significantly affected by fatty acid concentration when added to low-lipid pollen such as *Corymbia calophylla*. The addition of fatty acids did not improve longevity but a new knowledge in this area of fatty acids was that honeybees were more tolerant of linoleic acid than oleic acid in pollen diets.

In similar experiments, the most common of all substitute feedstuffs, soya bean flour did not improve longevity, nor did hypopharyngeal gland development respond to this type of protein. However, bees did respond positively to the low fat, high protein soya bean protein concentrate rather than the plain soya bean flours whether used as a defatted or full fat product.

A further study of the nutrition of bees during the study period revealed that from soya bean flour diets, little linoleic acid was accumulated in the body of bees. Bees fed sugar diets increased in lipid levels and again the only fatty acid not accumulated was linoleic acid. Manganese was the only element not accumulated like other minerals when the bees were fed the flour diets. These two elements may have limited the success of supplementary diets both in bee nutrition research and in the field in past and current feeding practises.

In order to maximise the efficiency of bees, populations have to be strong to collect nectar which bees turn into honey. Fruit and nut industries such as the almond industry which directly benefit from honeybee pollination can achieve major improvements in yield, if bees are placed into crops at their maximum population. Thus the findings from this project are very important to beekeepers and to research professionals who are involved in developing artificial feedstuffs for honeybees in order for bees to breed into populous colonies.

Another source of protein without using the traditional source such as from soya bean flour has to be identified. Any new diet will need to be tested against natural pollen where head weight can determine any physiological benefit and analysis of body nutrients will determine if all elements, particularly linoleic acid and manganese are accumulated.

This project was funded from industry revenue which is matched by funds provided by the Australian Government. This report, an addition to RIRDC's diverse range of over 1700 research publications, forms part of our Honeybee R&D program, which aims to improve the productivity and profitability of the Australian beekeeping industry.

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Peter O'Brien

Managing Director

Rural Industries Research and Development Corporation

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Rural Industries Research and Development Corporation (RIRDC) (Australia)

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Executive Summary

What the report is about

A survey was conducted nationally of bee-collected pollens which were analysed for fatty acids. During the survey (see also DAW-100A), 73 different fatty acids were identified from all pollen samples. Only five of these fatty acids: palmitic, stearic, oleic, linoleic and linolenic were common to all 577 pollens sampled.

European honeybees have evolved with plant species that have pollen that contain higher concentrations of lipid, particularly linolenic acid where concentrations are higher than Australian native plant pollen which were found in this research to be typically higher in the other essential fatty acid linoleic acid (e.g. *Eucalyptus*).

A second part of the research focussed on two of the five commonly found fatty acids, namely oleic and linoleic by supplementing them in varying concentrations into a low fat pollen collected from *Corymbia calophylla* (Marri; Redgum). This feedstuff was fed to honey bees to determine if there were any effects on longevity or physiological aspects such as nutrient levels and hypopharyngeal gland development within the honey bee.

The research is important from the point of view of flower resources that the bee industry rely when drought and climate change make it difficult for beekeepers to procure sufficient pollen flows that sustain and grow bee colonies. Artificial means of supplying protein and minerals to colonies needs to occur to retain maximum population levels that stem from young bees being able to fully develop hypopharyngeal glands, that supply the abundant worker jelly required to feed the hundreds of larvae that hatch from eggs laid each day by the queen.

Who is the report targeted at?

The report is targeted to scientific researchers who are interested in adjusting and testing current feeding practices with the new knowledge gained from this research. For the curious beekeepers the research will only add to their own interest in this field as many do make and test their own supplementary feeds often without scientific basis.

Background

In times of drought or when apiaries are in environments that lack floral abundance of nectar and pollen, many beekeepers maintain their colonies by supplying artificial feedstuffs to colonies of bees. The artificial ingredient of choice is the high protein soya beans. Soya bean flour is available in a range of products that differ in fat content and have never undergone scientific testing in hives for longevity and physiological effects. These were tested in the same way as the fatty acid (oleic and linoleic) enhanced pollen diets. Lupin flour was also tested as a possible import replacement for soya bean flour.

No studies of the fatty acid content of Australian plant pollen have been conducted until the RIRDC funded three research projects (DAW 91A, DAW100A & DAW 105A). The knowledge is essential if studies on honey bee nutrition are to be conducted and artificial diets are to be formulated.

Aims/Objectives

The objectives were to conduct an extensive literature review of the fatty acid composition of pollen and honey bee body tissue, then carry out a comprehensive Australia-wide survey of bee-collected pollens for their fatty acid composition. A second objective was to determine the effect of an antimicrobial fatty acid (linoleic) and a common lipid, oleic on honey bee longevity and hypopharyngeal gland activity. A third objective was to use cage experiments to ascertain the influence of high or low fats in soya bean flours on honey bee performance.

Methods used

Beekeepers utilise plants from the genus eucalyptus as their major source of honey. Two of the common fatty acids (oleic and linoleic) were added to a low-lipid eucalypt pollen that was bee-collected from *Corymbia calophylla* (redgum, marri). This diet was fed to honeybees confined in cages (mini colonies containing 1,400 bees) to ascertain their effect on longevity and hypopharyngeal gland development. Soy flour and lupin diets were tested in the same way.

Longevity was assessed over six weeks by measuring daily mortality and hypopharyngeal gland development was by measuring head weight which was verified using histological sectioning of the head. The value of the diets was assessed by weekly measurements (with gut removed) of the bees' body for protein, lipid, fatty acids and minerals.

Results/Key findings

Honeybees that were fed diets of redgum pollen had the greatest longevity of the 22 diets tested and those bees fed diets of pure sugar had the shortest life. Honeybees fed a protein concentrate (low fat) from soya bean flour had the longest life of the flours tested.

The addition of fatty acid (oleic and linoleic) to pollen at different concentrations caused significant differences in longevity but did not increase longevity over the controls (pollen only). However, the addition of oleic acid to pollen greater than 2% caused bees' longevity to decrease, a poor head weight response and a failure of the queen to lay eggs. The addition of linoleic acid greater than 6% to pollen diets had a similar response. As the percentage of oil was increased in both fatty acid additions, total consumption of the diet decreased.

In dietary comparisons, defatted and full-fat soya bean flours gave similar longevity and despite large differences in fat content, the response to diet of head weight was negligible and no response was elicited by the queen to lay eggs. Adding pollen to the flour diets improved longevity whereas the addition of pollen to lupin flour caused an increase in honeybee mortality.

Honeybees fed flour diets (soya bean, lupin) failed to accumulate linoleic acid in their body, which was in contrast to honeybees fed pollen diets. Manganese was another element that was poorly accumulated by honeybees fed both soya bean and lupin flour diets when compared with pollen diets. Bees fed sugar diets increased body fat, however, linoleic acid, was not accumulated.

Observations of honeybee behaviour inside the cages indicate that emerged honeybees, only a few days old, reorganise themselves into a caste sequence that differed to that normally followed by a colony where its repertoire follows from being a nurse bee to field bee.

Implications for relevant stakeholders

The development of artificial feedstuffs is dependent on more research into the effect on physiological aspects of bees viz hypopharyngeal gland development of the various elements that feedstuffs are composed. There are many 'successful' commercial feedstuffs in the market place, 'successful' only in that the honey bees are seen to consume the product supported by very little science-based evidence. The associated effects on the hypopharyngeal gland and nutrient accumulation in the bee's body are not known. Several proprietary brands of feedstuffs (including overseas product) should be scientifically tested/evaluated here in Australia.

The research process is typically a lengthy analysis of experimental work and longer term thinking in this area of nutrition by beekeepers (drivers of research) should be always be contemplated.

Recommendations

Further research into adding linoleic acid and manganese into feedstuffs to elevate body levels of these elements to be within normal limits attained when bees are fed on pollen. Further research should also test the other common fatty acids such as linolenic acid, palmitic acid, stearic acid and some of the less common fatty acids.

Introduction

The project examined the effect of oil-enhanced pollen on honeybee longevity, life-span, hypopharyngeal gland development (through head weights) and nutritional status of honeybees following the consumption of 22 diets prepared from pollen, soya bean and lupin flours.

The report is broken into four parts. The first describes an observation of behaviour inside cages of honeybees that may have had an influence during the cage experiments. The second tests the diets by measuring longevity and life-span. The third, is the measurement of head weights to further assess the effectiveness of diets on physiological processes. The fourth, is an examination of the nutrient status of bees fed the 22 diets.

Observation of “hairless” phenotype in a caged single cohort of honeybees as a potential indicator of behavioural status.

Honeybees kept in cages for nutrition experiments can be used to demonstrate the effect of various feedstuffs on honeybee physiology such as longevity and development of the hypopharyngeal gland (Haydak 1970). Thus the normal external sources of protein (pollen either stored in or entering the colony) are prevented from influencing the result.

During the series of caged-bee experiments containing approximately 1400 bees/cage (with no flight cage) hairless bees were observed. This phenotype of bee was distinctively hairless, displaying noticeable wing tears and was present in all cages supplied with different diets (Fig. 1-1A). All other bees within the cages resembled young hairy bees, normally described as nurse bees (Fig. 1-1B).

The hairless bees were deemed to resemble older bees whilst the hairy bees resembled young nurse bees. Similar hairless bees are seen in the field by Western Australian beekeepers, especially associated with foraging on Banksia flowers where bees have to squeeze past dense arrays of stamens to access the flowers' nectaries.

It was hypothesized the hairless bees were of forager status and would, as a consequence, have lighter head weights than the nurse bees of the same age which should still have a well developed hypopharyngeal gland (i.e. have a heavier head weight).

Foraging honeybees have lighter head weights due to the reduction in size of the hypopharyngeal gland (Dade 1962). Head weight mass and hypopharyngeal gland acini size are positively correlated ($r^2 = 0.80$; $N = 540$ bees) as shown by researchers at the Australian National University (Dr I Wallis, pers. com.).

The literature also reports on a phenomenon where 'shiny-black hairless' bees, similar to that seen in this study, were observed in experimental hives in the USA (Rothenbuler *et al.* 1969). They were described as being caused by an unknown pathogen at the time, but later this phenomenon was tentatively associated with virus-like particles (Kulincevic *et al.* 1969). Burnside (1933) described a similar pathogen that produced hairless bees and referred this adult bee disorder to 'paralysis' that Gochnauer (1978) described as a viral 'black bee syndrome' or hairless-black syndrome (HBSV) in which a non-occluded virus was isolated (Rinderer 1974). The virus was similar in morphology to chronic bee-paralysis virus (CBPV) and serologically indistinguishable (Rinderer and Green 1976).

A large number of researchers who have studied honeybees in caged bee experiments do not record this observed phenotypic change in any publication and it is described here for the first time.



Figure 1-1. An example of the two types of bees of the same age observed at the conclusion of a 42 day cage feeding experiment. Heads have been removed. A (top) bees with hairless patches, some with wing tears; B (bottom) normal hairy nurse bees from the same cage.

Longevity of honeybees fed linoleic and oleic acid enhanced redgum (*Corymbia calophylla*) pollen diets; high and low fat soya bean flour diets and lupin and flour diets mixed with redgum pollen

Australian beekeepers manage their honeybees (*Apis mellifera*) on eucalyptus floral resources that are typically low (by world averages) in pollen lipids. Eucalypts from eastern Australia have lipid concentrations below 2%. In Western Australia, two of the major eucalypt species utilised by beekeepers, *Corymbia calophylla* and *E. marginata* have pollen that are also low in lipid at 0.88 and 0.59% respectively (Manning and Harvey 2002).

Honeybees evolved in Europe where floral pollens are generally richer in lipids. Pollen from the United States of America, particularly in California, and North America also have a high lipid content, averaging 4.96% (Todd and Bretherick 1942) and 9.2% (Standifer 1966b), respectively. By comparison, Australian eucalypt pollens are depauperate in lipids and this might affect honeybee longevity.

Length of life of the worker bees is one of the three primary factors that govern honey production besides average daily brood production and individual productivity of worker bees (Woyke 1984). Kunert and Crailsheim (1988) found that the shortest life-spans occurred in periods when nectar and pollen were plentiful.

There have been relatively few studies on honeybee longevity following the bees' consumption of pollens from different species (containing different protein and lipid levels). A few studies on longevity exist where artificial diets have been tested (Winston *et al.* 1983). Schmidt *et al.* (1987) found that the longevity of bees fed pollen from wind-pollinated species was less than those fed sugar-only diets (no

protein), perhaps indicating the absence of pollen attractants, presence of pollen deterrents, toxic compounds (e.g. in *Typha latifolia*) or a poor nutrient balance. Honeybees fed other pollen had increased longevity. For example, bees fed dandelion (*Taraxacum officinale*) pollen had life-spans of 6.4 days greater than diets containing only sugar, whilst bees fed pollen from *Rubus* sp., *Populus* sp1 and sp2 had life-spans of 28.4, 38.0 and 28.6 days greater than the sugar controls, respectively. These differences were significantly correlated to the amount of pollen consumed, protein concentration of the pollen and total amount of pollen protein consumed (Schmidt *et al.* 1987).

A study of survivorship of foraging bees by Visscher and Dukas (1997), outside of a cage experiment, flying under natural conditions, showed two week-old bees which had started to forage had a life-span of 7.7 days. The short life-span was due to the high likelihood of death by predators and indicates the usefulness of using caged-bee experiments to eliminate other factors that might cause variation in data when evaluating artificial honeybee diets.

Honeybees require carbohydrates, proteins, vitamins, minerals and water for growth and development of the colony. These elements are sourced mainly from pollen and nectar. However, in times of floral dearth, colonies of bees gradually use up stored resources within their hive and when depleted can survive for a period on the protein and lipids stored in their body. During this period, the queen bee stops laying eggs and the colony population weakens. For beekeepers, this process is destructive for their business. Beehives need strong, populous colonies in order for the bees to store surplus nectar, which is then harvested.

For many years, artificial sources of protein and lipid have been researched to substitute for the occasions when these dearth periods occur. These are fed to colonies to maintain or increase their populations. The main source of ingredient has been soya bean flour. In Australia, this is an imported product which carries some expense compared to a locally grown source such as lupin. As a replacement source of protein, lupin flour is an untested alternative. It is a locally grown product and if suitable could replace imported soya bean flour. It would also remove the possibility of honey contamination with genetically modified material. Siede *et al.* (2003) showed that nearly 6% of honey varieties displayed at a show in western Germany contained traces of soya bean flour of which 2.8% tested positive for genetically-modified (GM) soya bean with 1% of honey samples suggesting the presence of transgenic glyphosate-tolerant soya bean material. Pollen substitute made from soya beans and fed to bees was said to be the main reason for the contamination. Besides the consumer issues about genetic modification, GM soya bean flours could contain detrimental concentrations of trypsin inhibitors that can significantly reduce the protein content of the hypopharyngeal gland (Sagili *et al.* 2005).

Soya bean flour has long been used as the main ingredient in honeybee supplementary feeds (Haydak 1949, Siede *et al.* 2003, Spencer-Booth 1960, Stace 2005). Australian beekeepers, because of supply problems, have resorted to using soya bean flours whether they are high-fat (full-fat) or medium-fat (defatted) flour. Beekeepers also use these flours for bee feed without any additional ingredient being mixed with it. However, an added percentage of pollen can provide some attractiveness and palatability to the raw product.

The soya bean flour is nevertheless collected by honeybees in times of dearth when placed in an apiary (Somerville 2005a). No research in Australia has investigated the life expectancy of honeybees after feeding on this product (whether low, medium or high fat) or when both of these flours are augmented with honeybee-collected pollen.

This experiment examined the relationship between the longevity and life-span of young emerged honeybees, which had little initial contact with protein (from pollen), and the concentration of two fatty acids added to *C. calophylla* pollen. It was hypothesised that honeybee longevity would improve as the concentration of fatty acid increased in the test pollen. However, it was recognised that a concentration would be reached where there would be an impact upon longevity as protein levels decreased as described by Schmidt *et al.* (1987). It was expected that the fat content of soya bean flours would have a similar relationship with longevity and life-span of honeybees.

The effect of diet on head weights (hypopharyngeal gland) of honeybees

- A. LINOLEIC AND OLEIC ACID ENHANCED REDGUM (CORYMBIA CALOPHYLLA) POLLEN DIETS**
- B. SOYA BEAN AND LUPIN FLOUR DIETS AND MIXES CONTAINING REDGUM POLLEN**

The worker honeybee head contains three large glands: the hypopharyngeal gland (Figs. 3-1 and 3-2), mandibular gland and postcerebral gland (Dade 1962). The hypopharyngeal gland is the largest of the glands in the head cavity consisting of 1000 or more pear-shaped lobules (Wang and Moeller 1969) where they almost fill the space in front of the brain as well as sending branches to the back of the brain (Dade 1962).

In young bees, the well-developed hypopharyngeal glands are responsible for the synthesis of a food rich in protein (jelly) which is fed to larvae, drones and the queen bee. The distribution of jelly from nurse bees to nestmates is rapid. Crailsheim (1992) found that when 100 nurse bees in two hives were injected with a radioactive amino acid which incorporates into the hypopharyngeal gland during protein synthesis, their jelly was found in 2409 individuals in one hive and 3756 in another hive overnight.

The synthesized food is derived from pollen and besides feeding larvae, bees use it to build up their own body stores and feed the secretion to other adult bees (Crailsheim 1992, Hrassnigg and Crailsheim 1998). The activity of the hypopharyngeal gland is affected by various brood stages and is significantly higher after young bees are presented with larvae of all ages than eggs, capped brood or a mixture of all stages (Huang and Otis 1989).

The proteinaceous food the worker bees produce from the hypopharyngeal gland is known as royal jelly. Haydak (1970) described two types of secretions produced by nurse bees: a watery-clear secretion from bees averaging 12 days of age and a milky-opaque secretion by 17 day old bees. The jelly consists of 67% water, 12.5% protein, 11% sugars and 5% fatty acids which is fed to larval queen bees and is initially similar to brood food fed to worker larvae but changes by day four when it is modified by the addition of pollen and honey (see Schmidt and Buchmann 1997). The unique and chemically most interesting feature of the jelly is its fatty acids (Schmidt and Buchmann 1997).

The hypopharyngeal gland reaches its maximum size in young bees (four to six days old) and thereafter becomes greatly shrunken, almost to the point of disappearance in (older) foraging honeybees (Dade 1962). The age at which maximum size of the hypopharyngeal gland develops coincides with increased pollen consumption up to the nursing age of three to ten days and remains elevated in bees aged from 10 to 18 days (Hrassnigg and Crailsheim 1998). Schmidt *et al.* (1987) showed the greatest rate of pollen consumption was during the first 10 days of emergence which thereafter, decreased sharply, especially after 21 days (Hrassnigg and Crailsheim 1998).

In older bees, the seemingly atrophied glands actively secrete two enzymes. The first, invertase (Simpson *et al.* 1968), is an enzyme that inverts nectar sucrose into two sugars: dextrose and levulose; the second enzyme is glucose oxidase which oxidises glucose to gluconolactone stabilising the principle acid in honey, gluconic acid (White 1997). The process also liberates hydrogen peroxide, which protects the ripening process of nectar from spoilage and in some honeys when they are diluted, the peroxide has a medical application (Molan 2001).

Gland size is negatively correlated to juvenile hormone biosynthesis (Huang *et al.* 1994) so that these shrunk glands are associated with increased rates of juvenile hormone biosynthesis. Juvenile hormone is therefore linked to the different tasks performed by young and old bees, though Huang *et al.* (1994) found gland development was not strictly linked to the bees' age-dependent behaviour. However, Robinson (1992) states that the juvenile hormone does play a major role in age polyethism in honeybee workers. In cage experiments using single cohorts, there was evidence of age polyethism inferred through head weight measurement.

Various methods of measuring the development of the hypopharyngeal gland have been established. An arbitrary scale method was described by Maurizio 1954 in which a number (1 to 4) was given to each stage of development (Stage 4 is maximum development), and this was also used by Simpson *et al.* (1968). Other researchers have measured the size of the acini (grape-like sphericals) of the gland at different stages or bee age (Crailsheim and Stolberg 1989, Crailsheim *et al.* 1992, Huang *et al.* 1994, Moritz and Crailsheim 1987), the volume of the lobules, diameter of the secretory globules and nuclei (Wang and Moeller 1969), the weight of the gland (Fluri *et al.* 1982) or the protein synthesis activity of the gland (Brouwers 1982, Huang and Otis 1989). Costa and Cruz-Landim (2002) measured the change of 19 enzymes of the hypopharyngeal gland of newly emerged bees, nurse bees and forage worker bees.

The glands from nurse bees have the highest rates of protein synthesis and have large diameter acini (Brouwers 1982). Elevated hypopharyngeal activity is maintained in bees from 9 to 19 days of age and substantially declines after day 20, but reestablishes briefly for the production of invertase (Huang and Otis 1989). Crailsheim and Stolberg (1989) showed acini diameter peaked at day eight for emerged bees and decreased to day 25. The size of the hypopharyngeal gland significantly ($P < 0.001$) decreased as the age of the bee increased (Huang *et al.* 1994). Fluri *et al.* (1982) found bees aged from 3-21 days old had heavier glands (dry weight) than newly emerged bees and bees aged 26-40 days old. Haydak (1959) showed worker bees attained the highest dry weight and nitrogen content of the heads and abdomens at the age of six days.

Sustaining brood-rearing activities in honeybee colonies during periods of dearth by artificial feeding has received considerable attention. Since the 1880s, some form of artificial diet has been formulated to replace the natural pollen diet of bees (Standifer *et al.* 1973). The use of artificial feedstuffs enabled colonies of honeybees to grow and stay alive in times of pollen shortage so they then can take full advantage of the main nectar flows (Spencer-Booth 1960).

Soya bean flour has been the most commonly used substitute protein in artificial feedstuffs for honeybees and is reported to be beneficial in increasing honey production when used as a supplement (Shoreit and Hussein 1993). In a review of honeybee diets, Spencer-Booth (1960) quoted a source that said soya bean flour was as effective as pollen for hypopharyngeal gland development. Haydak (1949) showed that brood could be raised to the sealed stage to at least 20 days when fed soya bean flour.

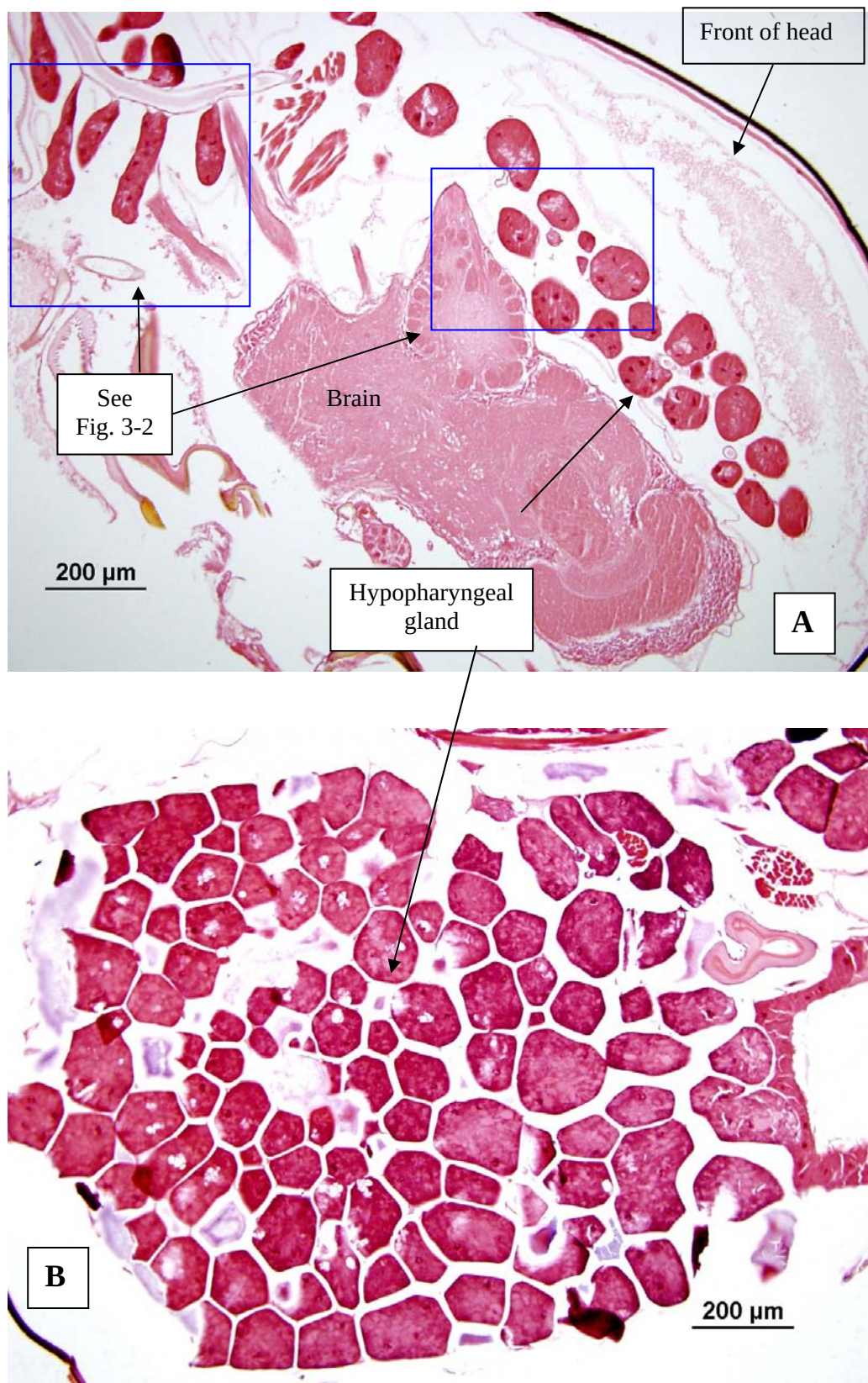


Figure 3-1. Lateral (A) and anterior (B) histological Sections of the honeybee head showing the hypopharyngeal gland.

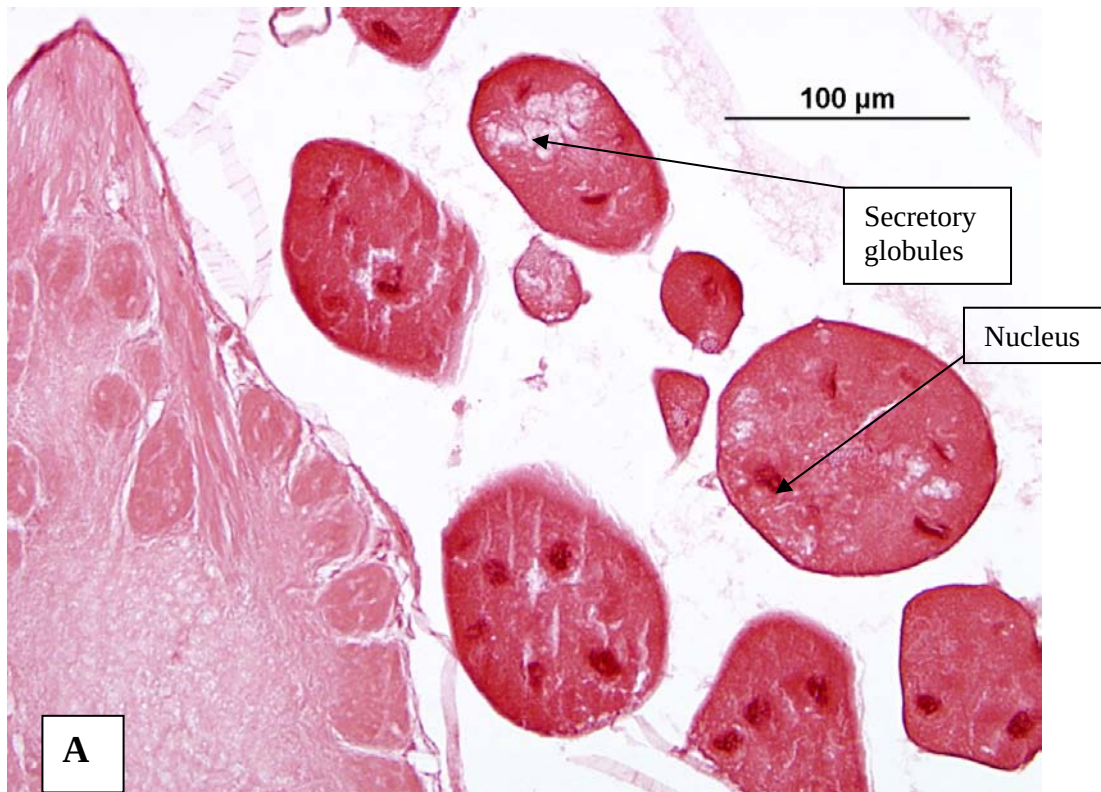


Figure 3-2. Close up of Sectioned hypopharyngeal gland (A) and duct towards mouth parts (B) (see boxes Fig. 3-1).

It was hypothesized that the addition of a certain amount of linoleic or oleic acid to *C. calophylla* pollen would be beneficial to honeybee longevity (see Section 2 above). In this Section, the bees fed identical diets used in (Section 2 above) were tested for any response of the hypopharyngeal gland to diet by weighing heads of individual bees. The enhanced-fat pollen diets, when fed to bees, were expected to

have a similar relationship to diets made or mixed from soya bean flours commercially produced with various grades of fat concentration. The soya bean flours can be low (protein concentrate), medium (defatted) and high fat (full-fat) flour which are used widely in the beekeeping industry. Beekeepers add powdered bee-collected pollen to flour to increase its palatability.

Nutrient composition of honeybees

- A. RESPONSE TO DIETS OF LINOLEIC AND OLEIC FATTY ACID ENHANCED RED GUM (*CORYMBIA CALOPHYLLA*) POLLEN**
- B. RESPONSE TO PURE DIETS OF SOYFLOUR (HIGH, MEDIUM AND LOW FAT) AND LUPIN FLOUR**

Honeybees are extremely polylectic (Schmidt *et al.* 1987) visiting many different plants for pollen or nectar. Pollen provides all the nutrients required by honeybees for growth and development. Honeybees can collect other foods such as black rust (*Uromyces* sp.) spores (Schmidt *et al.* 1987) and in times of pollen dearth, when extreme environmental conditions prevail, bees can be found collecting the fine dust from flour mills. Pollen consumption by bees and brood rearing are significantly correlated (Herbert and Shimanuki 1978b) and the digestibility of pollen is relatively efficient, averaging 70.2% (Roulston and Cane 2000).

The nutritional value of pollen from different plant species varies widely and because most plants flower seasonally, the total body protein for example, can significantly differ over time (Rani and Jain 1996). At one extreme, some pollen can bring about a real lengthening of life-span, hypopharyngeal gland and fat body development while other pollen types may shorten the life-span and have no effect on the bee's physiological condition (Maurizio 1950).

Pollen consists of three layers: the exine which consists of a matrix of cellulose and sporopollenin which can be sculptured with spines and pores, the internal layer composed of cellulose and pectic acids and enclosing the protoplasm and is known as the intine. External to the exine is a semisolid layer of lipids, proteins and sugars (Peng *et al.* 1985). The lipid component of the pollen can range from 0 to 11.2% with a mean of 2.52% in Australia where endemic plant pollens have much less lipid than exotic species, 1.78% and 4.13%, respectively (Somerville 2005b).

The lipoprotein, vitellogenin is the most abundant storage protein in female insects and is produced in the fat body located in the abdomen (Toth and Robinson 2005). When honeybees are receiving no supplies of pollen over an extended period, the greatest loss of nitrogen is from the abdomen (Haydak 1937). Lipids are also stored in the fat body and are a component with amino acids in the synthesis of vitellogenin, which is transported in the haemolymph. Vitellogenin contributes some 32-40% of the protein in the haemolymph in those bees in the hive (1-20 days old) and decreases to 15-26% in field bees [24-40 days old] (Fluri *et al.* 1982).

Most insects obtain amino acids from their foods by ingesting protein (Nation 2002) and for honeybees, it is from pollen. Honeybees efficiently digest and utilise both the food substance and nitrogen of pollen (Schmidt and Buchmann 1985). Ten essential L-amino acids are essential for the growth of honeybees: arginine, histidine, lysine, tryptophan, phenylalanine, methionine, threonine, leucine, isoleucine and valine (de Groot 1953, Haydak 1970). Of all the amino acids, the predominant is proline and between colonies of bees, there is great variance in the concentration of all amino acids (Crailsheim and Leonhard 1997).

The gastrointestinal tract of nine day old nurse bees contain the largest amount of pollen which declines to minimal amounts in foragers and is digested more efficiently by young bees than by foragers (Crailsheim *et al.* 1992). Freshly emerged bees have low levels of proteolytic enzymes but these rise to peak at day eight of life (Moritz and Crailsheim 1987; Szolderits and Crailsheim 1993). Proteolytic activity correlates to the protein content of the midgut and is associated with the size of the hypopharyngeal glands (Moritz and Crailsheim 1987).

From protein and lipids, most insects can synthesize carbohydrate (Nation 2002). This synthesis is important as royal jelly contains 11% carbohydrate (Schmidt and Buchmann 1997) which is produced by young bees. Adult honeybees can only raise brood for a relatively short time when fed a pure carbohydrate diet and during this time, protein is sourced from their own body tissue (Haydak 1935), particularly from the abdomen (fat body).

The macronutrient requirements for minerals have been established for only a few insects. The trace elements (such as sodium, zinc, iron, manganese and copper) have been little studied (Nation 2002). Small amounts of many minerals are required as metal ions in enzyme co-factors and metalloenzymes (Nation 2002). Iron is one element that is deposited in tissue (Haydak 1970).

The most important honeybee produced food, Royal jelly contains about 1% minerals composed mainly of potassium but also contains high levels of the trace elements zinc, iron, copper and manganese (Schmidt and Buchmann 1997). This brood food is secreted by the hypopharyngeal gland. The development of the gland responds to changes in bees' behaviour and with honeybee age and mirrored with this is pollen consumption. Pollen consumption is high when bees are young and low when bees are older. Minerals in pollen are apparently not essential for the development of the hypopharyngeal gland (Herbert and Shimanuki 1978b).

In earlier experiments, longevity and life-span and head weight were significantly affected by diets composed of pollen, pollen enhanced with fatty acids and flours. Therefore, analysis of the chemical components of the honeybee body may show some chemical relationship as to why these parameters may have been affected. This is the objective of this chapter.

Methods

General methodology

Honeybees

Newly emerged honeybees were obtained from drawn frames placed into nucleus colonies 21 days prior to an experiment testing lipid-enhanced pollen diets. One clean drawn frame was placed into 36 nucleus colonies to hatch as many bees as possible at the same time. Genetically, the queen bees were all sisters of the same age as Milne (1980) found analysis of variance on the length on life of bees in cages revealed highly significant differences between colonies sourced for bees.

Frames of bee brood were hatched in a warm room and the emerged bees were gently brushed into an empty cardboard cylinder every four hours for two days (except overnight, where the emerged bees were up to 12 hours old before being harvested). The cylinder of bees was gently rolled to mix bees. Handfuls of bees were gently scooped out by hand and released into a funnel on a stand whose end was fitted over the neck of a plastic bottle, which was placed on a two decimal Sartorius weighing balance. Approximately 30 g of bees were placed into each cage at each stage of the collection cycle. In total, approximately 145 g (c. 1400 bees) of honeybees were weighed out into 12 cages. A fertile queen bee was introduced into each cage.

Bees from the apiary had been marked with white paint on the dorsal thorax (Fig. 5-1) when they were about 12 hours to one day old. Approximately 400 emerged bees were marked and returned to a single nucleus hive. Fifty bees were removed weekly on the same day as 50 bees were removed from the 12 cages for measurements of head weight, protein, lipid, fatty acids and minerals. Each experiment (replicate) was conducted over six weeks, which was considered to be the average duration of a normal honey flow. The total time of each experiment was 63 days (21 + 42).

The data in Section 10 were obtained by visually examining the remaining bees from each cage for being hairy or hairless, being ascribed the status of 'young' or 'old' respectively. Heads were removed and weighed fresh.

The data in Section 13 were obtained by removing the gut (Fig. 5-2) from approximately 80% of the 20 903 bees which all had their heads removed for weighing. It does not include the 1,970 bees that were either gutted or had their heads removed in replicate two which were excluded from results because of the influence of fungal contamination of the sugar syrup had on the data. Each honeybee had its sting apparatus and gut removed prior to analysis (Herbert *et al.* 1970). By carefully pulling the sting with a pair of tweezers, the rectum comes out first, followed by the anterior intestine and the ventriculus (de Groot 1953).



Figure 5-1. Marked emerged bees in a hive in the apiary.

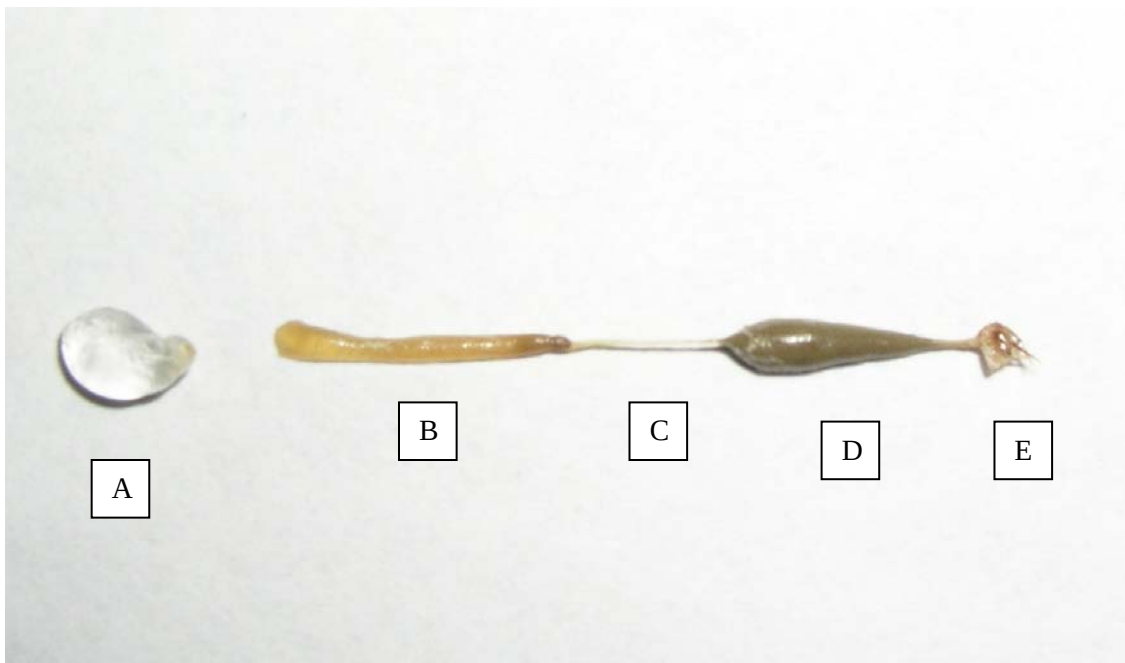


Figure 5-2. The gut removed from honeybees. L-R: honey stomach/sac (A), ventriculus (B), anterior intestine (C), rectum (D) and sting apparatus (E).

Cages

Twelve wooden cages were built (W 405 mm x L 405 mm x H 505 mm) each had a meshed (2 x 2 mm) sidewall and an enclosed access point made from muslin cloth (Fig. 5-3). Inside each cage was a smaller wooden cage (W 130 mm x L 240 mm x H 205 mm) which housed the newly emerged honeybees and their sugar, water and food supply (Fig. 5-4). The inner cages were heated from the exterior by thermal heating tapes set at 39°C and by a re-circulating fan heater set at 31°C that heated the room where the cages were located. Four shallow trays of water were placed on the floor of the room for humidity.

However, like Herbert *et al.* (1970), temperature and humidity varied and were recorded on Hastings Tinytalk™ data loggers. Each cage had a removable beeswax-coated piece of plastic foundation (L 145mm x H 105 mm) in which the bees could build comb and raise brood during the six week experiment. In prior tests, the cages and comb were successfully occupied by the small colony of bees. Wax foundation was drawn and bees raised brood (Fig. 5-5).

Pollen

The source of protein was from an endemic Western Australian tree, *Corymbia calophylla* (marri/redgum) which has an average protein concentration of 26.7% (Manning 2000) to 27.9% (Bell *et al.* 1983). The concentration of protein is high and showed no limitations of amino acids that would affect honeybee development as defined by De Groot (1953). The pollen is harvested in abundance by beekeepers, exported and used as an ingredient in the production of supplementary feedstuffs for the Australian beekeeping industry. Redgum pollen (RG) has an average lipid concentration of 0.88% (Manning and Harvey 2002) to 1% (Bell *et al.* 1983) and its fatty acid profile has been established (Manning 2001, Manning and Harvey 2002). Mineral concentration of the pollen was published by Manning (2000) and Bell *et al.* (1983). Crushed and sterilised redgum (cRG) pollen, that was irradiated at 20 kilograms and six years old (harvested fresh in 1997) at the start of the experiment, was used to fortify the soya bean and lupin flour diets. The cRG pollen had always been stored in a cool room at about 10°C.



Figure 5-3. The author with twelve cages used for the experiments.

Diets

Pollen trapped from *C. calophylla* was enhanced by thoroughly mixing 2% (i.e. 1 g fatty acid in 49 g pollen), 6%, 10% or 16% of linoleic acid to form 4 treatments. The mix was identical for oleic acid for the second concurrent set of four treatments. There were two controls in each experiment: 7-8 year old *C. calophylla* pollen that had been dried, crushed and irradiated (cRG); fresh bee-collected *C. calophylla* pollen (RG) which had been frozen since the beginning of the first experiment was mixed for the same period as used to mix the fatty acid incorporated treatments. There were two sugar controls in each experiment: one mixed from dry sugar (52%); a liquid commercial inverted sugar that was diluted to 52% from Sugar Australia.

Applied fatty acid

Linoleic (Sigma Chemicals Cat No 102187 Lot No 5207B) and oleic acids (Unilab Lab Reagent B/No H2H129) were used in concentrations of 2, 6, 10 and 16% in redgum (RG) pollen. The 2% pollen mix texture was crumbly and the 6 and 10% mix was a soft paste resembling the texture of peanut butter. The 16% mix was similar but very sticky. The nutritional qualities of each diet are given in Table 9-1. Each 50 g pollen-fatty acid mixture was placed into test tubes, which were set at an angle into the side of the cage to avoid spillage by bees. The feedstuffs were randomly assigned to the 12 cages and replicated sequentially for three times between October 2003 and February 2005 (Table 5-1). The number of bees gutted for analysis of nutrients was 11 053.

Flour - Soya bean and lupin

Soya bean and lupin beans were milled into flour by commercial processors. The high-fat soya bean flour (FF) was commercially sold as full-fat flour (Ben Furney flour mills, Dubbo, NSW; product code SF20) whilst the medium-fat flour (DF) was sold as defatted flour - non GM (Ben Furney Flour Mills, Dubbo NSW; product code SF25) and was subject to expelling and solvent extraction. Soya bean flour with the lowest fat (PRO) was commercially sold as Promine (product code HV; soy protein concentrate, Fort Wayne IN, USA). Lupin flour was prepared by milling *Lupinus angustifolius* var. *myallie* seed and was supplied by Dr John Snowden, Western Australian Department of Agriculture. Each flour type was enhanced with 10% cRG pollen. Defatted soya bean flour (DF) was additionally enhanced with 20 and 30% cRG pollen. The nutritional qualities of each diet are shown in Table 9-2, Chapter 9. Each feed mix was placed into test tubes as described above. The feedstuffs were randomly assigned to the 12 cages for each replicate (Table 5-1). Each diet was replicated sequentially three times between June 2004 and December 2004. The number of bees gutted for analysis of nutrients was 9850.

Sugar solution

A 52% sugar solution was fed into cages via an inverted bottle with a single 1.6 mm hole drilled into the plastic lid placed over a 40 mm hole in the top of the cage (Fig. 5-4). The sugar solution was fed *ad libitum*. The invert sugar was a commercially prepared product in liquid form, which was diluted to 52% prior to use.

Water supply

The caged bees were fed double-distilled water *ad libitum* in the same way as the sugar solution (Fig. 5-4).

Virus examination

The bees in Chapter 6 were subjected to a viral examination. Honeybee heads from a sample of bees remaining in the freezer after the conclusion of the experiment were examined by the virology laboratory at the Western Australian Department of Agriculture. Rinderer (1974) obtained virus suspensions after grinding heads of sick bees with a mortar and pestle. Eleven samples, each containing about 10 bee heads were examined by a veterinary pathologist using transmission electron microscopy.

Summary of experiments

Table 5-1 is the experimental schedule that shows the feedstuff type, when bees were born, and start and finish dates for the experiments.

Table 5-1. Experimental schedule.

Experiment	Feedstuffs tested	Bees born	Start date	Finish date	Comment
1	12 fatty acid enhanced pollen diets	16-17 Sept (spring)	9-Oct-2003	20-Nov-2003	
2	12 fatty acid enhanced pollen diets	4-5 Nov (summer)	27-Nov-2003	8-Jan-2004	Experiment repeated
3	12 fatty acid enhanced pollen diets	20-21 Jan (summer)	12-Feb-2004	25-Mar-2004	
4	12 Flour diets	1-2 Jun (winter)	24-Jun-2004	5-Aug-2004	
5	12 Flour diets	27-28 July (winter)	19-Aug-2004	30-Sept-2004	
6	12 Flour diets	12-13 Oct (spring)	3-Nov-2004	16-Dec-2004	
7	12 fatty acid enhanced pollen diets	21-22 Dec (summer)	13-Jan-2005	24-Feb-2005	Exp. 2 repeat



Figure 5-4. Cage used for the experiments. Left-hand side of cage (not in view) is made of mesh as is the removable floor of the cage. Diets were supplied via the test-tube. Bottles on top of cage supplied either distilled water or sugar syrup.

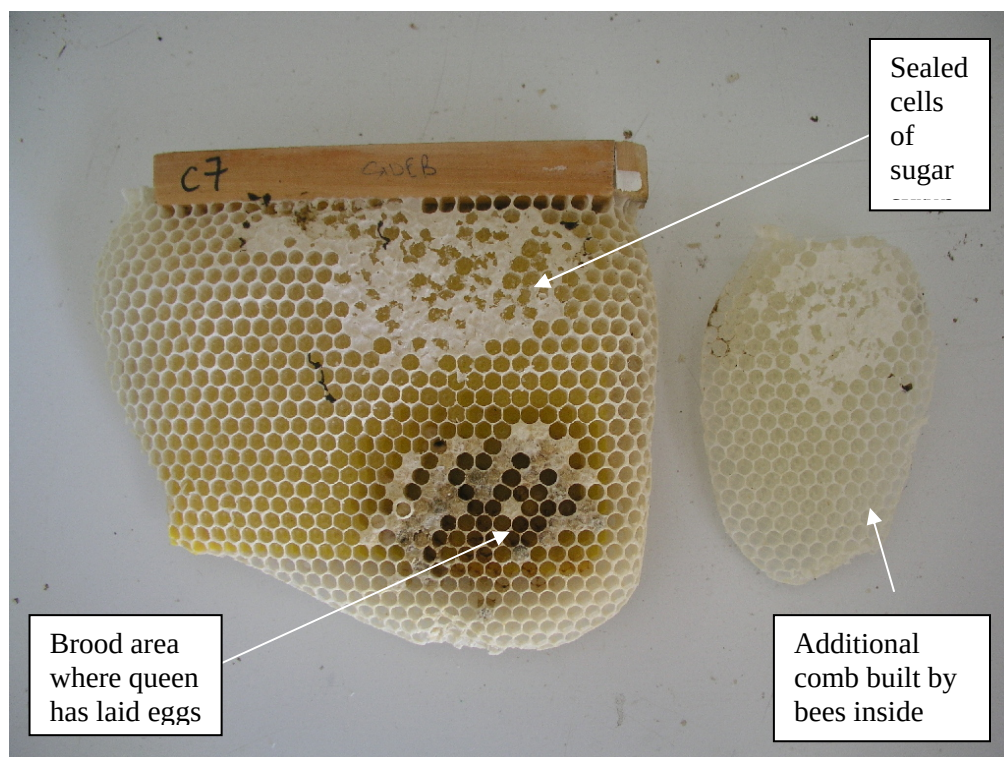


Figure 5-5. In test experiments, the bees readily drew the wax from the plastic foundation and raised brood.

Observation of “hairless” phenotype in a caged single cohort of honeybees as a potential indicator of behavioural status.

Methodology

The general procedures have been described above (Section 5).

To ascertain whether the observed visual differences of the bees in cages corresponded to any physical change within the bee, fresh head weights were obtained from a sub-sample of bees of hairy and hairless phenotypes that remained alive after 42 days in the last replicate, which was one of three (Experiment 7; Table 5-1, Section 5). The bees were fed fatty acid (oleic and linoleic) enhanced diets.

Following the conclusion of the six week experiment, all cages of bees were placed into a -20°C freezer for 24 hours. From each cage the bees were separated into groups based on whether they were the hairy or hairless phenotype. Heads were removed by scalpel and weighed.

Statistical methods

Means and standard deviations are given in the figures. Differences between the means of the various treatment groups were analysed with a simple analysis of variance model using the restricted maximum likelihood (REML) procedure in GenStat (2005) because of unequal numbers of bees in the groups.

The analysis was done using a factorial subdivision of the treatments into CRG, RG vs the rest (linoleic/oleic acid), where the rest have a factorial of rate (2, 6, 10, 16) x fatty acid (L,O). The data were constructed into factors: **AGE** (hairy/hairless); **PCT** (2, 6, 10, 16); **TREAT** (L (linoleic acid), O (oleic acid), CRG (crushed redgum), RG (fresh redgum pollen); **OTHER** (LO), CRG, RG; **FEED** (2%L, 2%O etc). The variate was fresh head weight (mg).

Hence, the simple REML model is $\text{FIXED} = \text{OTHER}/(\text{AGE}*\text{TREAT}*\text{PCT})$, random is left as blank (GenStat equation 1) which by default is the average within treatment bee variability.

To test linear and quadratic trends, two columns of variates were added to the data set: Linear x (2, 6, 10, 16) and Quadratic x^2 (0, 4, 36, 100, 256) and analysed using the model:

$\text{OTHER}/(\text{TREAT}*\text{AGE}*(x+x^2+\text{PCT}))$ random is left as blank (GenStat equation 2).

A binomial model, fitted using a generalised linear model in GenStat with a similar treatment model as in REML above, was used to examine the proportion of hairless bees in the population that were subjected to different fatty acids. The data were constructed into factors: **LorO** (yes or no [RG/CRG]); **TREAT** (linoleic, oleic or none [RG/CRG]); **PCT** (2, 6, 10, 16 or 0) and the variate, the percentage of hairless bees over total population for each diet. The data were analysed using the model:

$\text{LorO}/(\text{TREAT}*\text{PCT})$ (GenStat equation 3).

Results are given as predicted means from the models together with SE or 5%LSD. Differences between groups were considered to be significant at the 5% level of significance ($P = 0.05$).

Longevity of honeybees fed linoleic and oleic acid enhanced redgum (*Corymbia calophylla*) pollen diets; high and low fat soya bean flour diets and lupin and flour diets mixed with redgum pollen

Methodology

General procedures have been described in Section 5 (above). Twelve diets of pollen enhanced with two fatty acids (oleic and linoleic) were fed to bees in 12 cages and replicated three times. A further 12 diets, that contained flour from soya bean or lupin were fed to bees in the same way and replicated three times. The diet labels and fatty acid portion of the diets are given in Tables 7-1 and 7-2.

The protein, lipid, linoleic and oleic acid composition of the diets used in the series of experiments are given in Tables 7-1 and 7-2.

Statistical analyses

The effect of diet on fresh head weight of honeybees was analysed in SAS (2002-2003). Survivorship curves were determined by the Kaplan-Meier procedure (Kaplan and Meier 1958) where the Breslow test statistic was used for the comparison of the curves (Breslow 1975). A Cox Proportional Hazard regression model was used where bees removed from the cages for head weight measurements were treated as censored cases.

Table 7-1. Protein, lipid, linoleic acid and oleic acid content of the fatty acid enhanced redgum (RG) pollen diets. Pollen (cRG), cane and invert sugar diets made up the rest of the 12 cage experiments.

Diet label	Diet composition	Protein (%)	Lipid (%)	Linoleic acid (mg/g)	Oleic acid (mg/g)
2%L	Redgum pollen + 2% Linoleic acid	23.2	2.70	13.2	5.51
6%L	Redgum pollen + 6% Linoleic acid	22.6	7.20	28.0	11.2
10%L	Redgum pollen + 10% Linoleic acid	21.7	11.1	45.2	18.2
16%L	Redgum pollen + 16% Linoleic acid	20.4	17.3	74.3	30.4
2%O	Redgum pollen + 2% Oleic acid	24.0	1.10	5.13	13.9
6%O	Redgum pollen + 6% Oleic acid	23.3	7.20	5.25	30.6
10%O	Redgum pollen + 10% Oleic acid	22.2	11.7	7.29	48.4
16%O	Redgum pollen + 16% Oleic acid	20.4	14.2	11.3	87.6
RG	Redgum pollen	24.4	1.6	2.12	0.72

The factors in the model were daily temperature, humidity and an indicator variable for each diet group (i.e. zero or one) and an indicator variable for each replicate (zero or one), totaling 30 terms: temperature, humidity, foodCRG, foodRG, food2L, food6L, food10L, food16L, food2O, food6O, food10O, food16O, foodPRO, foodPRO10, foodDF, foodDF10, foodDF20, foodDF30, foodFF, foodFF10, foodLUP, foodLUP10, foodCS, foodinvertsugar, Exp 1, Exp 3, Exp 4, Exp 5, Exp 6, Exp 7 (Experiment 2 was removed from the data set because of sugar contamination - see results).

Table 7-2. Protein, lipid, linoleic acid and oleic acid content of the soya bean flour, lupin flour diets and diets augmented with crushed and irradiated redgum (cRG) pollen. A cane sugar diet made up the 12 cage experiments.

Diet label	Diet composition	Protein (%)	Lipid (%)	Linoleic acid (mg/g)	Oleic acid (mg/g)
PRO	Promine™	62.4	0.60	1.50	2.55
PRO10	Promine™ + 10% cRG	58.7	0.50	3.53	2.60
DF	Defatted	54.7	1.80	10.9	4.11
DF10	Defatted + 10% cRG	52.4	2.00	11.5	3.82
DF20	Defatted + 20% cRG	49.9	1.80	11.5	3.69
DF30	Defatted + 30% cRG	46.7	2.00	12.0	3.49
FF	Full fat	42.9	18.9	95.1	28.3
FF10	Full fat + 10% cRG	41.8	16.9	86.5	25.5
LUP	Lupin	42.2	6.90	29.5	21.2
LUP10	Lupin + 10% cRG	40.1	6.40	27.7	19.0
cRG	Crushed and irradiated redgum pollen	29.0	2.60	15.7	1.56

The effect of diet on head weights (hypopharyngeal gland) of honeybees

- A. LINOLEIC AND OLEIC ACID ENHANCED REDGUM (*CORYMBIA CALOPHYLLA*) POLLEN DIETS
- B. SOYA BEAN AND LUPIN FLOUR DIETS AND MIXES CONTAINING REDGUM POLLEN

Methodology

General procedures have been described in Section 5 (above). Twelve diets of pollen enhanced with two fatty acids (oleic and linoleic) were fed to bees in 12 cages and replicated three times. A further 12 diets, that contained flour from soya bean or lupin were fed to bees in the same way and replicated three times. In total, seven experiments were conducted, one of which was repeated. The diet labels and fatty acid portion of the diets are given in Tables 7-1 and 7-2 (above). At the same time as the cage experiments commenced, approximately 500 bees of the remaining emerged bees were marked with a white dot on the dorsal thorax and returned to a hive in an apiary (see Fig. 5-1, Section 5).

Each week of each six week experiment, a sample of 50 bees was collected from each cage and the single hive in an apiary, labelled in a plastic bag and immediately frozen at -20°C. When time permitted, each bag of frozen bees was emptied onto paper tissue and defrosted. Each bee was gutted for nutrient measurements and heads were removed by scalpel and weighed.

Fresh weights (mg) were recorded weekly to associate the normal development of the hypopharyngeal gland with head weight. Samples of heads with a weight between each head weight category viz: >7 mg, >8 mg, >9 mg, >10 mg, >11 mg, >12 mg and > 13 mg. Bee heads only from experiment 6 were used for histology and were placed in Bouin's fixative.

The protein, lipid, linoleic and oleic acid composition of the diets used in the experiment are shown in the methodology Section 7 (above).

Histology of the development of the hypopharyngeal gland

The emerged bees returned to the apiary were assessed for normal gland development by histology. A random sample of one head from each size category (see above) was cut vertically with a scalpel from the anterior face between the eyes and the separate sides of the head were embedded in Paraplast™

tissue embedding medium. Sections of the head were cut at a thickness of 5 μm with a metal knife on a manual rotary Leica RM2235 microtome and stained with haematoxylin and eosin (H & E), a general purpose stain that showed cytoplasmic components as pink and nuclei as blue-purple (Bancroft and Stevens 1977). As one of the better diets identified in the project, a sample of heads from bees fed the crushed redgum pollen diet (cRG) were also examined by histology at Week 1, Week 3 and Week 6. The histological work was undertaken by Mr Gordon Thomson, Histology, Murdoch University.

The histology slides were photographed and examined for hypopharyngeal gland development by comparison to the illustrations in Wang and Moeller (1969).

Statistical Analyses

The effect of diet on fresh head weight of honeybees was analysed by SAS (1999-2001) and consisted of three parts.

1. The mean head weights of the emerged bees from each experiment were compared to test for differences across experiments and seasons.
2. The growth rates of the mean head weight of the apiary bees were compared across experiments and season to test for differences.
3. The growth rates of the mean head weights of bees under the various diet treatments were compared, while considering the variation due to season and experiment.

Experiment 2 was noted to have been exposed to poisoning from contaminated sugar syrup. However, the emerged bees and bees that returned to the apiary were not exposed to the contamination. This data was still useful in the analysis.

1. Emerged bees

To check whether there was a significant difference in the mean head weight across seasons and across experiments, an analysis of variance was performed. The emerged head weight of the i^{th} observation from the e^{th} experiment was modelled as:

$$h_{e,0,i} = I + S_e + E_e + \varepsilon_{e,0,i}$$

where S_e and E_e are constants representing the effect of season and experiment on the emerged head weight and $\varepsilon_{e,0,i}$ are normally distributed errors.

The analysis was performed in the SAS system for Windows by using the GLM procedure. As there was only one season associated with each experiment, the experiment effect was considered to be nested within the main effect of the season.

2. Standardisation

The mean emerged head weights varied over season and experiment. So as not to confound the variation in head growth of the apiary bees, or bees under any of the diet treatments, with the variation due to seasonal and experimental effects of the head weights at birth, the data were standardised by applying the transformation:

$$\nabla h_{d,e,t,i} = h_{d,e,t,i} - E(h_{e,0})$$

where $h_{d,e,t,i}$ is the head weight and $\nabla h_{d,e,t,i}$ are adjusted head weights of the i^{th} observation taken at time t from bees under diet treatment d in the e^{th} experiment, and $E(h_{e,0})$ is the expected emerged head weight of a bee in the e^{th} experiment.

Thus this transformation simply shifts the data for each experimental group by the expected head weight at emergence. Therefore, under this transformation the expected value of the adjusted head weight, under diet d and experiment e , $E(\nabla h_{d,e,0})$ will be:

$$E(\nabla h_{d,e,0}) = E(h_{d,e,0}) - E(h_{e,0}) = 0$$

The effect this transformation has on the data is seen in Figure 8-4, where a “by replicate” plot of the mean head weights of the apiary bees along with the emerged bees (at day 0) is given for both the raw and standardised data.

3. Apiary bees

To investigate the rate of change of head weights of the apiary bees and to test if season and/or experiment effects this rate, a linear model was fitted to $\nabla h_{a,e,t,i}$ of the form:

$$\nabla h_{a,e,t^*,i} = I_a + T_a t^*$$

where I_a and T_a are parameters to be determined and t^* is the number of days after day 7. That is, $t^* = t - 7$.

The advantage of using such a model is that the two parameters have a useful physical interpretation. The parameter I_a captures the change that occurs in the first 7-day period after birth, while the parameter T_a gives a measure for the rate of change of the mean head weight after day 7.

More complex models were also tested. An adjusted R-squares of the linear model (20.8%) was compared with that of a quadratic (20.7%) and a cubic model (21.9%). The added additional complexity did little to improve the R-square values and so little extra variation was accounted for by these models. Moreover, as the linear model is most useful for interpretation purposes, it was considered optimal for our purposes.

In addition to the base model, terms were also added to account for effects across season and experiments. The full model fitted is of the form:

$$\begin{aligned} \nabla h_{a,e,t^*,i} = & I_a + T_a t^* + S_{a,e} + E_{a,e} \\ & + (S * T)_{a,e} t^* + (E * T)_{a,e} t^* \end{aligned}$$

The terms $S_{a,e}$ and $E_{a,e}$ account for the variation in the head weights during the first seven day period that is due to the season or experiment within the season, respectively. The terms $(S * T)_{a,e}$ and $(E * T)_{a,e}$ account for the variation in the growth rates due to the season and experiment within a season.

4. Head weights of caged honeybees fed diets

The standardised data, as defined in (2. above) was used for this analysis so that the variation in head weights due to diet was not confounded with that due to head weight at birth (i.e. of the emerged bees). The adjusted mean head weight for the bees under each of the diet treatments are plotted in Figures 8-4 to 8-10. The adjusted head weight in these figures is the difference between the head weight and the mean head weight at emergence.

An iterative procedure linear model was fitted to the data that allowed for the variation due to season and experiment. The full model chosen was:

$$\begin{aligned}\nabla h_{d,e,t^*,i} = & I + Tt^* + D_d + S_e + E_e \\ & + (T * D)t^* + (D * S)_{d,e} + (D * E)_{d,e} \\ & + (S * T)_e t^* + (E * T)_e t^* + (D * S * T)_{d,e} t^* + (D * E * T)_{d,e} t^*\end{aligned}$$

where $\nabla h_{d,e,t^*,i}$ is the difference in head weight from the average emerged head weight, and t^* is the number of days after day seven. The interpretation of each term of the model is shown in Table 8-1.

This particular model is useful for our purposes as it allows us to compare both the head weight after seven days and the overall growth rates. For a given experiment e and diet d we have:

$$\text{headweight after 7 days} = I + D_d + S_e + E_e + (D * S)_{d,e} + (D * E)_{d,e}$$

and

$$\begin{aligned}\text{growth rate} = & T + (T * D) + (S * T)_e + (E * T)_e \\ & + (D * S * T)_{d,e} + (D * E * T)_{d,e}\end{aligned}$$

Table 8-1. Explanation of model terms.

Term	Interpretation
D_d	variation in head weights due to the various diets
S_e	variation in head weights due to the different seasons
E_e	variation in head weights due to an experiment within a particular season
T	the growth rate of head weights, independent of other the factors
$(T * D)_d$	variation in growth rate due to the various diets
$(S * T)_e$	variation in growth rates due to the change of seasons
$(E * T)_e$	variation in growth rate due to experiment within a season
$(D * S)_{d,e}$	variation in the effect of diet on head weight between seasons
$(D * E)_{d,e}$	variation in the effect of diet on head weight between the experiments
$(D * S * T)_{d,e}$	variation in the effect of diet on head weight growth between seasons
$(D * E * T)_{d,e}$	variation in the effect of diet on head weight growth between the experiments

Nutrient composition of honeybees

- A. RESPONSE TO DIETS OF LINOLEIC AND OLEIC FATTY ACID ENHANCED RED GUM (*CORYMBIA CALOPHYLLA*) POLLEN.
- B. RESPONSE TO PURE DIETS OF SOYFLOUR (HIGH, MEDIUM AND LOW FAT) AND LUPIN FLOUR.

Methodology

General procedures have been described in Section 5 (above). Twelve diets of pollen enhanced with two fatty acids (oleic and linoleic) were fed to bees in 12 cages and replicated three times. A further 12 diets, that contained flour from soya bean or lupin were fed to bees in the same way and replicated three times. In total, seven experiments were conducted, one of which was repeated. The diet labels and fatty acid portion of the diets are given in Tables 7-1 and 7-2 (Section 7). At the same time as the cage experiments commenced, approximately 500 bees of the remaining emerged bees were marked with a white dot on the dorsal thorax and returned to a hive in an apiary (see Fig. 5-1, Section 5).

Each week of each six week experiment, a sample of 50 bees was collected from each cage and from the single hive in an apiary. Samples were placed into a sealable plastic bag, labelled and immediately frozen at -20°C. When time permitted, each bag of frozen bees was emptied onto paper tissue and defrosted. Each bee head was removed by scalpel and weighed and each bee was gutted for nutrient measurements (this Section). The area of comb in each cage at the conclusion of each experiment was also measured.

Applied fatty acid

The nutritional composition of each diet is shown in Table 9-1. The fatty acid profile of the two oils that were added to the pollen diets are shown in Table 9-2. The first experiment commenced in October 2003 and the final was completed in February 2005.

Flour - Soya bean and lupin

The nutritional composition for each diet is shown in Table 9-3. The first experiment commenced in June 2004 and the final was completed in December 2004.

Honeybee analysis

Honeybees (10) with their guts removed were analysed for mineral, protein, lipid and fatty acid composition each week for six weeks (depending upon each population's period of survival) for each diet treatment. A composite sample of wax scales collected from the abdomen of bees fed each of three diets: pollen + oleic, pollen + linoleic and redgum pollen were analysed for fatty acid composition (Table 13-4, Section 13).

Fatty acid Analysis

The same procedure was followed in methodology. Only those fatty acids exceeding 0.10 mg/g in body concentration are discussed.

Protein Analysis

The nitrogen content of the bees was determined by complete combustion of the bees (AOAC Official Methods 2000a). The percentage of nitrogen was then multiplied by a factor of 6.25 to give crude protein levels in the samples.

Lipid Analysis

The fat level was determined by a modification of the AOAC method 27.4.04 (2000b) where hexane was used as the extracting solvent.

Mineral Analysis

The bees were dissolved in concentrated nitric/perchloric acid in a block digester (200-210°C). After cooling, the digests were diluted with deionised water and analysed for minerals (P, K, Na, Ca, Mg, S,

Fe, Mn, Zn and Cu) by ICP-AES (Mc Quaker *et al.* 1979). The results for Na, Ca and Mg are not reported here because of their small variation in concentration over time and for diet.

Table 9-1. Protein (%), lipid (%), fatty acid (mg/g) and mineral content of the fatty acid enhanced pollen and redgum (RG) pollen diets. Cane and invert sugar diets made up the rest of the 12 cage experiments.

Diet	Protein	Lipid	Myristic	Palmitic	Palmitoleic	Unk3
RG + 2% L	23.2	2.70	0.24	2.04	0.06	0.09
RG + 6% L	22.6	7.20	0.25	3.07	0.07	0.10
RG + 10% L	21.7	11.1	0.30	3.60	0.11	0.08
RG + 16% L	20.4	17.3	0.42	4.64	0.20	0.08
RG + 2% O	24.0	1.10	0.79	2.81	0.76	0.09
RG + 6% O	23.3	7.20	1.57	4.32	1.75	0.50
RG + 10% O	22.2	11.7	2.40	5.82	2.76	0.75
RG + 16% O	20.4	14.2	4.03	9.18	4.85	0.02
RG	24.4	1.6	0.15	1.17	0.00	0.07
cRG	29.0	2.60	0.28	6.50	0.04	0

Diet	Stearic	Oleic	Linoleic	Linolenic	Unk5	Unk6	Lignoceric
RG + 2% L	0.34	5.51	13.2	2.10	0.10	0.04	0.08
RG + 6% L	0.45	11.2	28.0	3.89	0.18	0.08	0.16
RG + 10% L	0.57	18.2	45.2	5.31	0.14	0.06	0.14
RG + 16% L	0.85	30.4	74.3	8.42	0.12	0.06	0.07
RG + 2% O	0.52	13.9	5.13	1.37	0.07	0.03	0.05
RG + 6% O	0.85	30.6	5.25	1.49	0.10	0.05	0.09
RG + 10% O	1.25	48.4	7.29	1.97	0.10	0.04	0.06
RG + 16% O	2.01	87.6	11.3	2.81	0.11	0.03	0
RG	0.23	0.72	2.12	0.87	0.07	0.03	0.05
cRG	0.54	1.56	15.7	2.03	0.54	0.29	0.43

Diet	K (%)	P (%)	S (%)	Cu (mg/kg)	Fe (mg/kg)	Mn (mg/kg)	Zn (mg/kg)
RG + 2% L	0.53	0.41	0.29	23	130	36	91
RG + 6% L	0.51	0.39	0.28	22	130	35	87
RG + 10% L	0.49	0.38	0.27	21	130	34	82
RG + 16% L	0.47	0.36	0.25	20	120	32	80
RG + 2% O	0.53	0.41	0.29	23	140	37	89
RG + 6% O	0.51	0.39	0.28	22	130	36	87
RG + 10% O	0.49	0.38	0.27	21	130	34	84
RG + 16% O	0.45	0.35	0.25	20	110	32	77
RG	0.54	0.41	0.30	22	140	38	89
cRG	0.54	0.42	0.30	22	130	39	89

Table 9-2. Fatty acid (mg/g) profile of the oil added to pollen diets.

Unk1- between C14:1 & C15; Unk2 – between C16 & C16:1; Unk3 – between C16:1 & C17; Unk4 between C18:1 & C18:2; Unk5 – between C20:1 & C20:2; Unk6 – between C22:1 & C22:2.

Diet oil	Myristic	Unk 1	Palmitic	Palmitoleic	Unk 2	Unk 3	Stearic	Oleic
Linoleic acid	2.00	0	23.9	1.17	0	0	4.64	213.5
Oleic acid	38.9	5.93	72.3	43.6	10.2	0	16.2	729.8

Diet oil	Unk4	Linoleic	Linolenic	Gondoic	Unk5	Behenic	Unk6	Lignoceric
Linoleic acid	1.77	445.1	39.5	2.65	0	0.63	0	0
Oleic acid	1.80	31.5	3.85	2.56	0	0	0	0

Statistical Analyses

Differences between the means of the various treatment groups (12 cage diets and one unknown pollen diet by apiary bees) were analysed with a simple analysis of variance model using the restricted maximum likelihood (REML) procedure in GenStat (2005) because of the unequal numbers of bees in the groups formed. The analysis used a factorial subdivision of the treatments into an ANOVA model of *treatment x time* (XDATA) where a line (xdata) was fitted to each treatment and tested statistically (treatment x data). Non-linearity was also tested which required the p-value to be non-significant for the treatment.XDATA interaction after the fixed term treatment.xdata was fitted.

The predictions from REML analysis were based on the following equation:

$$\text{Constant} + \text{treatment} + \text{XDATA} + \text{treatment.XDATA} \quad \text{Equation 1}$$

And the overall linear slope for each diet was tested using the following equation:

$$\text{Constant} + \text{treatment} + \text{xdata} + \text{treatment.xdata} \quad \text{Equation 2}$$

Table 9-3. Protein (%), lipid (%), fatty acid (mg/g) and mineral content of the flour diets. Pollen (cRG), cane sugar diets made up the rest of the 12 cage experiments.

Diet	Protein	Lipid	Myristic	Palmitic	Palmitoleic	Unk3
PRO	62.4	0.6	0.03	0.98	0	0
PRO10	58.7	0.5	0.07	1.77	0.02	0
DF	54.7	1.80	0.04	3.27	0.03	0
DF10	52.4	2.00	0.06	3.79	0.03	0
DF20	49.9	1.80	0.08	3.93	0.04	0
DF30	46.7	2.00	0.10	4.25	0.13	0
FF	42.9	18.9	0.18	21.46	0.15	0.13
FF10	41.8	16.9	0.16	19.85	0.13	0.12
LUP	42.2	6.9	0.23	7.69	0.02	0.05
LUP10	40.1	6.4	0.12	3.55	0.06	0.03

Diet	Stearic	Oleic	Linoleic	Linolenic	Unk5	Unk6	Lignoceric
PRO	0.36	2.55	1.50	0.22	0	0	0
PRO10	0.41	2.60	3.53	0.73	0.06	0	0
DF	0.81	4.11	10.9	1.43	0	0	0
DF10	0.77	3.82	11.5	1.50	0.08	0	0.10
DF20	0.77	3.69	11.5	1.49	0.11	0	0.11
DF30	0.75	3.49	12.0	1.60	0.15	0.08	0.15
FF	5.99	28.3	95.1	16.63	0	0	0.19
FF10	5.42	25.5	86.5	15.14	0.04	0	0.17
LUP	3.51	21.2	29.5	3.77	0	0	0.25
LUP10	1.04	19.0	27.7	4.25	0.05	0	0.31

Diet	K (%)	P (%)	S (%)	Cu (mg/kg)	Fe (mg/kg)	Mn (mg/kg)	Zn (mg/kg)
PRO	2.52	0.79	0.50	11	100	42	32
PRO10	2.32	0.75	0.47	13	100	42	42
DF	2.17	0.66	0.41	20	160	46	57
DF10	1.96	0.62	0.40	21	150	47	71
DF20	1.82	0.61	0.39	21	160	47	82
DF30	1.63	0.58	0.38	22	140	46	91
FF	1.78	0.65	0.35	11	69	35	41
FF10	1.63	0.62	0.34	13	75	36	49
LUP	1.02	0.43	0.28	6	52	57	35
LUP10	0.98	0.43	0.28	8.1	64	56	45

Results

Observation of “hairless” phenotype in a caged single cohort of honeybees as a potential indicator of behavioural status

Results

Characteristics of honeybee phenotypes

Initial head weight when honeybees emerged prior to the experiment is shown in Figure 10-1. Fresh head weight data from the redgum pollen fed cages (controls) (there were no bees remaining in the two sugar controls at 42 days) were pooled and graphed (Fig. 10-2). The frequency distribution shows two distinct groups of bees: hairless and hairy despite all of the bees being the same age. The heavier-headed hairy bees in Figure 10-2 are described as being 'overaged nurses' in single cohorts by Robinson (1992).

The whole data set (N = 2,699) when graphed showed the same response (data not shown) as the redgum fed bees graphed in Figure 10-2. The shift in head weights from emerged bees which initially filled the cages (Fig. 10-1) to that 42 day later (Fig. 10-2) was remarkable given the length of time the bees were caged.

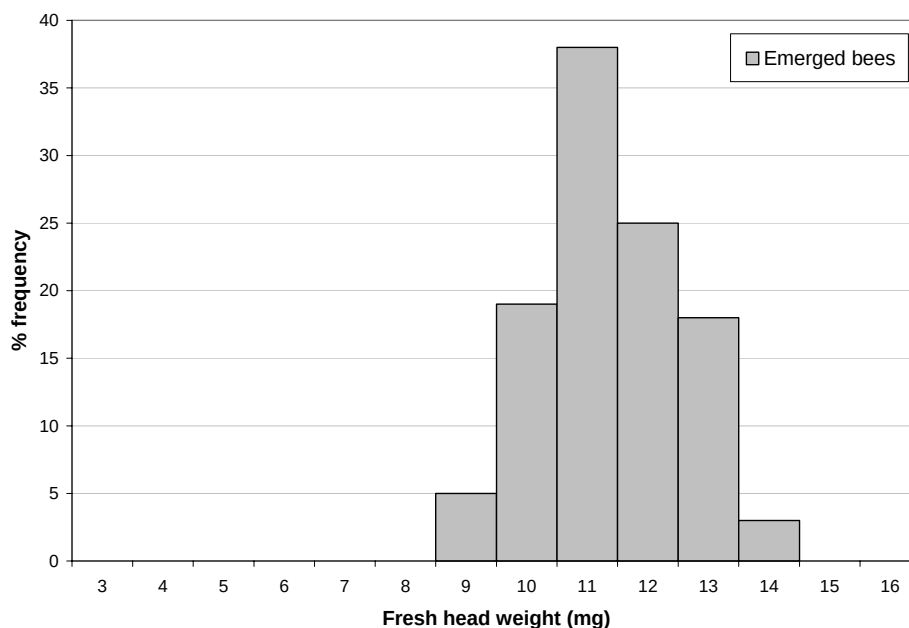


Figure 10-1. Frequency of fresh head weight sample of emerged bees (N = 108) at the beginning of a feeding experiment. Average head weight = 10.9 mg.

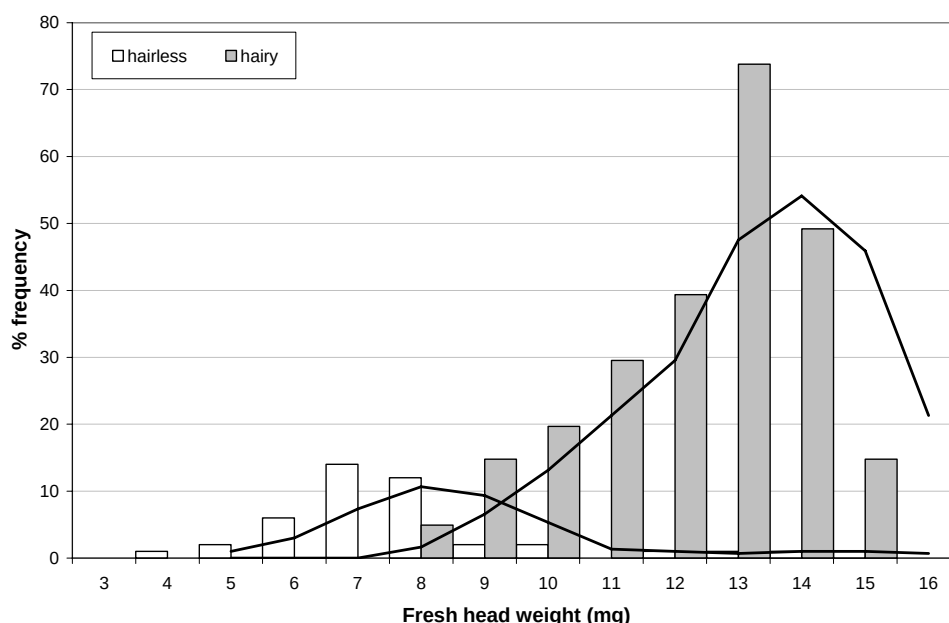


Figure 10-2. Frequency of fresh head weights of bees fed RG pollen (N = 289) from a sub-sample of N = 93 bees at the conclusion of a 42 days feeding experiment. Lines illustrate smoothing +3 moving average.

Hairy and hairless bees versus head weight

Predicted means showed a significant difference ($P < 0.001$) between the head weights of hairless (6.70 ± 0.07 mg, N = 425) and hairy bees (10.2 ± 0.09 mg, N = 500), respectively (5%LSD = 0.19). The hairless bees observed in the experiment had the lightest head weights, which correspond to head weights expected of foraging bees (older bees). The lighter head weights of the hairless bees may have been due to the resorption of the hypopharyngeal gland. Head weight was capable of responding to diet, especially for a diet of pollen containing 6% linoleic or oleic acid (Fig. 10-3).

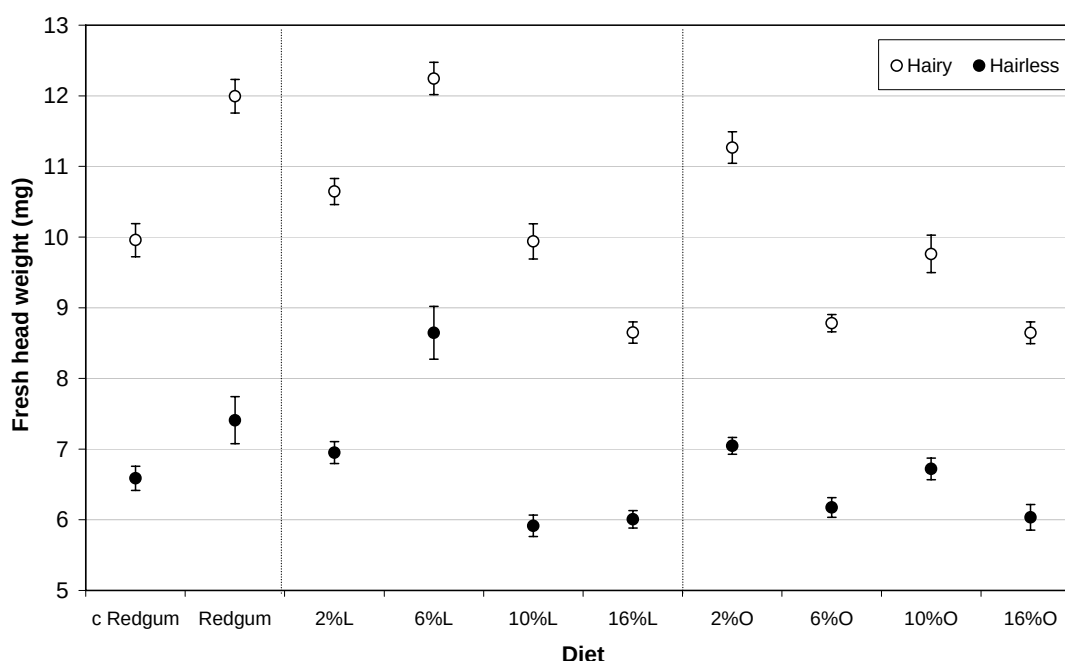


Figure 10-3. Head weights of hairless and hairy honeybees fed a redgum pollen diet mixed with various rates of linoleic acid and oleic acid. Mean $\pm$ S.E. (5% LSD = 0.588).

For both diets where linoleic and oleic acids were mixed with RG pollen, the head weights of hairy and hairless bees were clearly separated and followed a similar trend to each other in response to diet (Fig. 10-3). However, from REML equation 1, a significant 3-way interaction was found ($P = 0.018$) where the response curves were not the same across both types of bee (hairy and hairless) and for both fatty acid diets.

Effect of diet on phenotype

An analysis by REML of FEED*AGE showed there was a significant difference ($P < 0.001$) between hairy and hairless bees and diet.

Both groups of bees (hairless and hairy) responded to diet in a similar manner. The addition of 6% linoleic acid to diet led to an increase in head weight whilst a 6% addition of oleic acid gave a reduced head weight (Fig. 10-3). Using equation 2, a significant difference was found between the quadratic trends of both types of bee by fatty acid (linoleic or oleic) ($P = 0.003$). Linear trends seemed similar ($P = 0.452$) suggesting overall there is a common decay of head weight as fatty acid concentration increased (Fig. 10-3).

Proportional shifts in hairy and hairless populations with diet

The proportion of hairless bees in the cage population after 42 days was 20.8 % (464 hairless vs 2,235 hairy). From the binomial model, the predicted percentage of hairless bees in cages fed oleic acid was higher (21.8 ± 1.34 %, $N = 4$) than those fed linoleic acid (15.6 ± 1.05 %, $N = 4$) and significantly different ($P < 0.001$). Cage bees fed redgum pollen showed a similar percentage (12.8 ± 1.39 %, $N = 2$) of hairless bees as linoleic acid-fed bees (data not shown). A significant difference ($P < 0.001$) was found when different rates of fatty acids were added to the diet and a significant interaction ($P < 0.001$) was found from equation 3. Generally, hairless bees were in higher percentages amongst populations of bees fed oleic acid enhanced pollen diets. The phenotype reversal for the 10% rate is unexplained (Fig. 10-4).

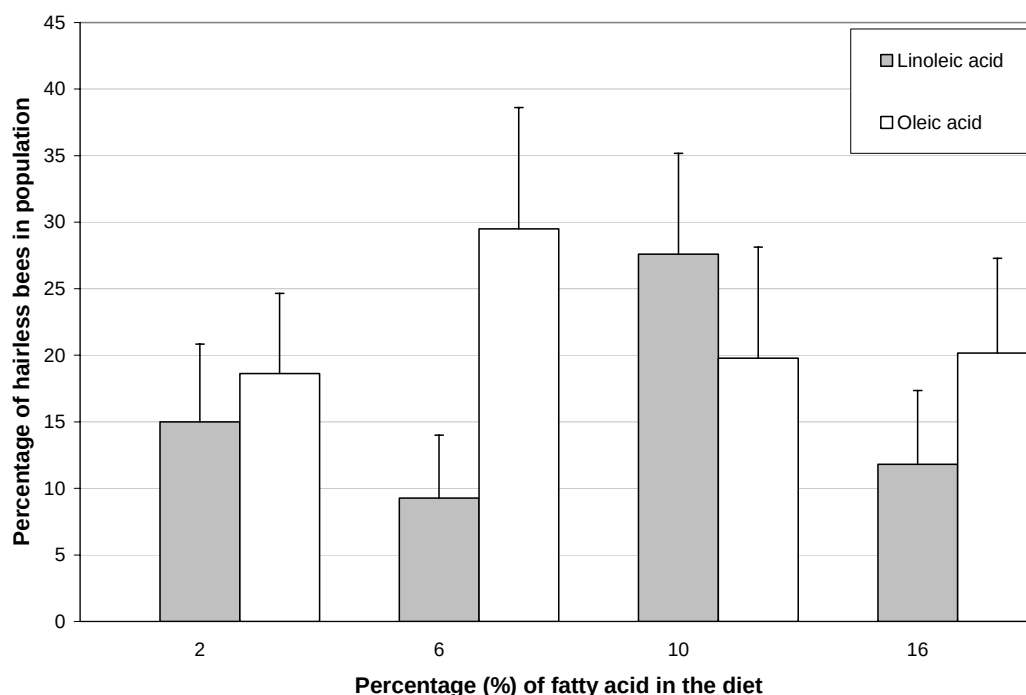


Figure 10-4. Mean percent of hairless bees in the cage population ($\pm 5\%$ LSD) after a 42 day cage experiment.

Microscopy

Results from electron microscopy of head samples examined for viral particles showed none present in 11 samples, each containing up to 10 heads, in a report from the Animal Health Laboratories, Department of Agriculture and Food, Western Australia.

Longevity of honeybees fed linoleic and oleic acid enhanced redgum (*Corymbia calophylla*) pollen diets; high and low fat soya bean flour diets and lupin and flour diets mixed with redgum pollen.

Results

Controls

The survival curve for honeybees fed cRG and cane sugar are shown in Figure 11-1. Approximately half the cRG bees were still alive after 42 days whereas there were almost none surviving on cane sugar (Fig. 11-2). The diet of cRG, despite its 6-8 year age, conferred long survivorship to the honeybees (Fig. 11-6, Table 11-1). Honeybees fed cane sugar without cRG pollen had a high mortality rate, a life-span of just over two weeks (Table 11-1) and had a hazard ratio 9.4 times greater than those bees fed a diet of cRG pollen (Fig. 11-6). Bees fed invert sugar were more adversely affected than if fed cane sugar (Fig. 11-6) and had the shortest life-span (Table 11-3) and the poorest survivorship (Fig. 11-3) of all the diets trialled.

Lupin flour

Bees fed lupin flour alone, showed a higher survival rate than if 10% pollen was added to the diet (Fig. 11-1). When pollen was added to the diet, the life-span was decreased by 4 days (Table 11-1). Bees fed either LUP or LUP10 diets had an increased hazard ratio than those bees fed the pure pollen (RG) diets (Fig. 11-6) of 4.9 and 7.1 times, respectively.

Linoleic and oleic acid

Bees fed diets of RG, 2%L and 6%L showed a similar survival curve (Fig. 11-2) whereas bees fed diets containing 10%L and 16%L had high mortality. Diets containing 2%O gave bees a superior longevity when compared to diets containing higher concentrations of oleic acid (Fig. 11-3). A comparison of life-span (Table 11-1) showed diets with linoleic acid ranked highest amongst the pure pollen diets with bees fed 2%L and 6%L diets completing 42 days in cages without a high death rate. This suggests some beneficial aspect or non-toxic nature of linoleic acid whereas bees fed oleic acid at a concentration of greater than 2% in their diets had high mortality, with life-spans (Table 11-1) less than the corresponding values for linoleic acid. The indication is that oleic acid in high concentrations perhaps confers an adverse palate to the diet. Bees fed diets of 6%O, 10%O and 16%O had an increased risk of death by 4.2, 6.7 and 9.5 times greater, respectively, than those bees fed a pollen diet (Fig. 11-6).

Soya bean flours

The survival of honeybees fed DF diets and those mixed with pollen showed similar survival curves (Fig. 11-4). DF soya bean flour fed as a pure diet produced bees with poorer longevity than if pollen was included in diets. A high-fat soya bean flour diet (FF) mixed with pollen showed similar longevity to both low-fat (PRO) and PRO10 diet. High-fat soya bean flour diet (FF) like the medium-fat soya bean flour (DF) showed a poorer longevity by comparison (Fig. 11-5). The addition of sequentially higher rates of pollen (10%, 20% and 30%) to diets of DF correspondingly reduced the hazard ratio (Fig. 11-6) but life-span remained identical for all three diets at 22 days (Table 11-1). The addition of 10% pollen to the high-fat (FF) soya bean flour diet increased life-span by five days (Table 11-1) and reduced the hazard ratio (Fig. 11-6).

The life-span of bees fed the low-fat soya bean flour diets (PRO and PRO10) were similar at 26 days (Table 11-1) and of all the soya bean flour diets, of which DF30 diet had the highest life-span of 22 days. The bees on Promine™ diets had the lowest hazard rate (Fig. 11-6).

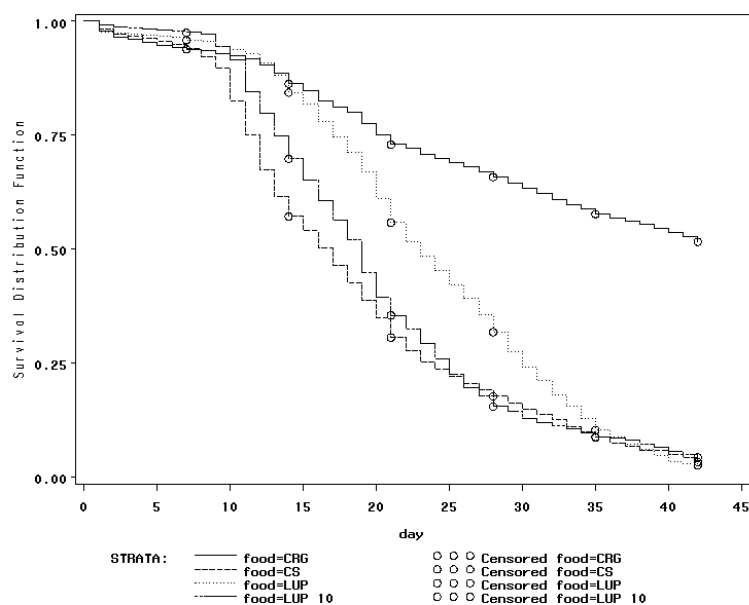


Figure 11-1. Survival curves for honeybees fed crushed and irradiated redgum pollen (cRG), cane sugar (CS), lupin flour (LUP) and lupin flour + 10% cRG pollen (LUP10). Data are for all three replicates combined.

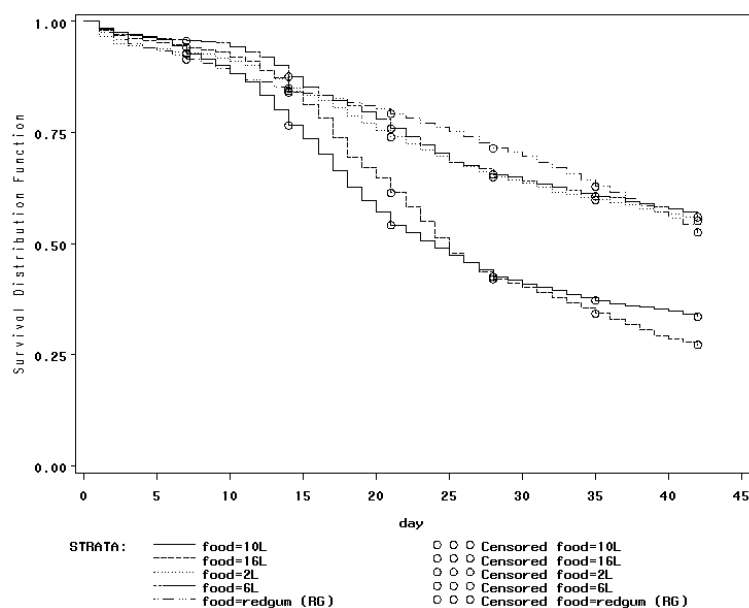


Figure 11-2. Survival curves for honeybees fed linoleic acid enhanced redgum pollen and redgum pollen (RG). Data are for all three replicates combined.

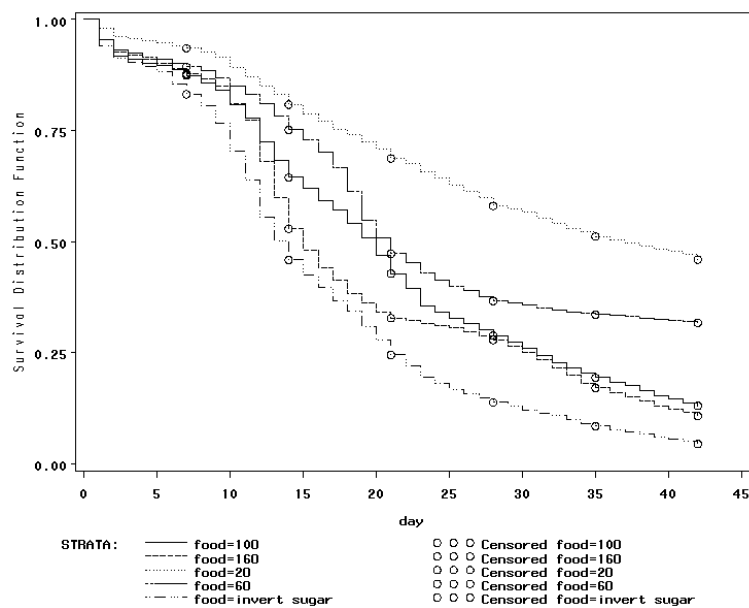


Figure 11-3. Survival curves for honeybees fed oleic acid enhanced redgum pollen (cRG) and invert sugar. Data are for all three replicates combined.

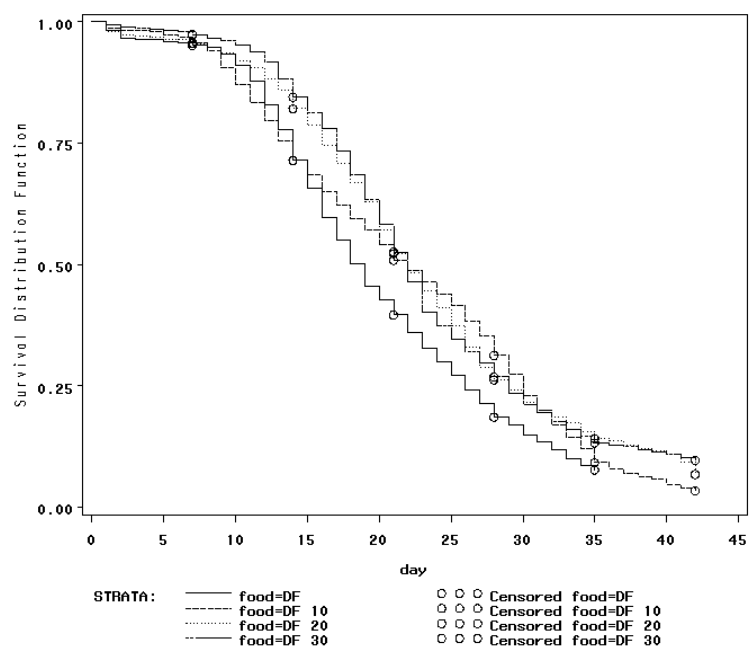


Figure 11-4. Survival curves for honeybees fed medium-fat soya bean flour (DF) and DF soya bean flour mixed with cRG pollen at 10, 20 and 30%. Data are for all three replicates combined.

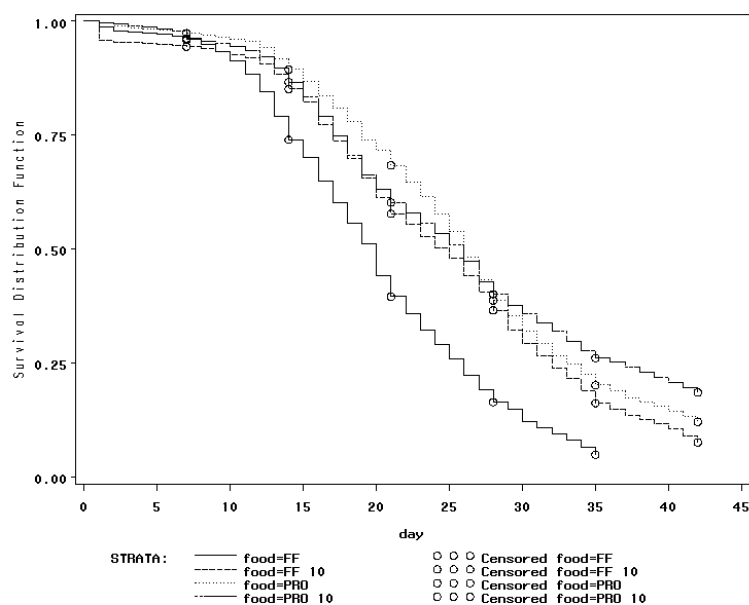


Figure 11-5. Survival curves for honeybees fed low-fat (PRO) and high-fat (FF) diets, and both diets mixed with 10% cRG pollen. Data are for all three replicates combined.

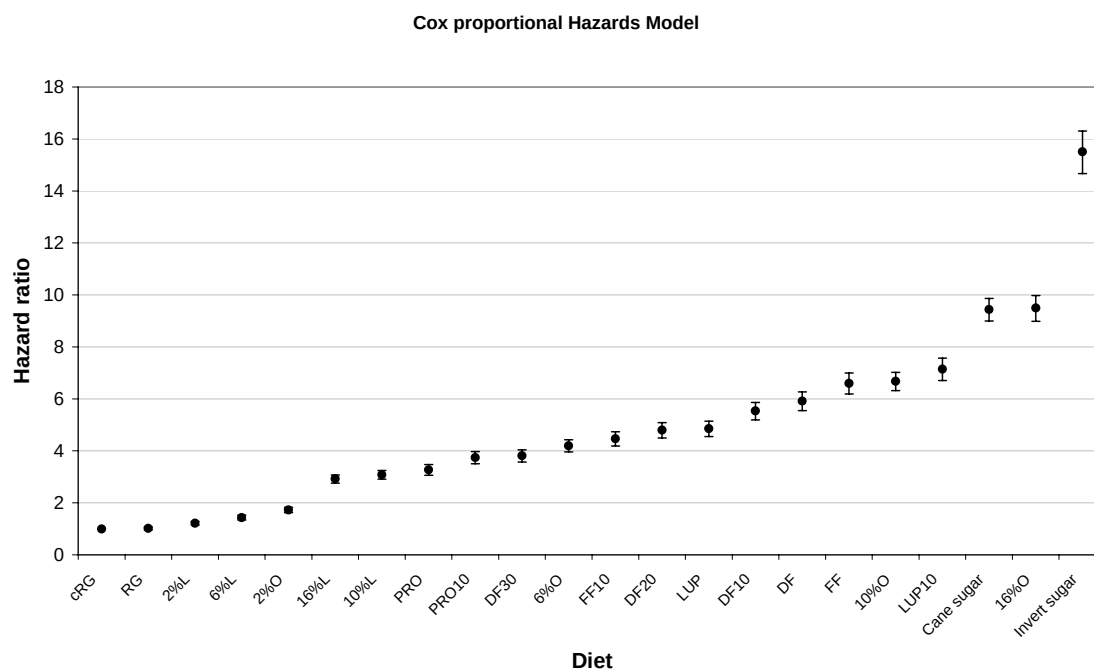


Figure 11-6. A Cox Proportional Hazard model of 22 diets with upper and lower 95% confidence limits. The comparison diet used was crushed and irradiated redgum pollen (cRG). The hazard ratio of a diet is the increased risk of dying by being fed that diet compared to a different diet.

Table 11-1. Diet ranking of the most successful diet (1) to least successful diet (22) according to a Cox Proportional Hazards model including temperature and humidity as factors. Diets marked with * are borderline statistically significant at the 5% level. “NA” means that 50% of the bees had not died by the end of the experiment at day 42.

Ranking	Diet	Not significantly different		Life-span
1	cRG	RG		NA
2	RG	cRG		NA
3	2%L			NA
4	6%L			NA
5	2%O			37
6	16%L	10%L*		25
7	10%L	PRO	16%L*	24
8	PRO	10%L		26
9	PRO10	DF30		26
10	DF30	PRO10		22
11	6%O	FF10		21
12	FF10	6%O		25
13	DF20	LUP		22
14	LUP	DF20		23
15	DF10			22
16	DF			19
17	FF	10%O		20
18	10%O	FF	LUP10*	20
19	LUP10	10%O*		19
20	Cane sugar	16%O		17
21	16%O	Cane sugar		15
22	Invert sugar			14

Diet consumption

There were significant differences in diet consumption ($P < 0.001$). The most consumed diet was fresh redgum (RG) pollen with 46.7 g consumed over six weeks. The least consumed diet was 16% oleic acid + pollen at 0.51 g/six weeks (Fig. 11-7). All flour diets were poorly consumed as were the pollen diets high in applied fatty acids, particularly with oleic acid (Fig. 11-7).

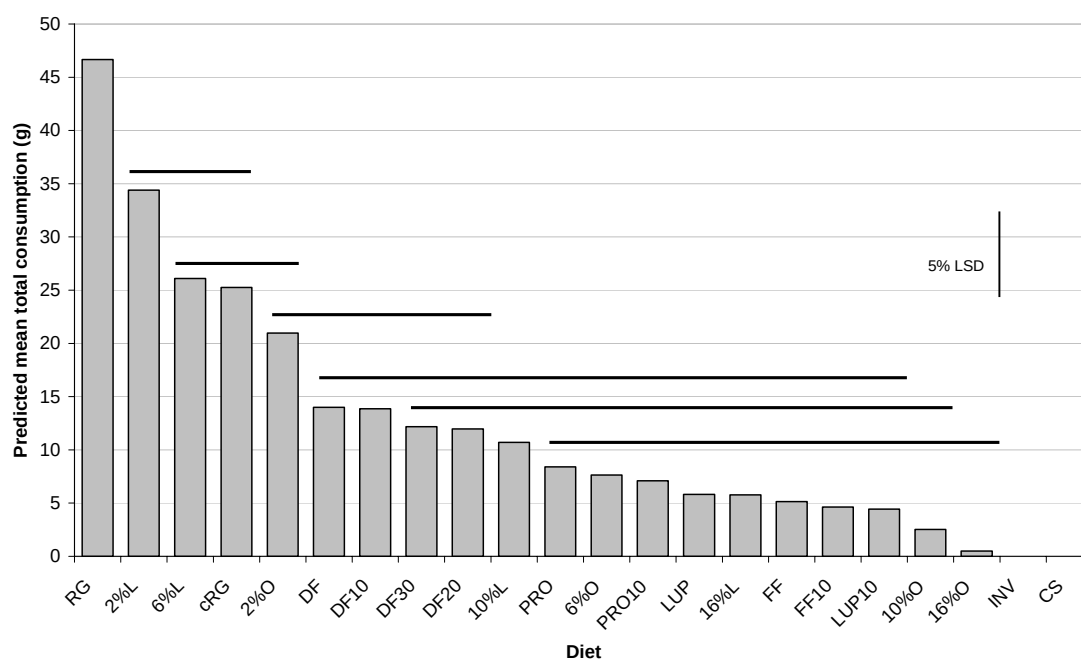


Figure 11-7. Predicted mean total consumption (g) of diets fed to honeybees. Invert (INV) and cane sugar (CS) fed bees had no protein supplement (5% LSD 9.67 g). The bar above the diet recognizes those diets that are not significant to each other at $P < 0.05$.

Contamination

A situation arose during experimentation, where accidental microbial contamination of the cane sugar control occurred. A diet using invert sugar remained unaffected. The first and second experiment used a cane sugar solution that was not boiled and was only mixed using boiled hot water. By the time the second replicate commenced, the sugar solution was > 80 days old and bees fed the cane sugar diet started dying rapidly after seven days with most of the population perished by day 22.

Bees in experiment 2 were 20 times more likely to die than the bees fed on cane sugar in experiment 7, so it was removed from the data set. Using a simple model which included all seven replicates and involving temperature, humidity and experiment as factors, it was found that the bees in replicate 7 were the most long lived, followed by replicates: 3, 6, 4, 5, 1 and 2. Samples of the contaminated sugar solution were sent to two laboratories for independent analysis. The cane sugar solution was identified as being '*heavily contaminated with a moderate mixed population of yeasts and fungal elements*'. Laboratory (1) identified the main contaminant in the stock sugar solution as *Candida guilliermondi* with *Aspergillus niger* also present. Laboratory (2) identified the main contaminant as *Pichia ohmeri*. The invert sugar used at the same time was free of contaminants.

The second laboratory was also sent samples of the honeybees' watery gut fluid from bees fed three different diets viz: redgum (RG), crushed redgum (cRG) and 6% linoleic acid from experiment 2. *Candida glabrata* was isolated from the gut of honeybees fed the RG diet; *Candida magnoliae* was isolated from the gut material from bees fed the 6%L diet and *Rhodotorula glutinis* was found in the gut fluid from bees fed the cRG diet. The inside wall of the cages of the bees fed a pollen diet were characteristically streaked (Fig. 11-8). No *Nosema apis* spores were detected in the guts, a gut parasite that beekeepers usually associate with faecal streaking.



Figure 11-8. Faecal streaking inside cage from a redgum (RG) diet with a contaminated sugar source. View from bottom of cage.

The effect of diet on head weights (hypopharyngeal gland) of honeybees

- A. LINOLEIC AND OLEIC ACID ENHANCED REDGUM (*CORYMBIA CALOPHYLLA*) POLLEN DIETS
- B. SOYA BEAN AND LUPIN FLOUR DIETS AND MIXES CONTAINING REDGUM POLLEN

Results

Head weights of emerged honeybees

Emerged honeybees showed significant differences in head weight (Fig. 12-1). Head weight of emerged honeybees was highest during winter and lowest in spring. A linear regression (equation 1) showed season and replicate within season were significant at the 5% level ($P < 0.0001$ and $P = 0.0034$, respectively). Replicate 3 was the odd set of data that caused a significant *replicate within season* interaction ($P = 0.0062$) where it differed from other summer experiments. When removed from the data set, *season* alone accounted for the variation between replicates ($P < 0.0001$). Replicate 3 may have differed because of prevailing conditions in the apiary that month. Pollen may have been different in quality and/or quantity which directly influenced the hypopharyngeal gland development within the head.

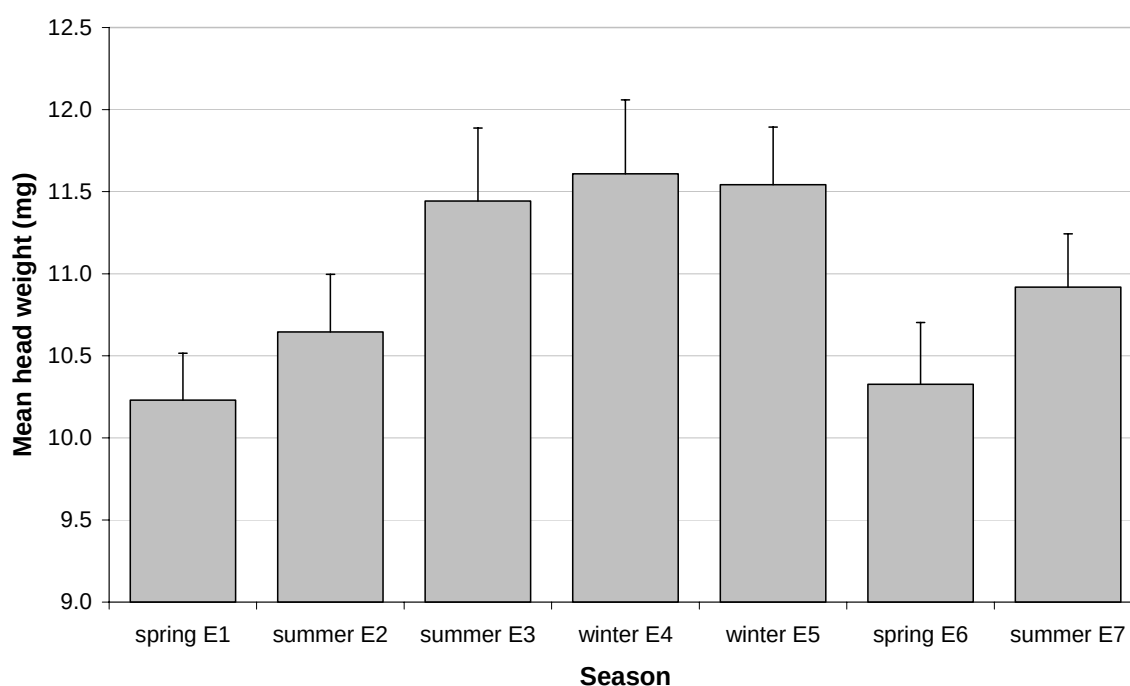


Figure 12-1. Mean head weight of emerged honeybees and 5%LSD over season (replicate) from bees born in September 2003 to December 2004. E1 to E7 = experiment number.

Honeybees from the apiary

Large differences in bee head weight were found between experiments and over the duration of the experiment with a mean increase in the head weight during the first week, before a general decrease in the mean head weight (Fig. 12-2).

Histological Sections of the heads of apiary bees are given in Figures 12-3A and 12-3B (low magnification) and Figures 12-4A and 12-4B (high magnification). Bee heads from experiment 6 (the only one used for histology) showed the rapid development of the hypopharyngeal gland especially between Week 1 and Week 2 (Figs 12-3A, 12-4A). By week 2, the mean head weight peaked (Fig. 12-2) and histology showed hypopharyngeal gland development fully developed in heads of larger weight. Full development of the gland was observed for week 3 and week 4 as mean head weight declined (Fig. 12-2). Bee heads from week 5 and week 6, showed some heads with depleted glands (Figs 12-3B, 12-4B), however some of the larger heads still showed the hypopharyngeal gland well developed (see week 6, 12 mg head weight, Figs 12-3B, 12-4B).

From the statistical analysis, the full model accounted for 49.5% of the variation in the head weight data of apiary bees. Each term in the model was significant ($P < 0.0001$) and therefore *season* and *experiment* had an effect on the change in head weight during the first seven days and in growth rate over the following days. Each of the parameters for *experiment within season* were significant ($P = 0.05$) and during the first seven days there was a significant difference in growth of the bee heads across experiments. The growth rate of the bees varied across *season*, however there was less variation *within a season* with only the third experiment being significantly different from the other summer experiments (see Fig. 12-1).

A comparison of the parameter estimates for the additive effect of *season* and the effect of *season* on growth rate is given in Figure 12-5. Summer experiments appear to have a much higher head weight recording at the end of the first seven days when compared to the winter and spring experiments. The head weight for all experiments began to decrease after day 7. Winter had the greatest decrease in head weight over time compared to the summer and spring experiments. During the six week experiment, the mean head weight during the summer months decreased at a much slower rate than the other seasons.

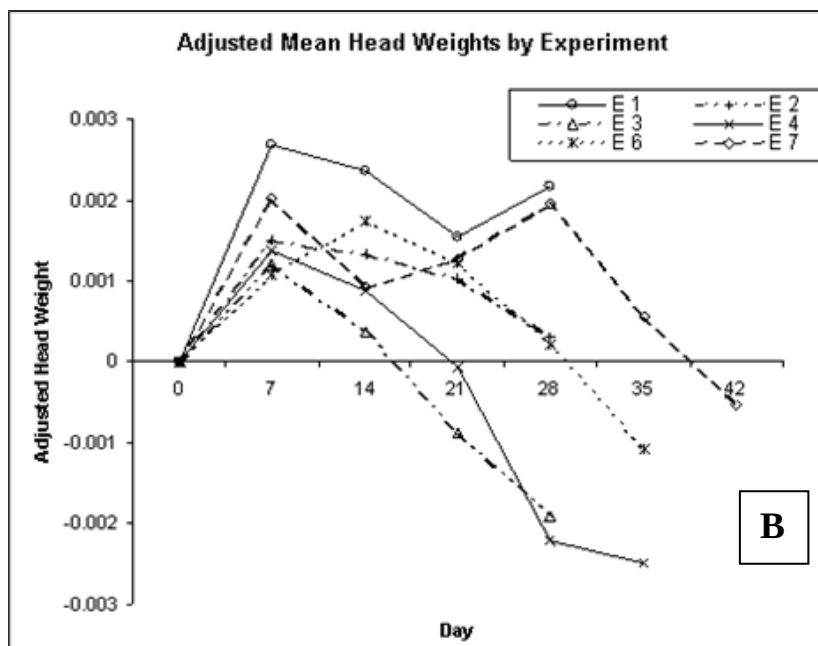
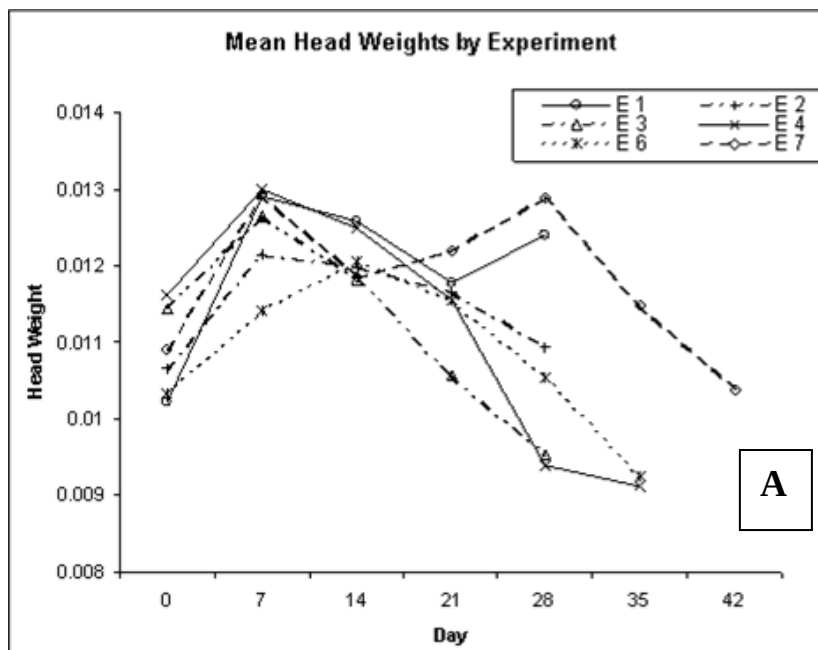


Figure 12-2. Seasonal changes in mean head weight (g) of honeybees from an apiary (A) and statistically adjusted (B). E1 (spring), E2 (summer), E3 (summer), E4 (winter), E5 (spring) and E7 (summer). There were no emerged bees available to be returned to the apiary in experiment 5 (E5).

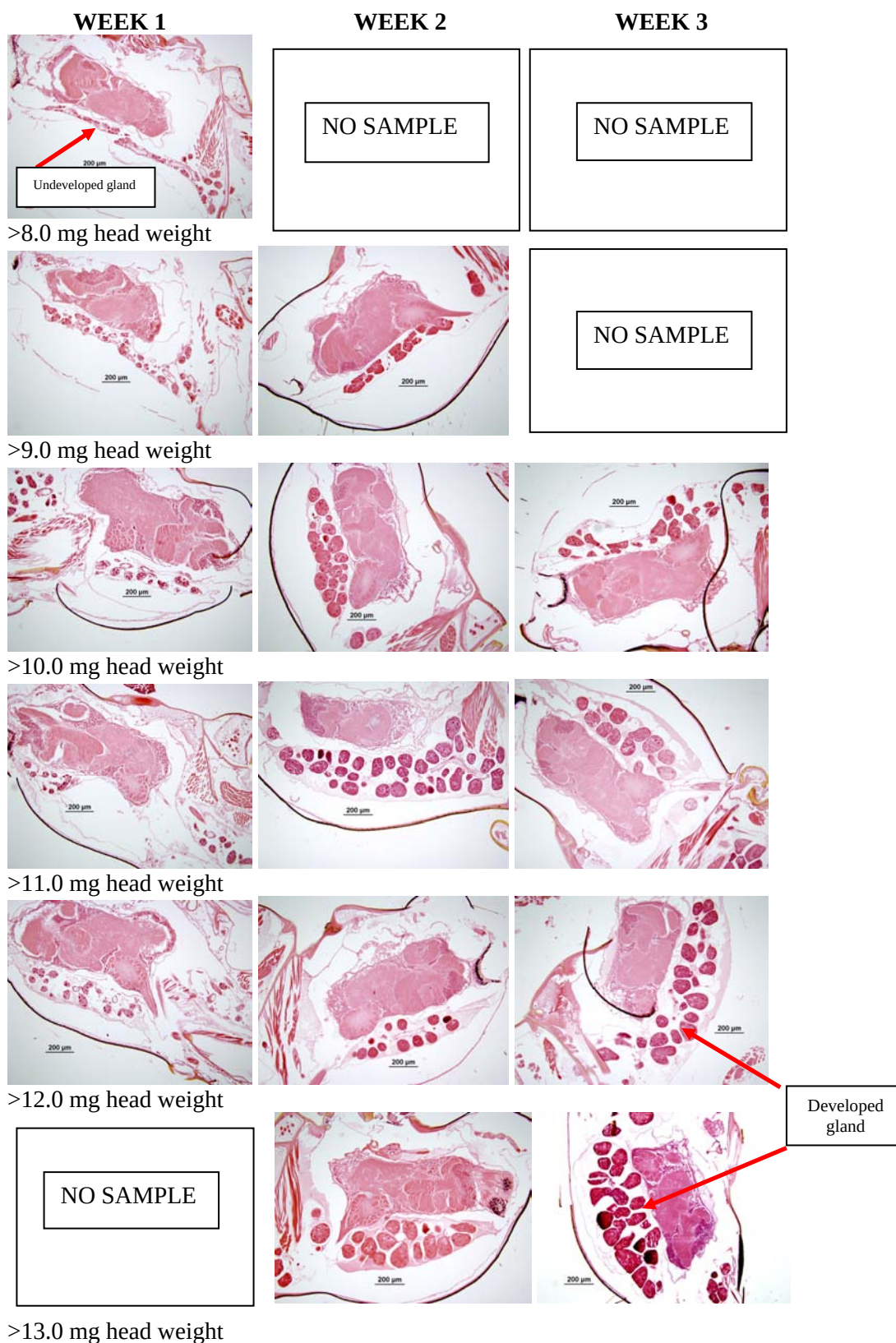


Figure 12-3A. Histological Sections at lower magnification of honeybee heads by head weight (mg) and by weekly sample (Week 1 to Week 3) from marked emerged bees returned to a hive in an apiary. No sample = samples were not available.

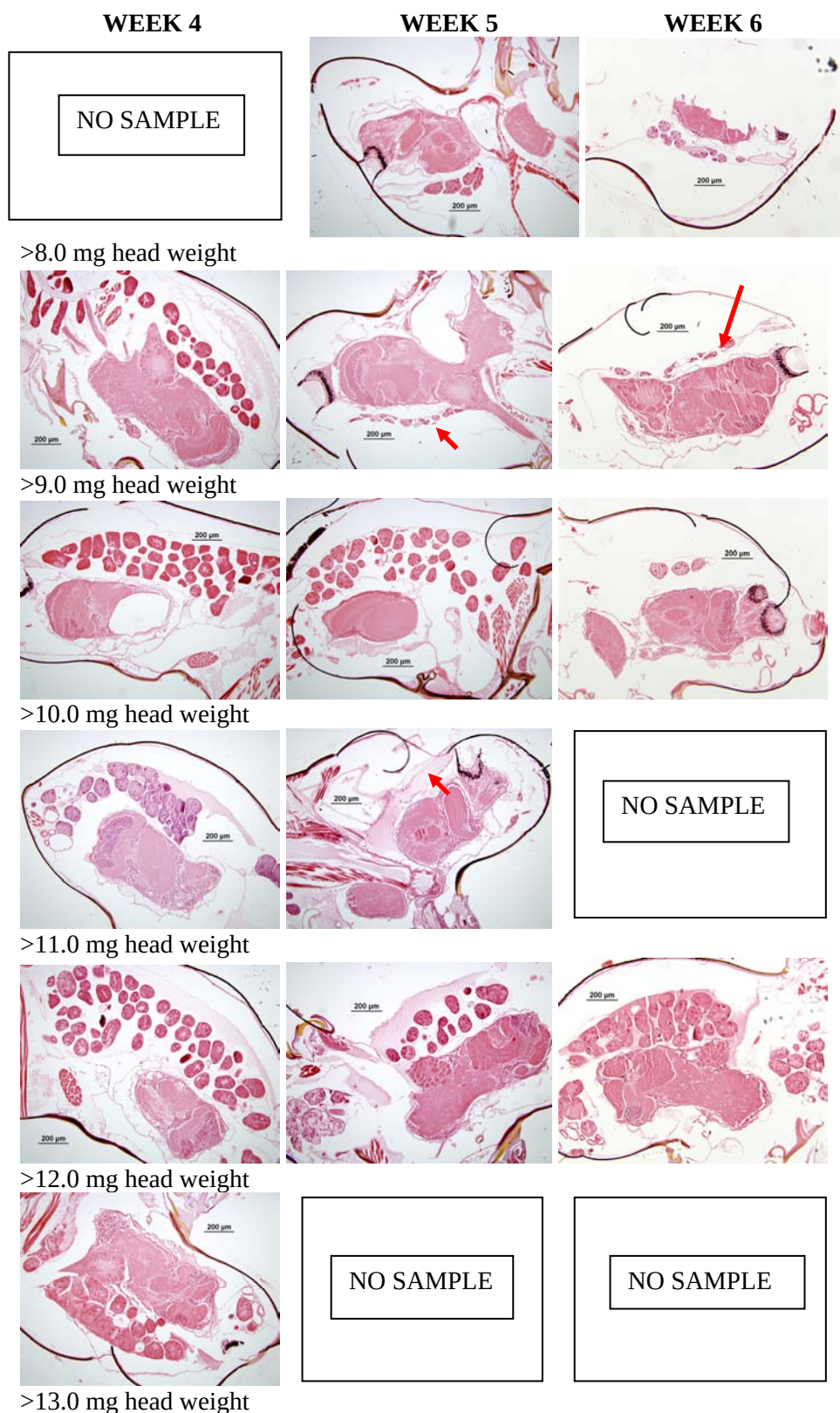


Figure 12-3B. Histological Sections at lower magnification of honeybee heads by head weight (mg) and by weekly sample (Week 4 to Week 6) from marked emerged bees returned to a hive in an apiary. No sample = samples were not available. Depleted glands arrowed.

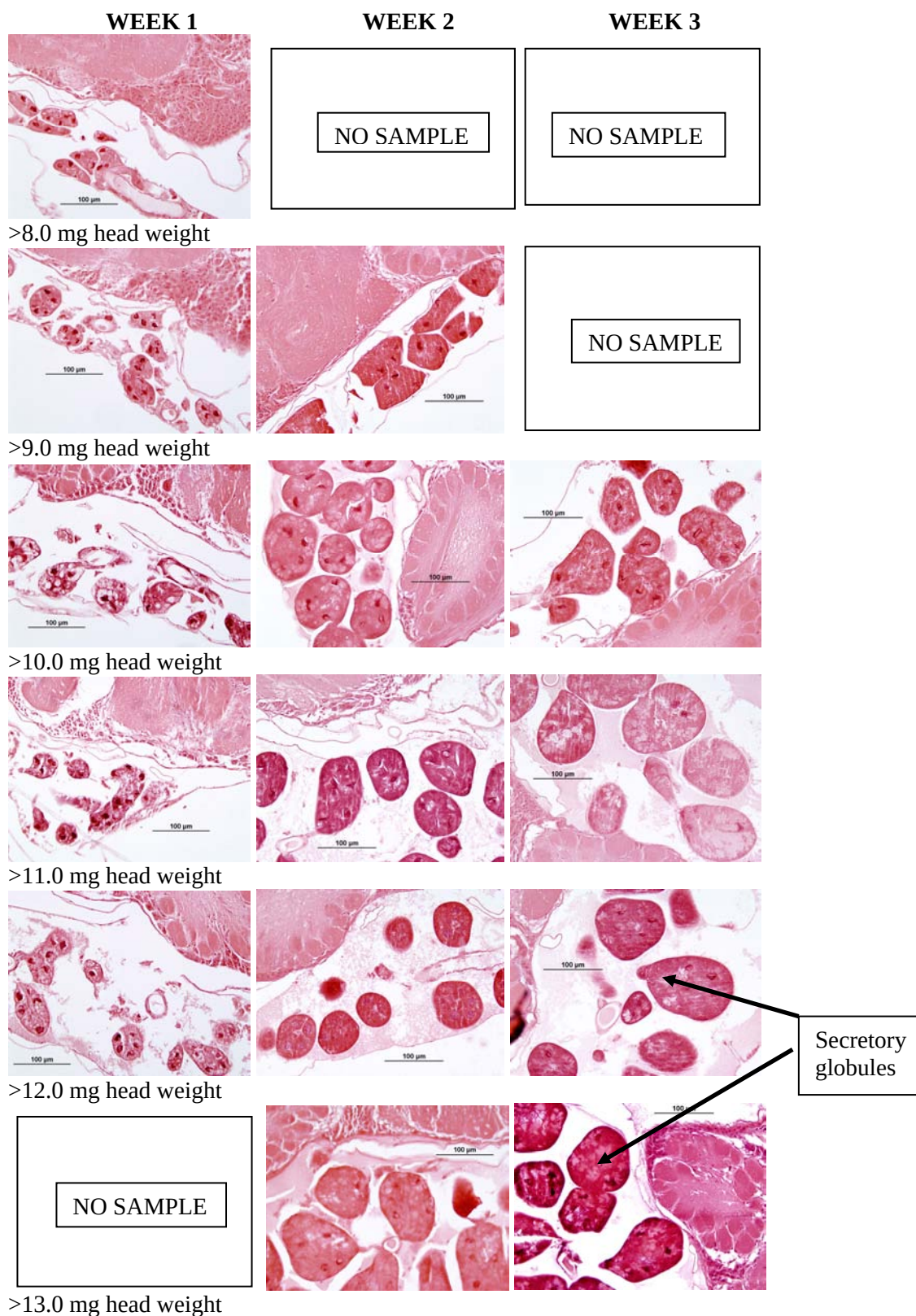


Figure 12-4A. Histological Sections at higher magnification of honeybee heads by head weight (mg) and by weekly sample (Week 1 to Week 3) from marked emerged bees returned to a hive in an apiary. No sample = where samples were not available.

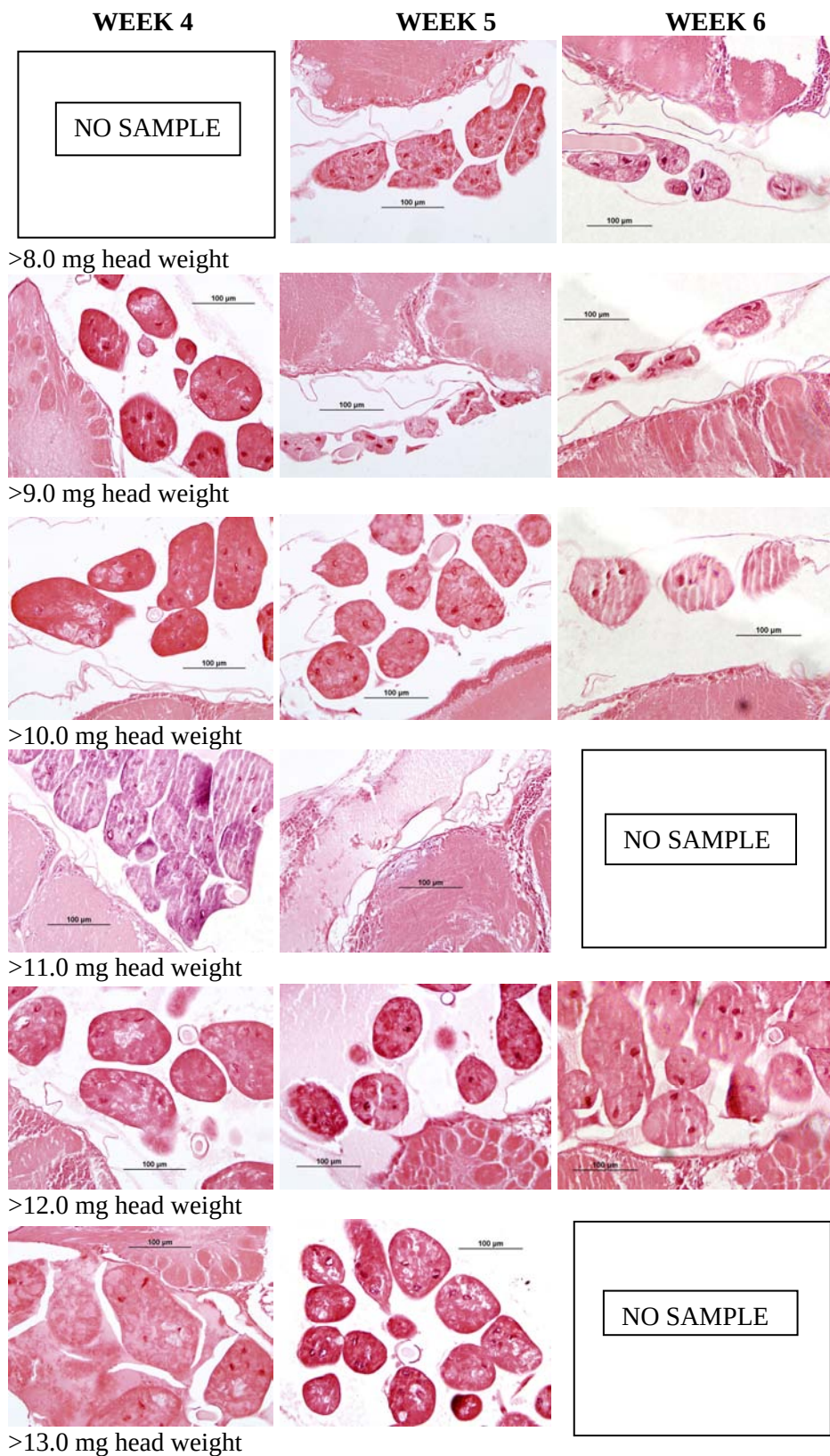


Figure 12-4B. Histological Sections at higher magnification of honeybee heads by head weight (mg) and by weekly sample (Week 4 to Week 6) from marked emerged bees returned to a hive in an apiary. No sample = where samples were not available.

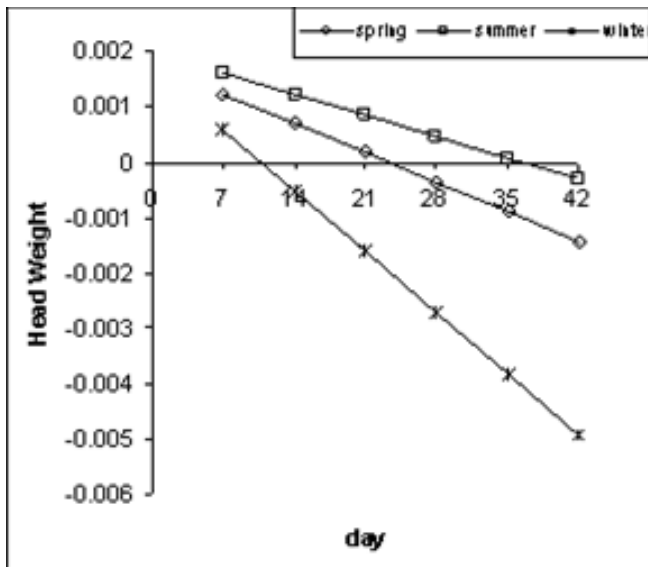


Figure 12-5. Regression model fits of the seasonal data.

Effect of diet on head weight

The model accounted for 34.8% of the variance and each term in the model was significant ($P < 0.0025$). Therefore *diet*, *season* and *experiment* did have an effect on head weight and head weight growth rate. There was an interaction between diet and the seasons and an experiment which indicated that the effect of a diet on head weight varied amongst seasons. A diet that had a certain effect over summer may have a different effect during a different season. These interactions made it difficult to compare the diets directly.

However, to gain an understanding of the effects of the diets, the parameters in the model were computed according to the standardized season of spring and experiment number 1. The estimates for the change in head weight are given in Table 12-1 along with the rankings of diet for each parameter in order of decreasing value. Although these rankings give an apparent order, care must be taken when interpreting these parameters as each estimate has an associated confidence interval and cannot be considered different if their confidence intervals overlap. Thus, Table 12-1 must be considered alongside the matrices of Tables 12-2 and 12-3 which show the pair-wise comparisons of the parameter estimates for each diet and the diet's impact on growth rate. Thus although Table 12-1 shows that 10%L diet gives the highest day seven value for all the diets, it is not significantly different from the diets: PRO10, DF20, FF10 and RG (see Table 12-2). The diet with the greatest growth rate was found to be diet 2%L, however it was not significantly different to diets PRO10, PRO, DF30 and RG (see Table 12-3).

Table 12-1. Model estimates ranking of head weight with diet type at day seven and growth rate for all diets.

Diet	Parameter		Ranking	
	Day 7	Growth Rate	Day 7	Growth Rate
2%L	-0.0028	0.000055	21	1
RG	-0.0021	0.000041	2	2
6%L	-0.0025	0.000041	9	3
2%O	-0.0026	0.000038	12	4
cRG	-0.0023	0.000036	6	5
6%O	-0.0027	0.000031	18	6
DF30	-0.0026	0.000023	14	7
PRO	-0.0028	0.000020	22	8
PRO10	-0.0023	0.000019	5	9
10%L	-0.0021	0.000005	1	10
FF	-0.0027	0.000003	17	11
DF10	-0.0025	0.000002	10	12
CANE	-0.0027	-0.000005	16	13
16%L	-0.0027	-0.000006	19	14
LUP	-0.0027	-0.000006	20	15
LUP10	-0.0024	-0.000010	7	16
16%O	-0.0027	-0.000012	15	17
DF20	-0.0023	-0.000014	4	18
FF10	-0.0022	-0.000015	3	19
10%O	-0.0026	-0.000022	11	20
DF	-0.0024	-0.000026	8	21
INVERT	-0.0026	-0.000088	13	22

Head weights of caged honeybees fed flour diets

In distinct contrast to the head weights of apiary bees, bees fed in cages showed a sharp decrease in head weight (Fig. 12-7) and was due to the time delay for emerged bees to locate the food source within the cage and having no older nurse bees present. Both the cane and invert sugar diets showed a continual and gradual decrease in head weight over time whereas redgum pollen (RG) gradually increased head weight after day seven and peaked at day 28. Crushed and irradiated pollen (cRG), that was seven years old caused, head weights to increase in weight from day 14 to day 42. Head weights for those emerged bees returned to hive in the apiary reached a peak in head weight at day seven and subsequently declined to day 42.

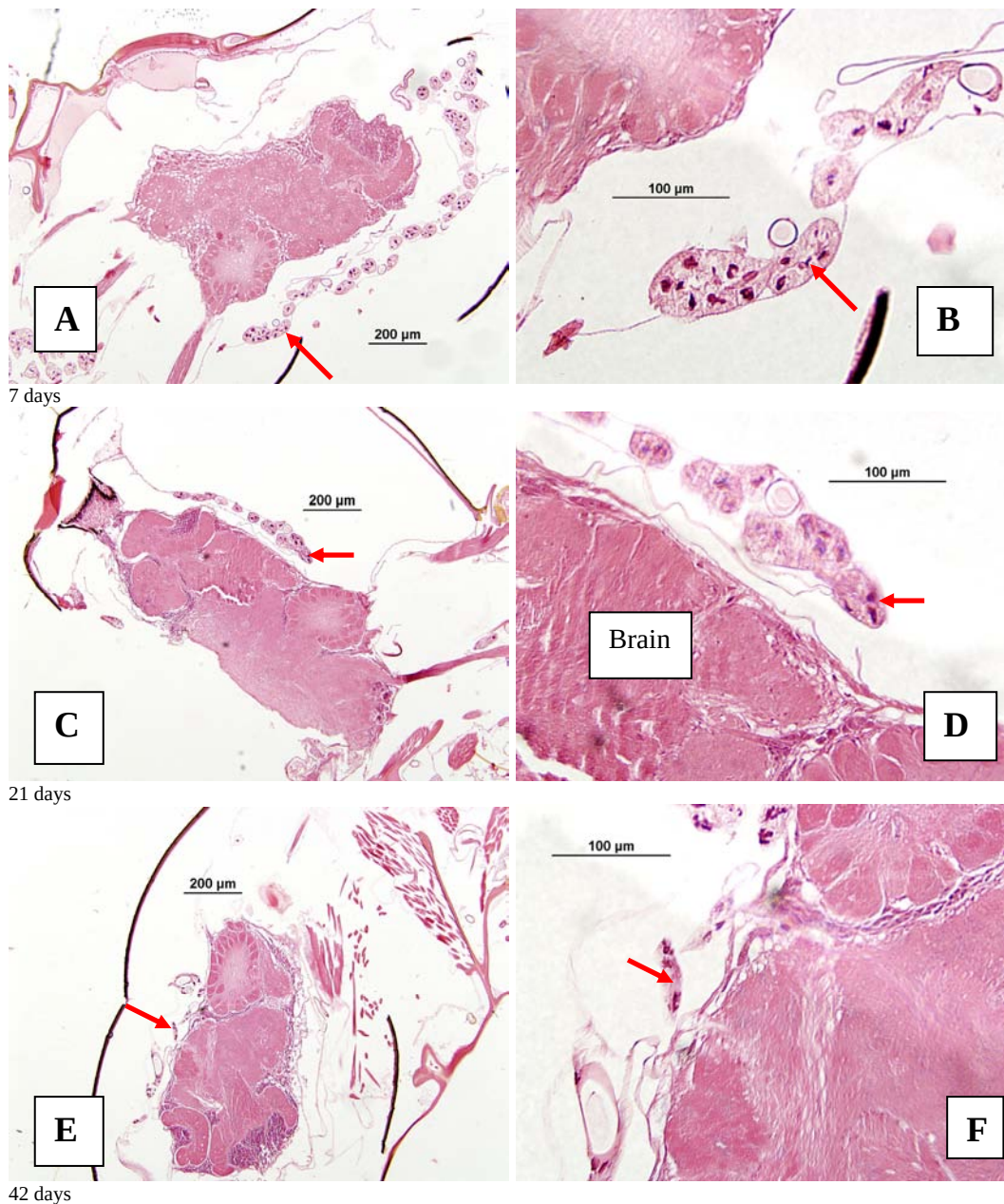


Figure 12-6. Histological Sections at low and high magnification of the honeybee head of emerged bees fed cRG diet at seven days (A and B), 21 days (C and D) and 42 days (E and F). Hypopharyngeal glands (arrowed). Glands present in week 1 and week 3 but nearly absorbed by week 6.

Histological Sections of heads where bees were fed one of the pollen diets (cRG) [primarily chosen because the diet showed the highest longevity] showed that over the experimental period, the hypopharyngeal glands never fully developed (see Fig. 12-6) like those illustrated in Figures 12-3A, 12-3B, 12-4A and 12-4B over the same time periods but did develop enough to supply royal jelly to the small colony to enable bees in 5 of the 6 experiments to reproduce (see Table 12-4).

A pure full-fat soya bean flour diet (FF) and one mixed with 10% cRG pollen both followed a similar declining head weight pattern (Fig. 12-8) without stimulating any significant hypopharyngeal development, whereas a low fat soya bean flour (Promine™), showed a slight increase in head weight over time (Fig. 12-10), particularly from the diet PRO10 where the addition of 10% cRG pollen improved head weight. Lupin flour on its own produced bees with lighter head weights than a diet which included 10% cRG pollen. The defatted soya bean flour (DF) on its own gave the lightest head weights of all the DF diets. The addition of between 10 and 20% cRG pollen to DF diets generally produced bees with improved head weights (Fig. 12-9).

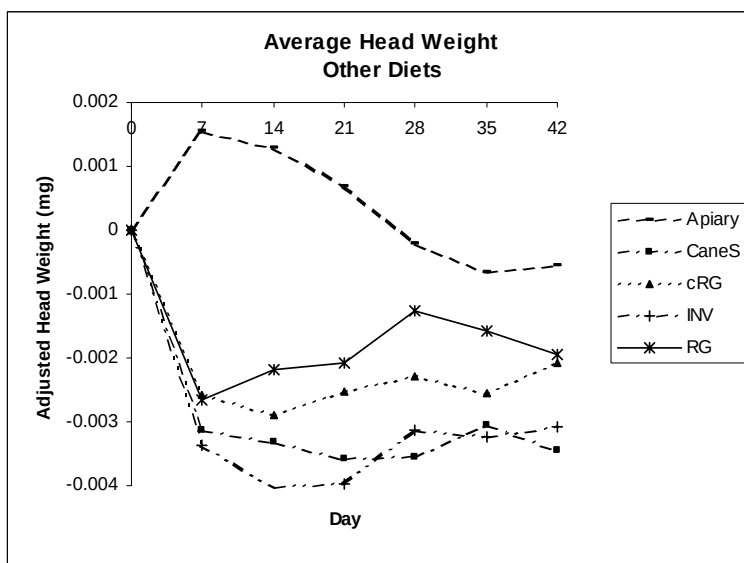


Figure 12-7. Mean head weight of honeybees fed cane sugar, invert sugar, redgum (RG) and crushed redgum pollen (cRG) compared with marked emerged bees returned to an apiary over the same experimental period.

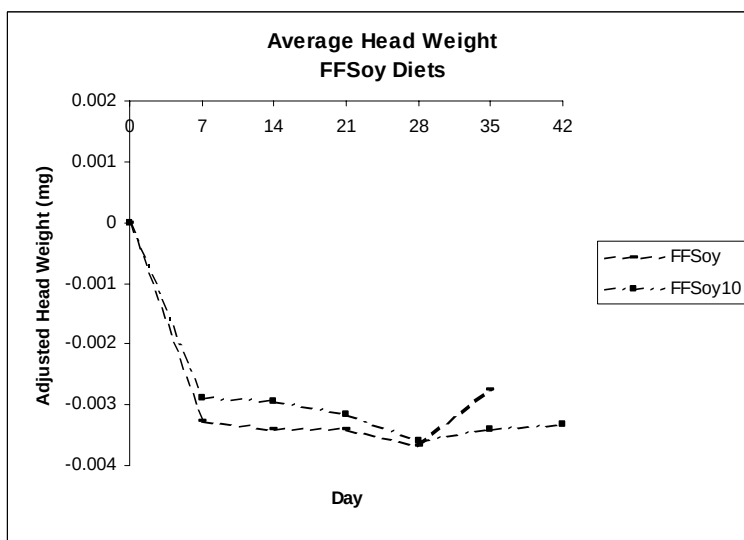


Figure 12-8. Mean head weight of honeybees fed full-fat soya bean flour and full-fat soya bean flour mixed with 10% cRG pollen.

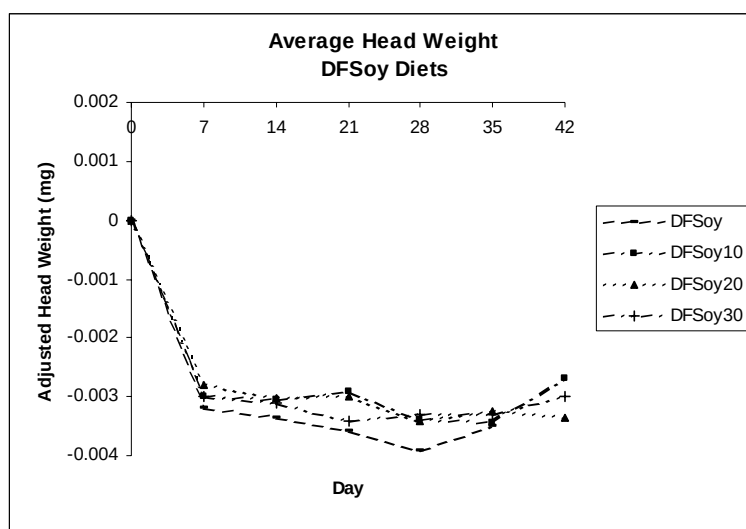


Figure 12-9. Mean head weight of honeybees fed defatted soya bean flour mixed with 10%, 20% and 30% cRG pollen.

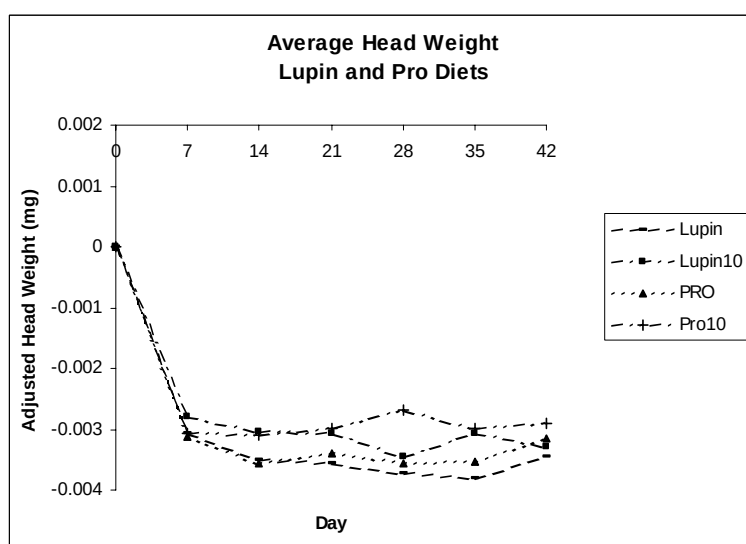


Figure 12-10. Mean head weight of honeybees fed lupin and Promine™ (soya bean) flours and diets with mixed with 10% cRG pollen.

Head weights of caged honeybees fed linoleic and oleic acid enhanced pollen diets

Pollen enhanced with 16% linoleic acid was associated with bees having lighter head weights than after being fed either 2, 6 or 10% diet. The 2%L and 6%L diets were both similar in being able to increase head weights after day seven (Fig. 12-11).

The effect of oleic acid diets on head weight differed to linoleic acid diets with only the 2%O diets clearly able to influence head weight (Fig. 12-12). Diets containing 6, 10 and 16% oleic acid only showed a slight increase in head weight after day 7.

Diet and brood production

In a number of the cages, the queen bee laid eggs, which developed into larvae, pupated and emerged at day 42. In Table 12-4, the data whether eggs, larvae, pupae or signs of an emerged bee were combined to simplify the table in order to see which diet enabled a caged population to reproduce. The results showed both pollen diets (RG and cRG) enabled bees to physically develop a functional hypopharyngeal gland and feed larvae which developed into newly emerged bees. Of the diets enhanced with fatty acids, only the 2%L, 6%L and 2%O diets encouraged the queen to lay eggs on the

supplied comb which then developed into larvae. Amongst the flour diets only one, Promine™ mixed with 10% cRG pollen enabled a small number of eggs to be laid (Table 12-4).

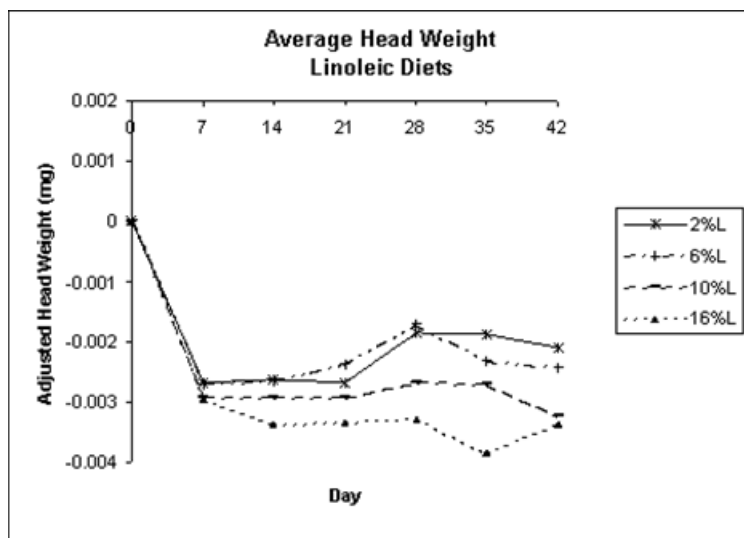


Figure 12-11. Mean head weight of honeybees fed redgum (RG) pollen enhanced with 2, 6, 10 and 16% linoleic acid.

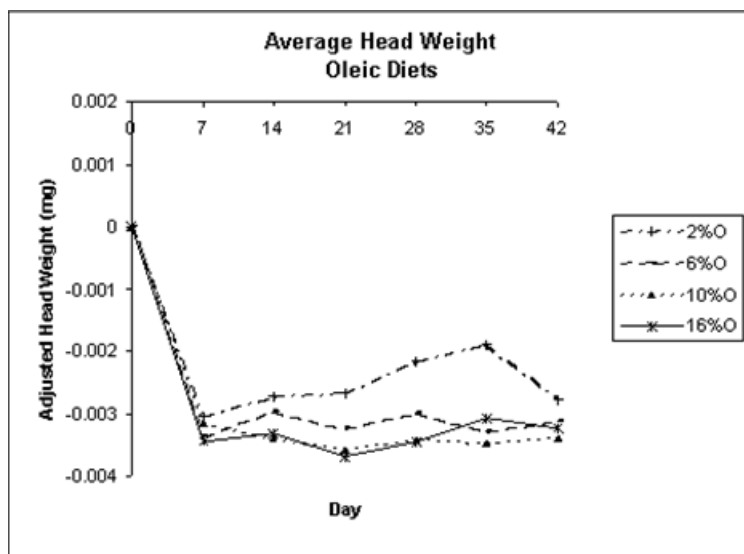


Figure 12-12. Mean head weight of honeybees fed redgum (RG) pollen enhanced with 2, 6, 10 and 16% oleic acid.

Table 12-2. Similarity of the D parameter (Diet) for each diet.

DIET	PRO	2%L	LUP	16%L	6%O	FF	CS	16%O	DF30	INV	2%O	10%O	DF10	6%L	DF	LUP10	cRG	PRO10	DF20	FF10	RG	10%L
PRO	X																					
2%L	X	X																				
LUP	X	X	X																			
16%L	X	X	X	X																		
6%O	X	X	X	X	X																	
FF	X	X	X	X	X	X																
CS		X	X	X	X	X	X															
16%O	X	X	X	X	X	X	X	X														
DF30	X	X	X	X	X	X	X	X	X													
INV	X	X	X	X	X	X	X	X	X	X												
2%O		X	X	X	X	X	X	X	X	X	X											
10%O		X	X	X	X	X	X	X	X	X	X	X										
DF10		X	X	X	X	X	X	X	X	X	X	X	X									
6%L				X	X	X	X	X	X	X	X	X	X	X								
DF		X		X	X		X	X	X	X	X	X	X	X	X							
LUP10		X		X	X		X	X	X	X	X	X	X	X	X	X						
cRG										X	X	X	X	X	X	X	X					
PRO10		X		X	X		X	X		X	X	X	X	X	X	X	X	X				
DF20		X		X	X		X	X		X	X	X	X	X	X	X	X	X	X			
FF10				X	X			X		X	X	X		X	X	X	X	X	X	X		
RG																	X	X	X	X	X	
10%L																	X	X	X	X	X	X

Table 12-3. Similarity of the T*D parameter (growth rate) for each diet.

DIET	INV	DF	10%O	FF10	DF20	16%O	LUP10	LUP	16%L	CS	DF10	FF	10%L	PRO10	PRO	DF30	6%O	cRG	2%O	6%L	RG	2%L
INV	X																					
DF	X	X																				
10%O	X	X	X																			
FF10	X	X	X	X																		
DF20	X	X	X	X	X																	
16%O	X	X	X	X	X	X																
LUP10	X		X	X	X	X	X															
LUP	X		X	X	X	X	X	X														
16%L	X	X	X	X	X	X	X	X	X													
CS	X	X	X	X	X	X	X	X	X	X												
DF10	X		X			X	X	X	X	X	X											
FF	X		X			X	X	X	X	X	X	X										
10%L	X	X	X	X	X	X	X	X	X	X	X	X	X									
PRO10			X			X			X				X	X								
PRO			X			X			X				X	X	X							
DF30			X			X			X				X	X	X	X						
6%O				X	X	X	X	X		X	X	X		X	X	X	X					
cRG							X	X			X	X		X	X	X	X	X				
2%O								X			X	X		X	X	X	X	X	X			
6%L											X	X		X	X	X	X	X	X	X		
RG											X	X		X	X	X	X	X	X	X	X	
2%L														X	X	X				X		X

Table 12-4. Number of cells with eggs, larvae, pupae or sign of emerged bees compared with diet. cRG pollen was common to all six experiments. NA = not applicable. *experiments where bees developed no brood are not shown.

Diet	No. of experiments	Number of cells with eggs, larvae, pupae or recently emerged bees*	Development stage reached
cRG	6	110, 355, 130, 237, 2	Emerged bees
RG	3	98, 286, 411	Emerged bees
2%L	3	255, 205	Emerged bees
6%L	3	220, 203	Eggs and larvae
2%O	3	92, 129	Emerged bees
PRO10	3	28	Eggs

Nutrient composition of honeybees.

- A. RESPONSE TO DIETS OF LINOLEIC AND OLEIC FATTY ACID ENHANCED RED GUM (*CORYMBIA CALOPHYLLA*) POLLEN.**
- B. RESPONSE TO PURE DIETS OF SOYFLOUR (HIGH, MEDIUM AND LOW FAT) AND LUPIN FLOUR.**

Results

Fatty acid analysis of oils used to enhance pollen diets

The linoleic and oleic oils used to enhance the fat content of pollen were not pure as they were laboratory grade and this is reflected in Table 9-2 (Section 9). A large concentration of oleic acid, almost 48% of the linoleic acid concentration, was present in the linoleic oil used to enhance pollen diets. The oleic oil was more pure and contained only 4.3% of the oleic acid concentration as linoleic acid.

Fatty acid analysis of wax scales from bees AND COMB PRODUCTION

Fatty acid analysis

The dominant fatty acid in the wax scales produced from the bees' abdomen was palmitic acid (Table 13-1). This was highest in concentration in wax produced from bees fed redgum pollen diets. Other dominant fatty acids were Unk5, oleic, lignoceric and Unk6. Palmitoleic and linolenic acids were not present in wax scales. Myristic, Unk1, palmitoleic, Unk3, linoleic or linolenic acids were not found in wax scales of bees fed redgum pollen diets (Table 13-1).

Comb production

The average area of comb produced was highest for bees fed pollen [458 cm² (RG), 412 cm² (cRG)]. The next highest was 2%L (163 cm²) with the rest of that diet sequence producing comb ranging in area from 33 to 87 cm². The bees fed oleic acid+pollen diets had a comb area that ranged from 20 to 104 cm² and bees fed sugar produced 41 cm² (cane sugar) and 58 cm² (invert sugar).

Table 13-1. Fatty acid (mg/g) profile of wax scales produced by bees fed pollen diets enhanced with linoleic acid and oleic acid and redgum pollen. Unk1- between C14:1 & C15; Unk2 – between C16 & C16:1; Unk3 – between C16:1 & C17; Unk4 between C18:1 & C18:2; Unk5 – between C20:1 & C20:2; Unk6 – between C22:1 & C22:2.

Diet	Myristic	Unk 1	Palmitic	Palmitoleic	Unk 2	Unk 3	Stearic	Oleic
Linoleic + redgum	0.65	0.29	117.6	0	0	2.29	3.84	20.9
Oleic + redgum	0.62	0.31	106.4	0	0	3.39	4.21	20.9
Redgum	0	0	131.9	0	5.71	0	5.33	34.7

Diet	Unk 4	Linoleic	Linolenic	Gondoic	Unk 5	Behenic	Unk6	Lignoceric
Linoleic + redgum	4.21	0.96	0	1.19	8.64	4.40	2.50	29.1
Oleic + redgum	7.00	1.04	0	1.29	14.3	4.19	6.01	31.3
Redgum pollen	13.0	0	0	2.20	40.7	6.14	19.6	39.4

Diets

The concentration of 20 nutritional components measured in emerged bees (prior to experimentation) are shown in Table 13-2. The majority of the elements increased in bodily concentration following exposure to provided food (see Figs 13-1 to 13-20).

Table 13-2. Mean and standard error of bodily elements of emerged bees (prior to experimentation) after their guts were removed.

Body measurement	Mean	S.E.	N
Protein	17.4 %	1.03	7
Lipid	1.74 %	0.24	7
Myristic acid	0.12 mg/g	0.01	7
Palmitic acid	2.24 mg/g	0.09	7
Palmitoleic acid	0.37 mg/g	0.05	7
UNK3	0.16 mg/g	0.02	7
Stearic acid	1.69 mg/g	0.04	7
Oleic acid	9.07 mg/g	0.40	7
Linoleic acid	0.66 mg/g	0.09	7
Linolenic acid	0.65 mg/g	0.04	7
UNK5	0.20 mg/g	0.03	7
UNK6	0.07 mg/g	0.01	7
Lignoceric acid	0.06 mg/g	0.01	7
Potassium	0.34 %	0.03	6
Phosphorus	0.26 %	0.02	6
Sulphur	0.17 %	0.01	6
Copper	6.16 mg/kg	0.52	6
Iron	22.7 mg/kg	1.76	6
Manganese	1.14 mg/kg	0.26	6
Zinc	25.7 mg/kg	2.55	6

Of the twenty components of the honeybee body measured (see Table 13-3), only four showed no significant difference between six treatments. These were the body concentrations of lipid, myristic acid, potassium and phosphorus. The rest of the components, viz. protein, the fatty acids (palmitic, palmitoleic, Unk3, stearic, oleic, linoleic, linolenic, Unk5, Unk6, lignoceric) and minerals (sulphur, copper, iron, manganese and zinc) were significantly influenced ($P < 0.04$) by the treatments tested.

Time did not influence most of the body components measured. However, the body content of lipid, palmitic acid, Unk3 acid, Unk5 acid, lignoceric acid, sulphur and zinc were significantly altered ($P < 0.05$) over the duration of the experiment (see Table 13-6). Copper was borderline significant ($P = 0.062$).

Table 13-3. Significance values for the effect of diet, time and linear trend of 20 body components measured in the honeybee.

Body component measurement	Effect of Treatment	Effect over time (XDATA)	Linear trend (xdata)
Protein	P<0.001	P=0.217	P=0.027
Lipid	P=0.147	P<0.001	P=0.458
Myristic acid	P=0.161	P=0.262	P=0.763
Palmitic acid	P=0.029	P<0.001	P=0.012
Palmitoleic acid	P<0.001	P=0.246	P<0.001
Unk3	P<0.001	P=0.004	P<0.001
Stearic acid	P<0.001	P=0.195	P=0.013
Oleic acid	P<0.001	P=0.287	P=0.062
Linoleic acid	P<0.001	P=0.808	P<0.001
Linolenic acid	P<0.001	P=0.688	P=0.045
Unk5	P<0.001	P=0.003	P=0.520
Unk6	P<0.001	P=0.293	P<0.001
Lignoceric acid	P=0.014	P<0.001	P=0.154
Potassium	P=0.114	P=0.403	P=0.003
Phosphorus	P=0.116	P=0.172	P=0.189
Sulphur	P<0.001	P=0.029	P=0.016
Copper	P<0.001	P=0.062	P=0.258
Iron	P<0.001	P=0.513	P<0.001
Manganese	P<0.001	P=0.134	P=0.092
Zinc	P=0.035	P<0.001	P=0.410

A significant ($P < 0.05$) linear trend was shown for a number of body components such as body protein, palmitic acid, palmitoleic acid, Unk3 acid, stearic acid, linoleic acid, linolenic acid, Unk6 acid, potassium, sulphur, and iron measured for the different treatments tested (Table 13-3). The linear trend of oleic acid was borderline ($P = 0.062$). The body components such as lipid, myristic acid, Unk5 acid, lignoceric acid, phosphate, copper, managanese and zinc showed no significant linear trend.

Protein

There were significant differences in protein levels with treatment ($P < 0.001$). The protein level in bees increased for all diets except sugar (Fig. 13-1). Protein levels of bees fed on sugar remained depressed compared to the other three diets and apiary bees. After day 21, protein uptake from flour diets decreased after initially increasing.

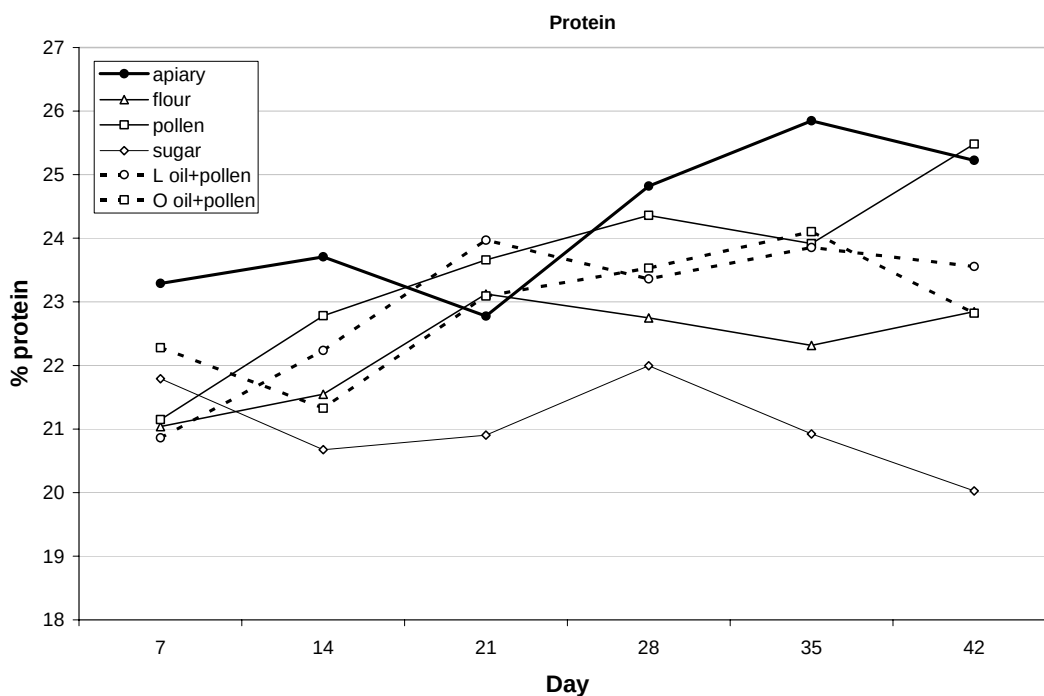


Figure 13-1. The predicted values of the concentration of body protein (%) over 42 days between different diets (5% LSD = 2.37 %).

Lipid

There were no significant differences in lipid levels with treatment ($P = 0.147$). Body lipid levels increased to peak at day 21 to day 28 before decreasing. The carbohydrate diet enabled bees to attain the highest level of lipid, but a rapid loss occurred after day 28 which was in contrast to the other diets and bees from the apiary (Fig. 13-2).

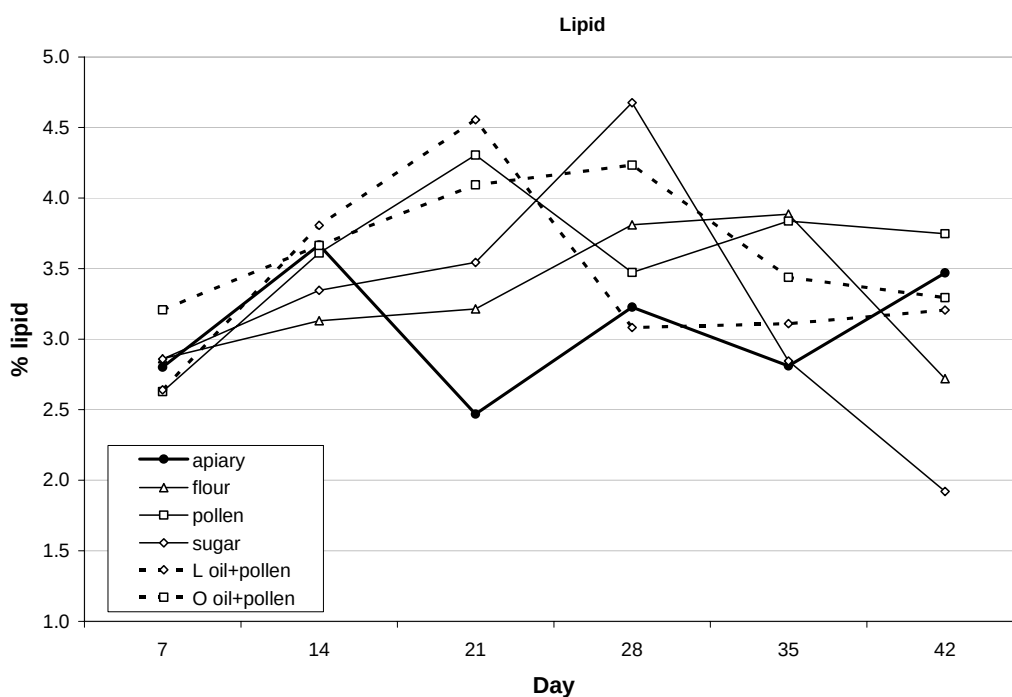


Figure 13-2. The predicted values of the concentration of body lipid (%) over 42 days between different diets (5% LSD = 1.27 %).

Fatty acids

Of the fatty acids measured from bee bodies, eleven were in sufficient concentration in the body of the bee to be of importance. These acids were: myristic, palmitic, palmitoleic, stearic, Unk3, oleic, linoleic, linolenic, Unk5, Unk6 and lignoceric and cover the five fatty acids commonly found in pollen.

Myristic acid (C-14)

There were no significant differences in myristic acid levels with treatment ($P = 0.161$). Generally, the concentration of myristic acid ranged from 0.06 to 0.08 mg/g over 42 days (Fig. 13-3A). As the apiary bees grew older from three weeks of age, the concentration of myristic acid increased. The amount of myristic acid excreted generally increased the longer the bees remained caged (Fig. 13-3Bi, Fig. 13-3Bii).

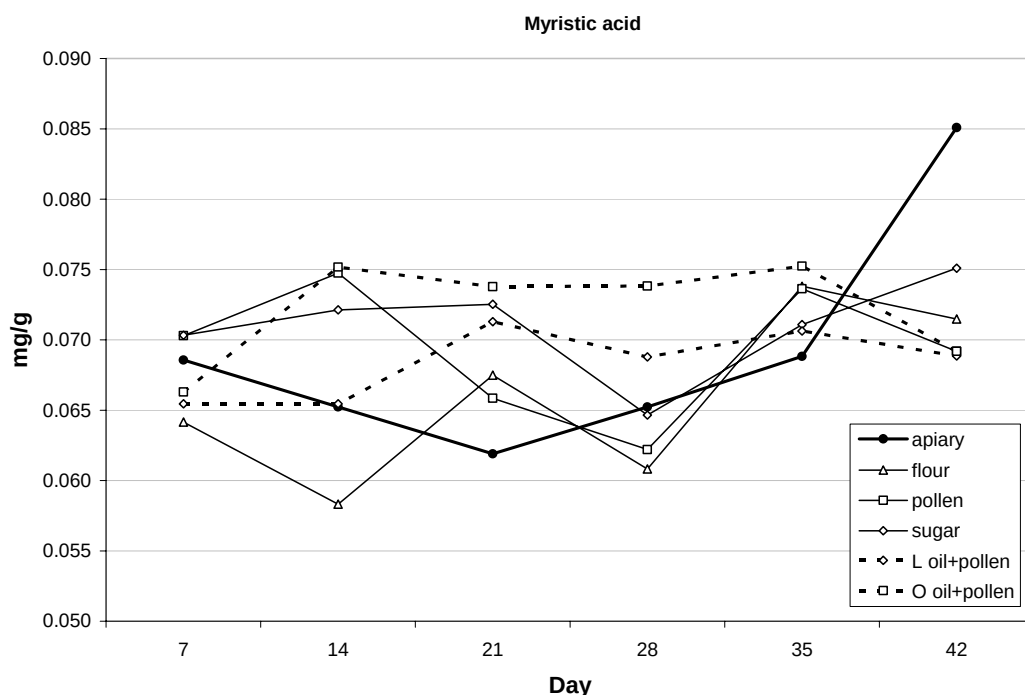


Figure 13-3A. The predicted values of the concentration of body content of myristic acid (mg/g) over 42 days between different diets (5% LSD = 0.02 mg/g).

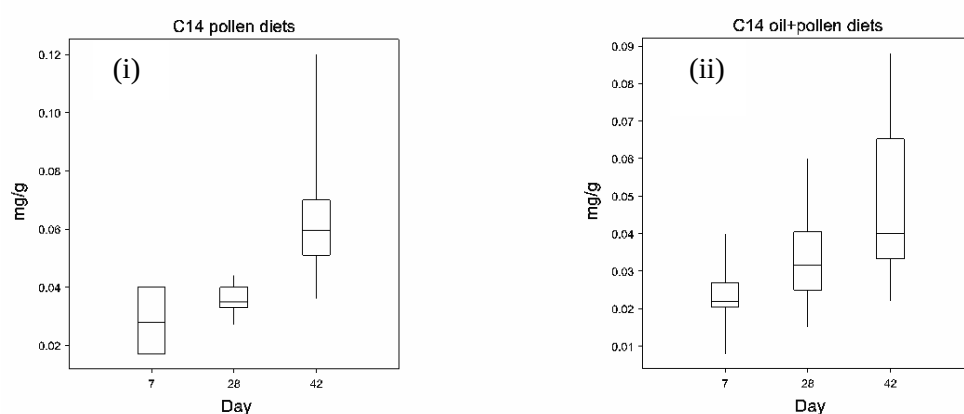


Figure 13-3B. Box plots of the concentration of myristic acid (mg/g) excreted into the gut of bees fed pollen (i) or both oil+pollen diets (ii) at day 7, 28 and 42.

Palmitic acid (C-16)

There were significant differences in palmitic acid levels with treatment ($P = 0.029$). The concentration of palmitic acid peaked from all diets except for flour diets and apiary bees at day 14 which steadily declined to day 42. The bees fed flour diets showed a steady increase in the fatty acid until day 35 where it reached a peak before declining (Fig. 13-4A). Bees from the apiary showed a similar pattern to that of bees in cages fed pollen and the two oil+pollen diets, but the concentration peaked earlier at day seven and then declined from the body tissue before increasing from day 35.

The concentration of palmitic acid in the gut increased over the time the bees were caged for the experiment (Fig. 13-4B) despite being a major component of wax scales produced for the extensive rebuilding of the plastic foundation during the early days of the experiment (Table 13-4). From pollen diets, the excretion concentrations of palmitic acid at day 28 were similar to that at day 7. This coincided with the period when bees produced wax scales which were high in palmitic acid for comb production.

Palmitoleic acid (C-16:1)

There were significant differences in palmitoleic acid levels with treatment ($P < 0.001$). There was significant divergence from day 21 where the palmitoleic acid concentration in bees fed both the pollen and both oil+pollen diets gradually decreased whereas concentrations remained high from flour and sugar diets (Fig. 13-5A). Excretion rates of palmitoleic acid increased only slightly (Fig. 13-5B) except for an increase in concentration at day 42 for pollen-fed bees (Fig. 13-5Bi).

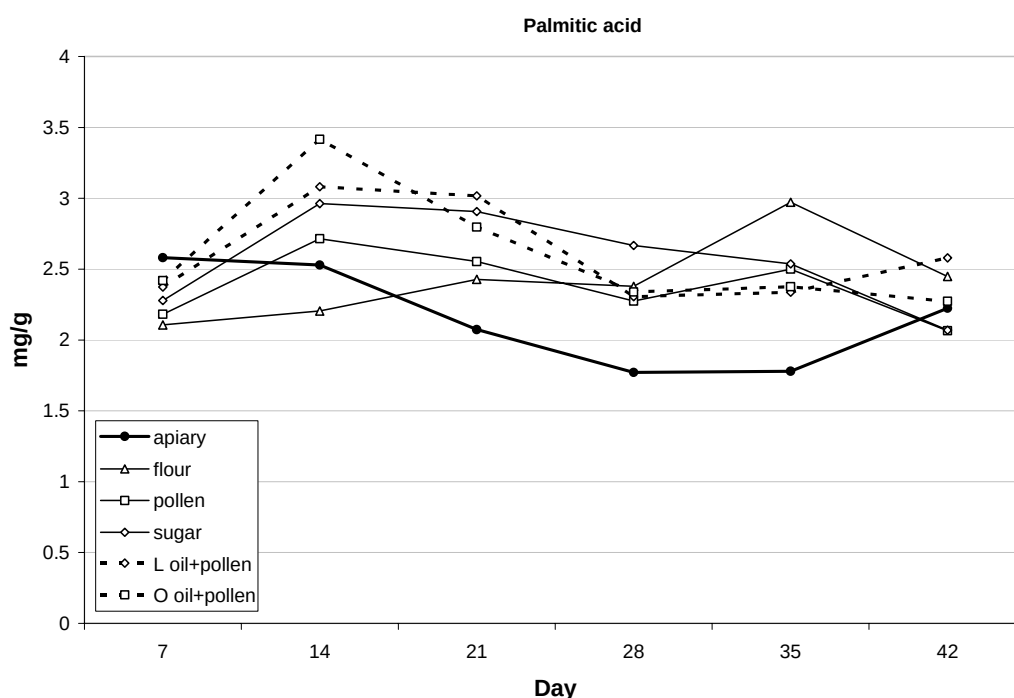


Figure 13-4A. The predicted values of the concentration of body content of palmitic acid (mg/g) over 42 days between different diets (5% LSD = 0.81 mg/g).

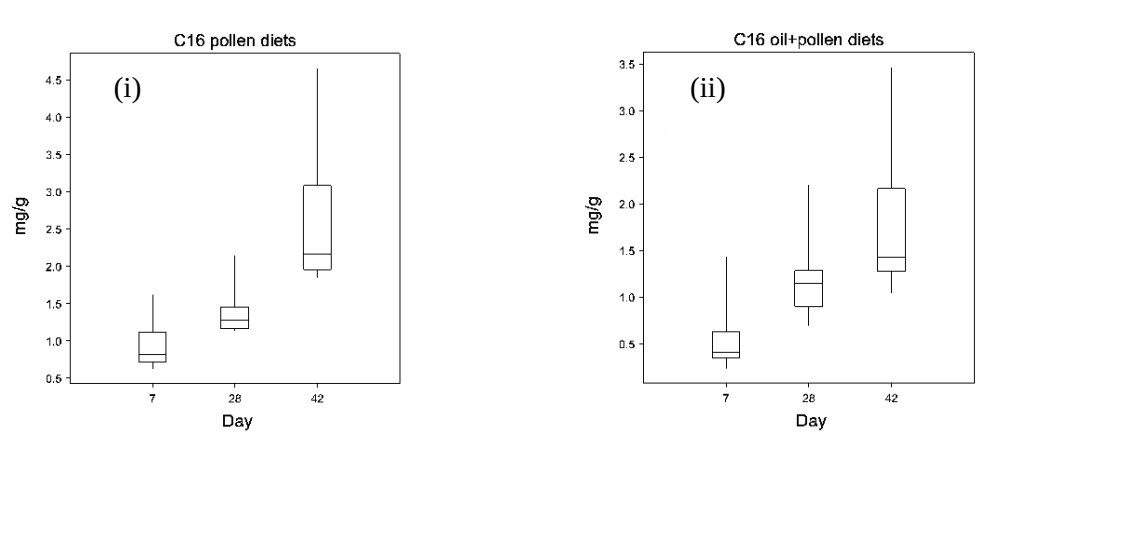


Figure 13-4B. Box plots of the concentration of body palmitic acid (mg/g) excreted into the gut of bees fed pollen (i) or both oil+pollen diets (ii) at day 7, 28 and 42.

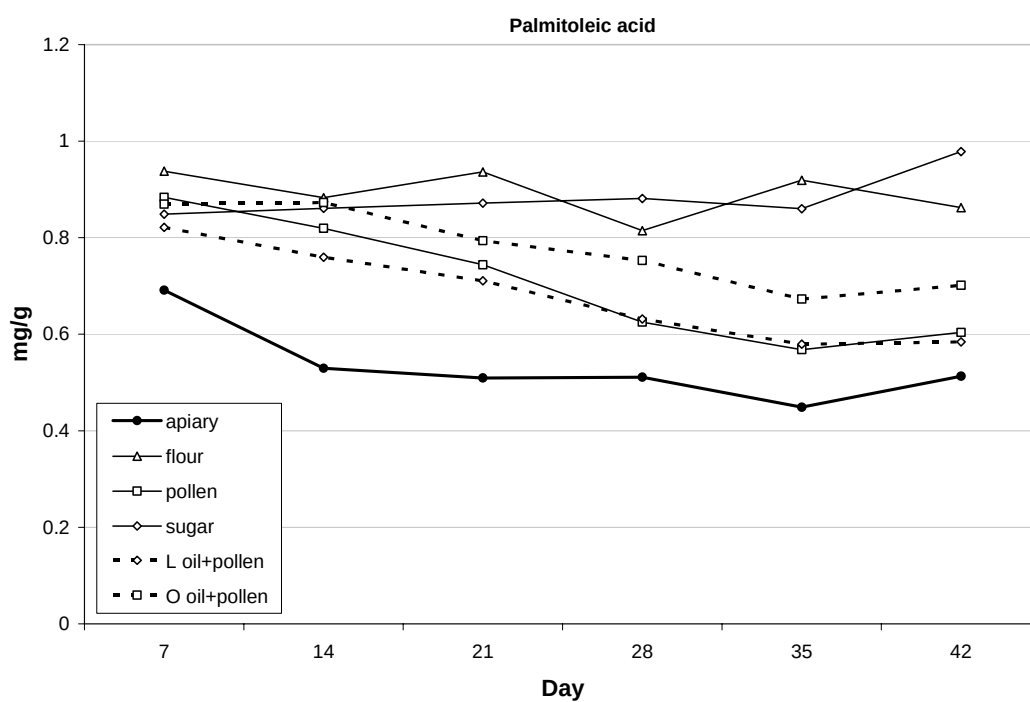


Figure 13-5A. The predicted values of the concentration of body content of palmitoleic acid (mg/g) over 42 days between different diets (5% LSD = 0.12 mg/g).

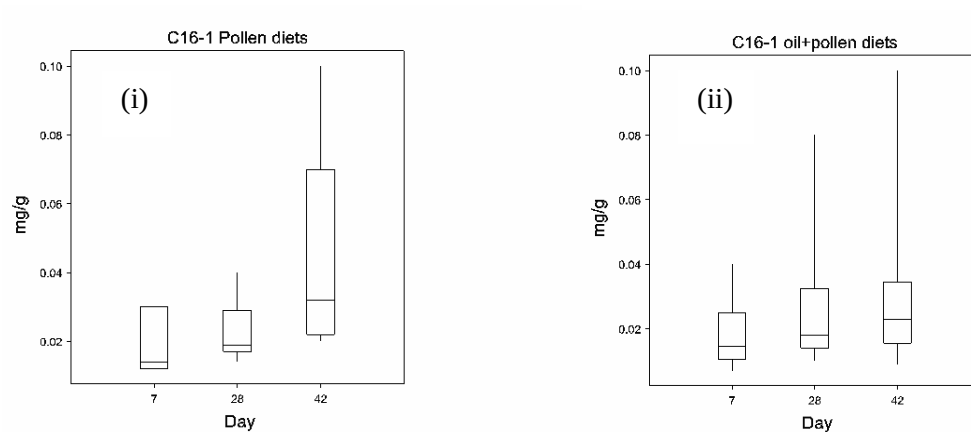


Figure 13-5B. Box plots of the concentration of body palmitoleic acid (mg/g) excreted into the gut of bees fed pollen (i) or both oil+pollen diets (ii) at day 7, 28 and 42.

Fatty acid – Unk3 eluting between fatty acids C16:1 and C17.

There were significant differences in Unk3 acid levels with treatment ($P < 0.001$). Bees fed on flour diets had Unk3 levels which peaked at day 14 and then decreased over the following four weeks to be the lowest of all treatments (Fig. 13-6A). Uptake of Unk3 was lowest from day seven to day 21 in bees fed sugar. Initial concentrations of Unk3 from bees in the apiary were much lower than levels in caged bees which steadily rose to the highest level of all treatments at day 42.

The Unk3 fatty acid was not present in the oils added to the pollen diet (Table 9-2, Section 9) or crushed and irradiated redgum pollen (Table 13-1). Unk3 was only present in full fat soya bean (FF) and lupin flour (LUP). This was unusual because it was present in fresh redgum pollen (Table 9-1, Section 9). The concentration of Unk3 excreted generally increased over the 42 day experiment (Fig. 13-6B).

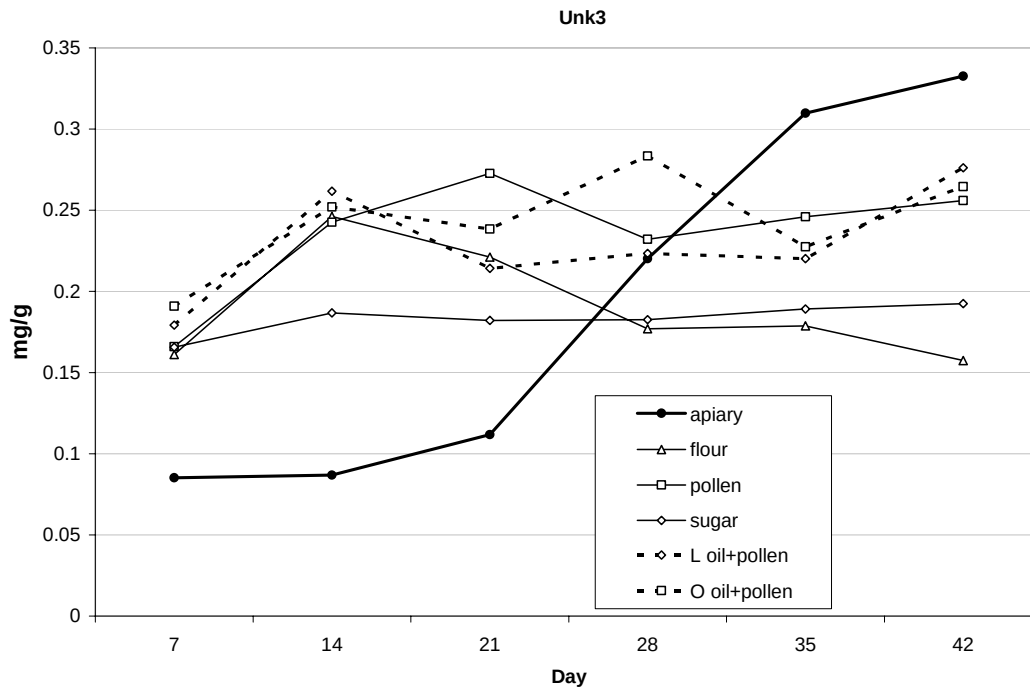


Figure 13-6A. The predicted values of the concentration of body content of Unk3 acid (mg/g) over 42 days between different diets (5% LSD = 0.09 mg/g).

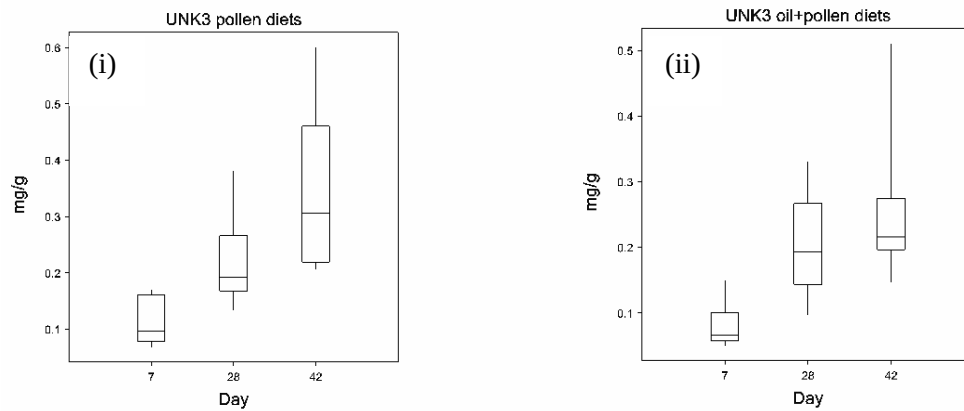


Figure 13-6B. Box plots of the concentration of Unk3 acid (mg/g) excreted into the gut of bees fed pollen (i) or both oil+pollen diets (ii) at day 7, 28 and 42.

Stearic acid (C-18)

There were significant differences in stearic acid levels with treatment ($P < 0.001$). The bees from the apiary had an elevated concentration of stearic acid which may have been due to differences in diet (Fig. 13-7A). Overall, the concentration of excreted stearic acid increased but dipped slightly at day 28 in bees fed pollen diets (Fig. 13-7Bi).

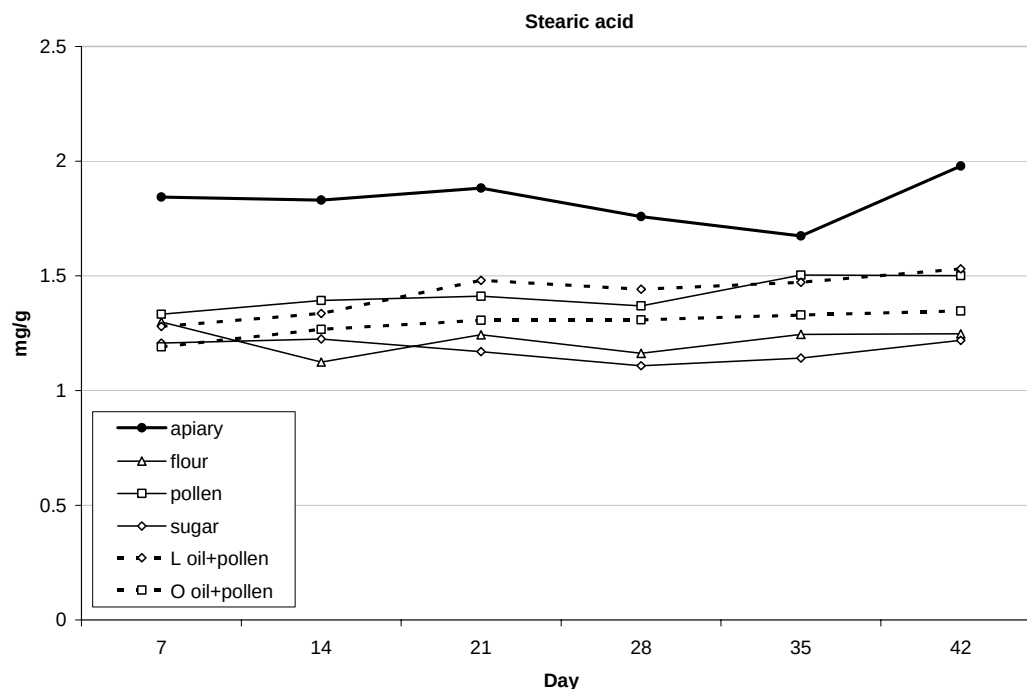


Figure 13-7A. The predicted values of the concentration of body content of stearic acid (mg/g) over 42 days between different diets (5% LSD = 0.20 mg/g).

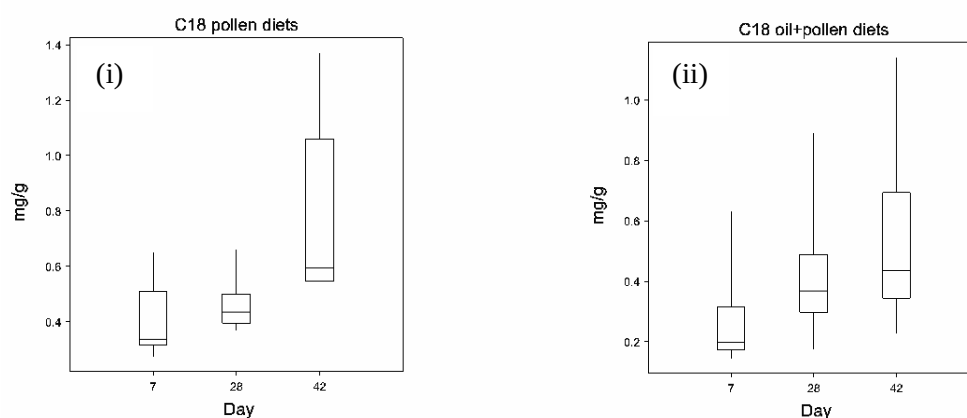


Figure 13-7B. Box plots of the concentration of body stearic acid (mg/g) excreted into the gut of bees fed pollen (i) or both oil+pollen diets (ii) at day 7, 28 and 42.

Oleic acid (C-18:1)

There were significant differences in oleic acid levels with treatment ($P < 0.001$). The oleic acid concentration was generally higher in bees fed flour diets. A similar gradual increase in concentration was measured in bees from the apiary albeit at lower concentrations. Oleic acid was the highest concentration of all fatty acids excreted by day 42 (Fig. 13-8B).

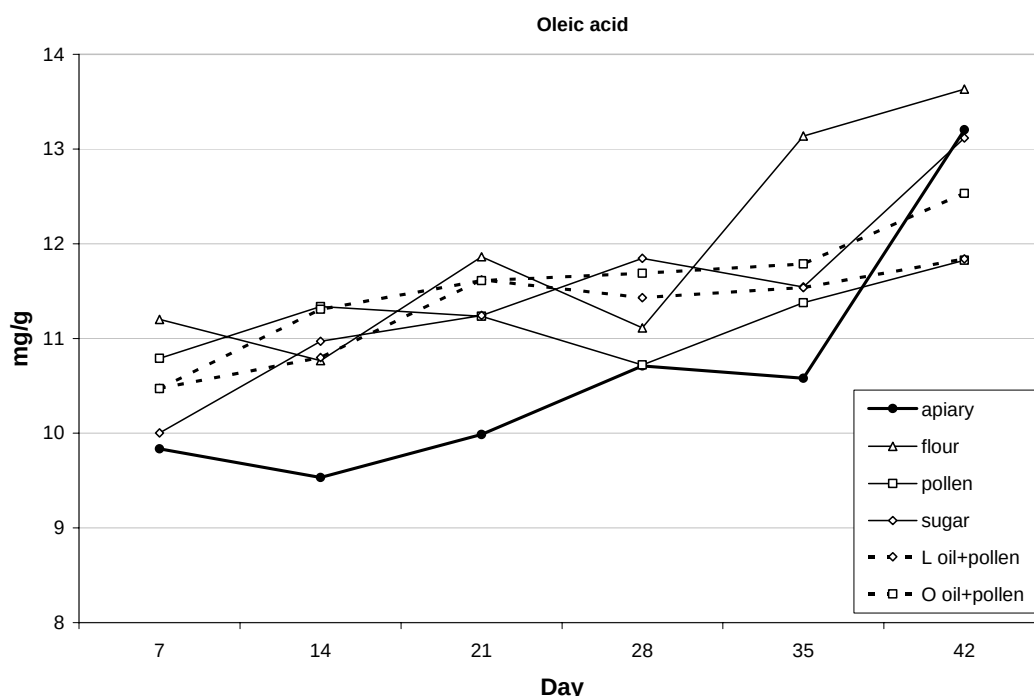


Figure 13-8A. The predicted values of the concentration of body content of oleic acid (mg/g) over 42 days between different diets (5% LSD = 1.28 mg/g).

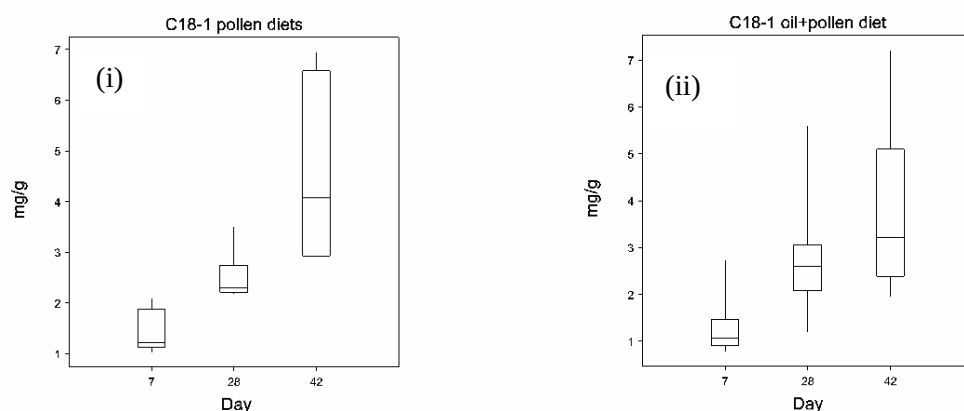


Figure 13-8B. Box plots of the concentration of body oleic acid (mg/g) excreted into the gut of bees fed pollen (i) or both oil+pollen diets (ii) day 7, 28 and 42.

Linoleic acid (C-18:2)

There were significant differences in linoleic levels with treatment ($P < 0.001$). The bees fed pollen and both oil+pollen diets showed an increasing concentration over the experimental period (Fig. 13-9A). In contrast, accumulation by bees of linoleic acid from the flour and sugar-only diets was weak.

The concentration of linoleic acid in bees from the apiary generally followed the same linear trend for pollen and both oil+pollen diets (Fig. 13-9A). Linoleic acid was excreted in increasing rates over the

length of time the bees were caged (Fig. 13-9B), except for bees fed pollen diets, where the concentration in the gut decreased at day 28 (Fig. 13-9Bi). This indicates that linoleic acid was being reabsorbed into the body because of an unknown bodily requirement.

Linolenic acid (C-18:3)

There were significant differences in linolenic acid levels with treatment ($P < 0.001$). The bee response to this fatty acid was similar for all diets except the concentration of linolenic acid was higher for bees fed flour diets than any of the other three diets (Fig. 13-10A). The apiary bees had a much higher body concentration of linolenic acid, similar to that of stearic acid (Fig. 13-7A). The concentration of linolenic acid in the gut decreased at day 28 for bees fed pollen diets (Fig. 13-10Bi) as it did for linoleic acid (Fig. 13-9Bi) and perhaps for the same reason. For the bees fed the oil+pollen diets, the amount excreted was less than bees fed pollen.

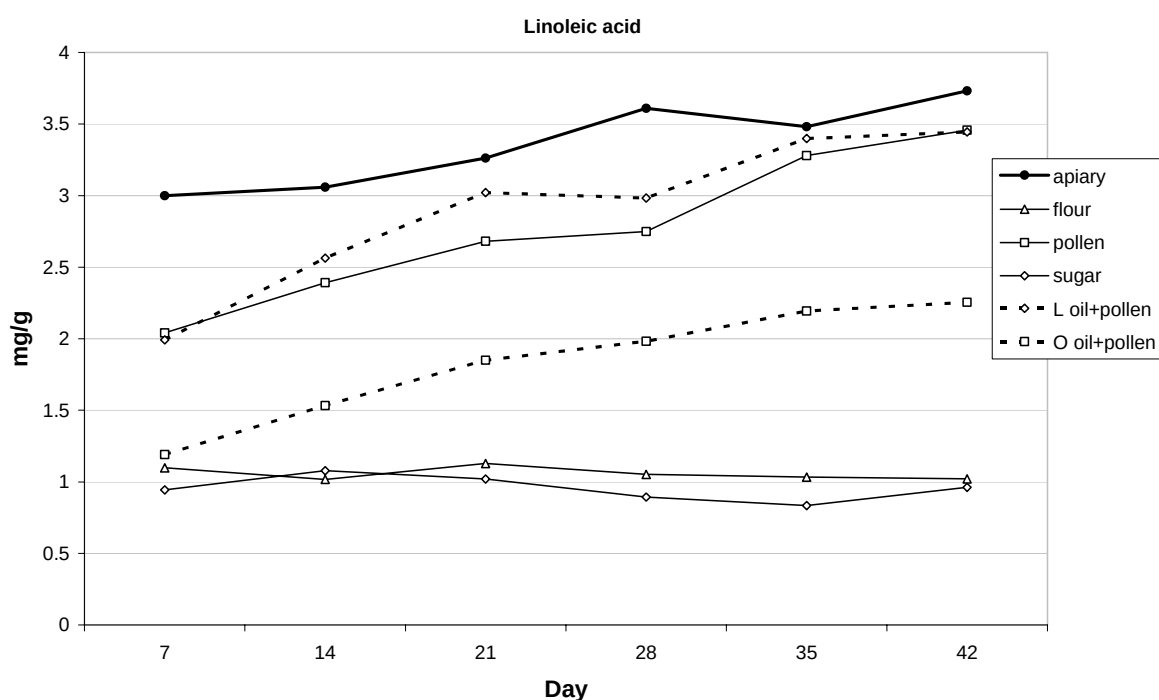


Figure 13-9A. The predicted values of the concentration of body content of linoleic acid (mg/g) over 42 days between different diets (5% LSD = 0.78 mg/g).

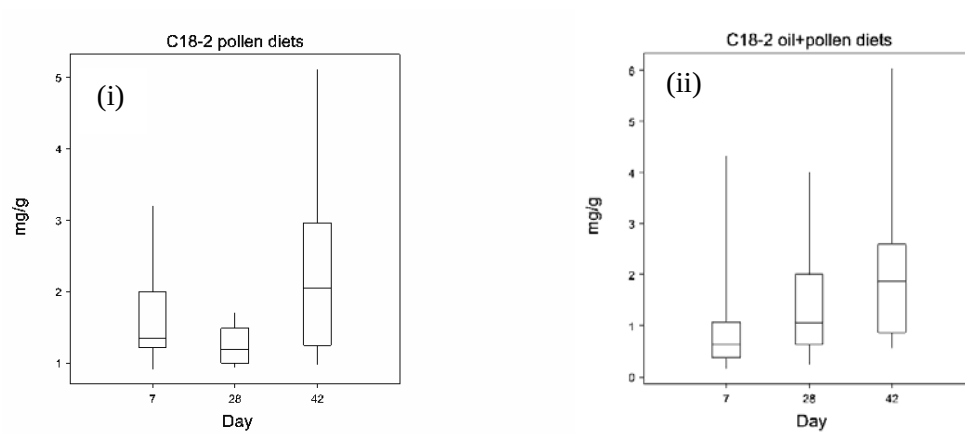


Figure 13-9B. Box plots of the concentration of body linoleic acid (mg/g) excreted into the gut of bees fed pollen (i) or both oil+pollen diets (ii) at day 7, 28 and 42.

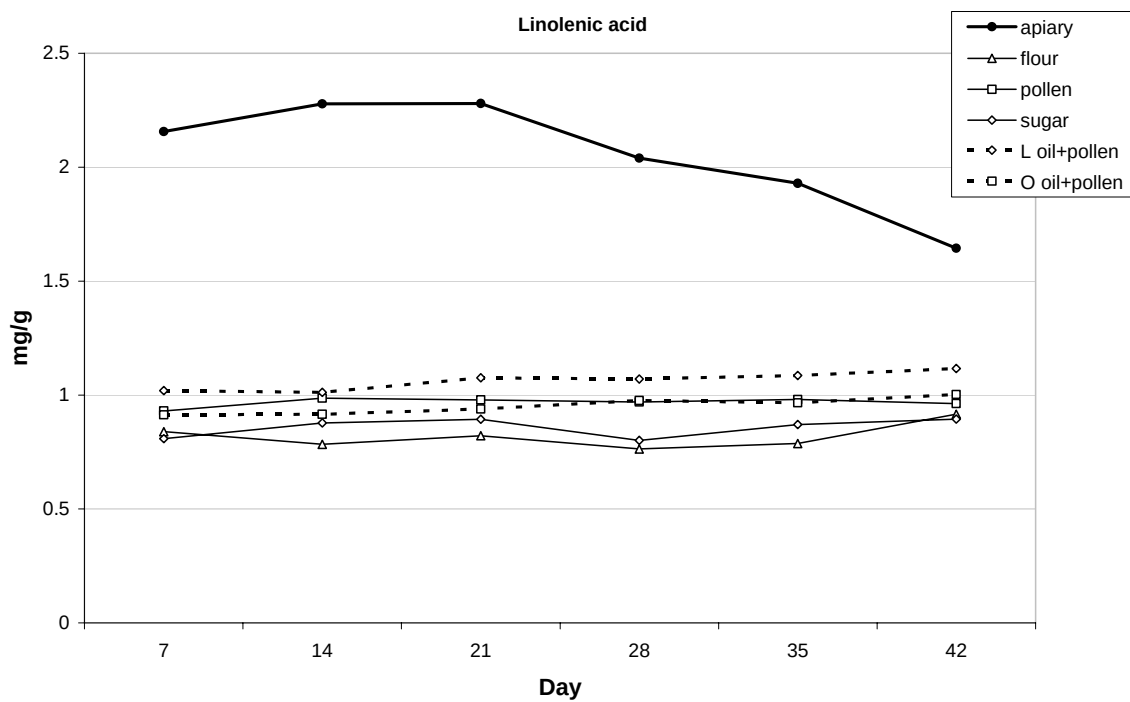


Figure 13-10A. The predicted values of the concentration of body content of linolenic acid (mg/g) over 42 days between different diets (5% LSD = 0.23 mg/g).

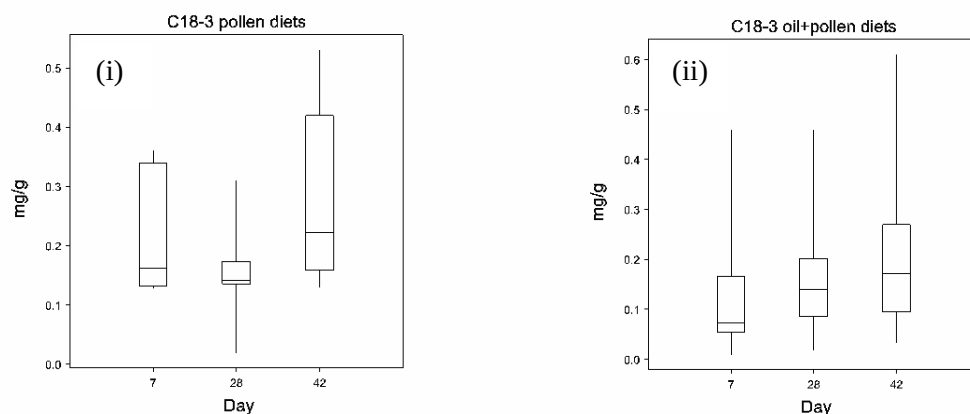


Figure 13-10B. Box plots of the concentration of body linolenic acid (mg/g) excreted into the gut of bees fed pollen (i) or both oil+pollen diets (ii) at day 7, 28 and 42.

Fatty acid – Unk5 eluting between C-20:1 and C-20:2

There were significant differences in body Unk5 acid levels with treatment ($P < 0.001$). The apiary bees had a gradual uptake of Unk5 acid albeit at a much lower level than caged bees which tended to peak in concentration at day 21 and between days 35-42. The excretion of Unk5 acid increased in concentration over the period of the experiment (Fig. 13-11B).

The fatty acid was not present in any of the oils added to the pollen (Table 9-2, Section 9), but was present in all pollen diets (Table 9-1, Section 9) and all flour diets enhanced with pollen (Table 9-3, Section 9) which meant that it was exclusively a fatty acid found in pollen and this was reflected in the higher amount excreted from bees fed pollen diets (Fig. 13-11Bi). Unk5 was a dominant fat found in wax scales of bees (Table 13-1).

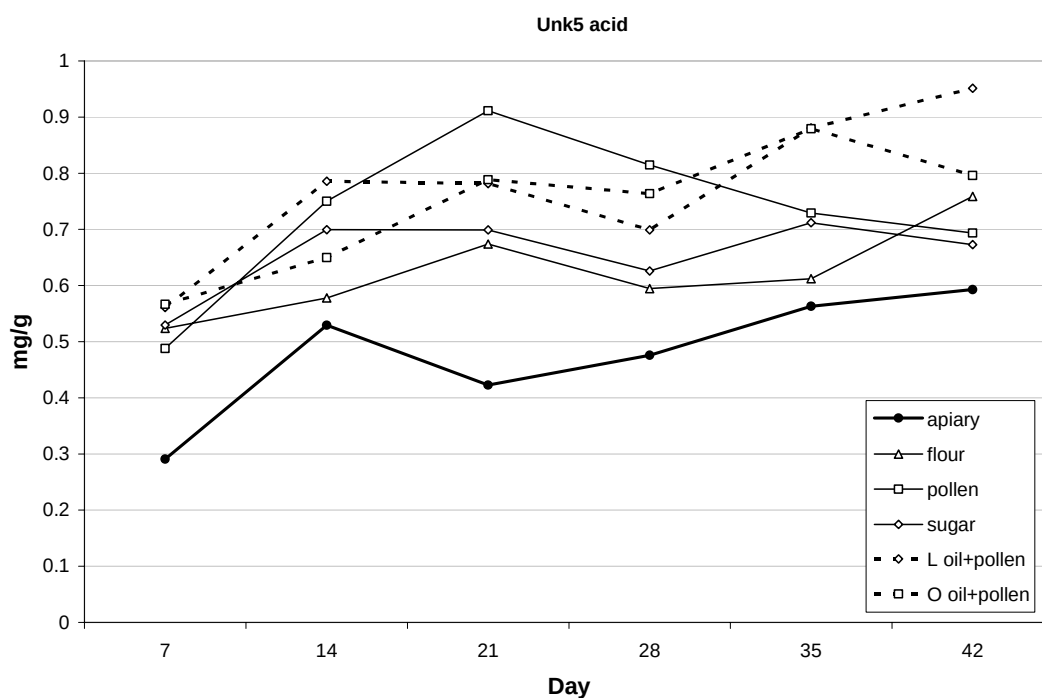


Figure 13-11A. The predicted values of the concentration of body content of Unk5 acid (mg/g) over 42 days between different diets (5% LSD = 0.27 mg/g).

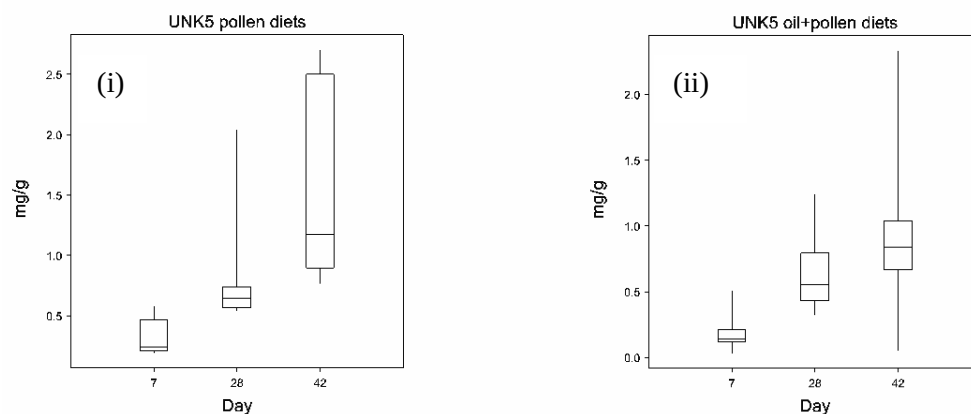


Figure 13-11B. Box plots of the concentration of Unk5 (mg/g) excreted into the gut of bees fed pollen (i) or both oil+pollen diets (ii) at day 7, 28 and 42.

Fatty acid – Unk6 eluting between C-22:1 and C-22:2

There were significant differences in Unk6 acid levels with treatment ($P < 0.001$). The pollen diets produced bees with a higher concentration of Unk6 than the other three diet types, with bees fed flour or sugar diets having the lowest concentrations in their bodies. The bees in the apiary showed a more rapid uptake at an earlier age to peak at day 14 before slightly decreasing (Fig. 13-12A).

Graphically, the excretion patterns between pollen and pollen+oil diets was similar to Unk5 but at lower concentrations (Fig. 13-12B). Like Unk5, Unk6 acid is another fat only associated with pollen as it was not present in the oils added to pollen (Table 9-2, Section 9) and was only detected in defatted soya bean flour diets mixed with 30% pollen (Table 9-3, Section 9). It was one of the dominant fatty acids in wax scales (Table 13-1).

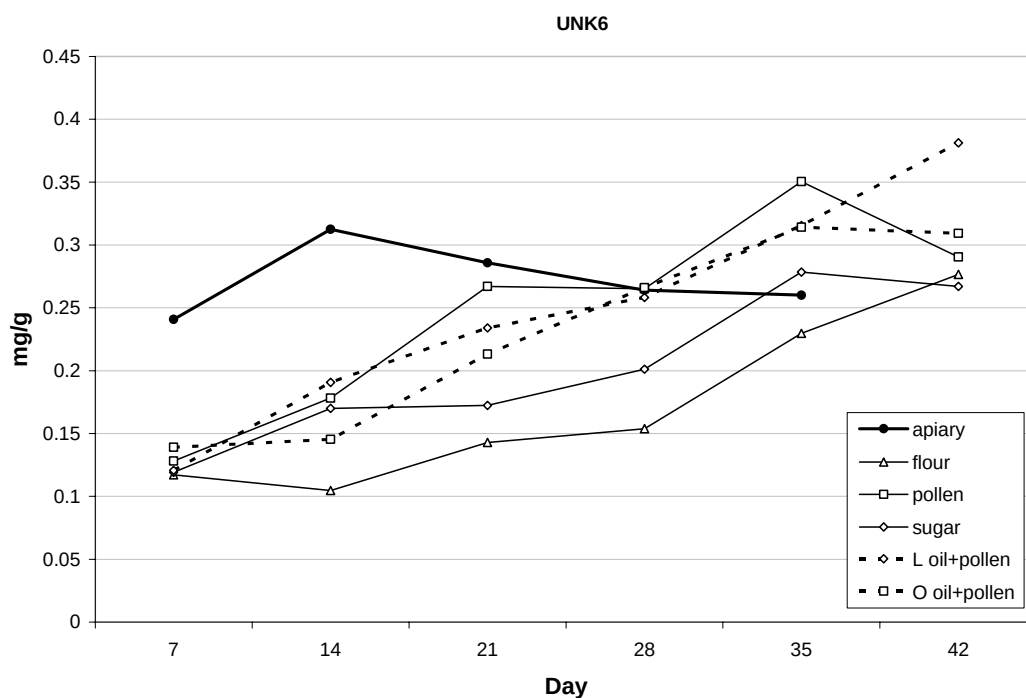


Figure 13-12A. The predicted values of the concentration of body content of Unk6 acid (mg/g) over 42 days between different diets (5% LSD = 0.05 mg/g). Data point for day 42 (apiary) is missing.

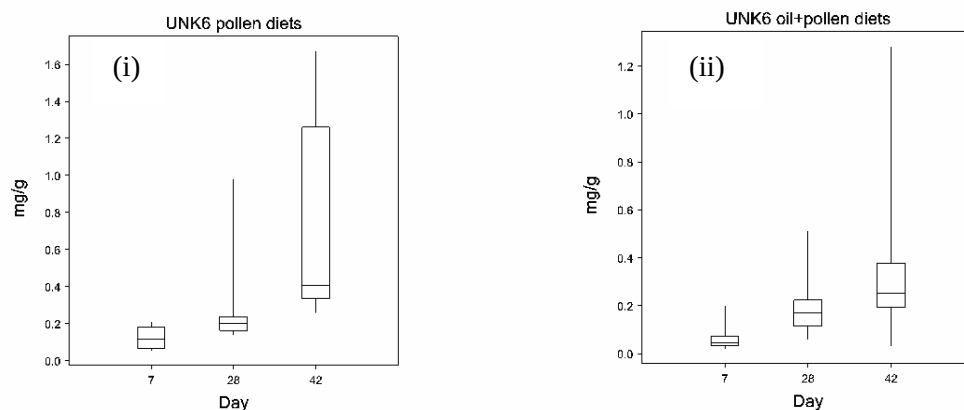


Figure 13-12B. Box plots of the concentration of Unk6 acid (mg/g) excreted into the gut of bees fed pollen (i) or both oil+pollen diets (ii) at day 7, 28 and 42.

Lignoceric acid (C-24)

There were significant differences in lignoceric levels with treatment ($P < 0.014$). The concentration of lignoceric acid rapidly accumulated in the first 14 to 21 days before generally decreasing in bees in all treatments (Fig. 13-13A). The flour diets allowed for a sustained elevated concentration following the peak level at day 21. The concentration of lignoceric acid in bees in the apiary showed an initial increase to day 14 which was followed by a decreased concentration which gradually recovered by day 42. Body concentration of lignoceric acid in bees from the apiary was generally lower than for caged bees.

The concentration of lignoceric acid increased in the gut during the experiment (Fig. 13-13B). Lignoceric acid was not present in the oils added to the pollen diet (Table 9-2, Section 9) and like Unk3, was only present in full fat soya bean and lupin flour and unlike Unk3, lignoceric acid was present in both crushed and irradiated redgum pollen and fresh pollen (Table 9-1, Section 9). The fatty acid was not present in Promine™ or defatted soya bean flour (Table 9-3, Section 9). Lignoceric acid was one of four dominant fatty acids found in wax scales of bees (Table 13-1).

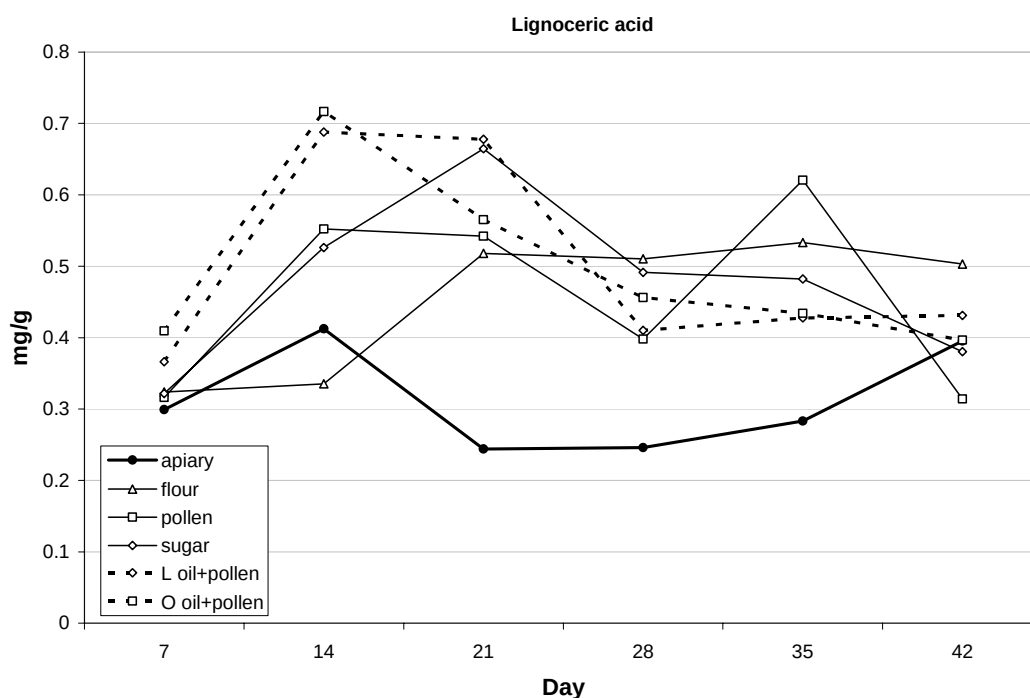


Figure 13-13A. The predicted values of the concentration of body content of lignoceric acid (mg/g) over 42 days between different diets (5% LSD = 0.29 mg/g).

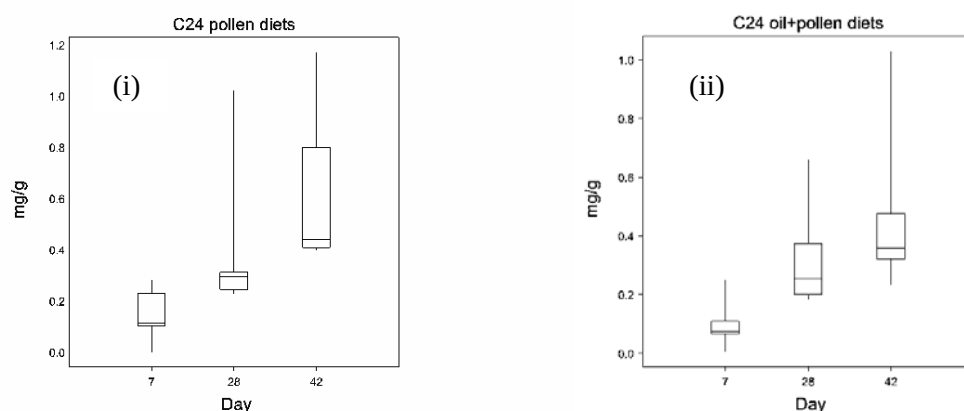


Figure 13-13B. Box plots of the concentration of lignoceric acid (mg/g) excreted into the gut of bees fed pollen (i) or both oil+pollen diets (ii) at day 7, 28 and 42.

Minerals

Ten elements were measured from bee bodies. Sodium, calcium and magnesium varied by only small concentrations during the experiment period and across diets. Therefore, the concentrations of these elements are not presented for the experimental period. The average ($\pm$ S.E.) concentrations for all diets were: Na ($0.03 \pm 0.00\%$ N=301); Ca ($0.02 \pm 0.00\%$, N=301) and Mg ($0.03 \pm 0.00\%$, N=301).

Potassium

There were no significant differences in potassium levels with treatment ($P = 0.114$). Generally, the bees fed on flour diets were low in potassium for most of the experiment. From day 28, bees on a sugar diet experienced a sharp decline in potassium. The bees from the apiary had low levels of potassium from day 14 to 21 (Fig. 13-14).

Phosphorus

There were no significant differences in phosphorus levels with treatment ($P = 0.116$). For the flour diets, the peak concentration was reached at day 28. Bees fed flour diets had the lowest concentration of phosphorus for most of the duration of the experiment. The sugar diet and apiary bees showed a similar trend to each other until day 28, when levels sharply decreased for sugar diets (Fig. 13-15). Phosphorus in apiary bees rose to their highest level at day 35.

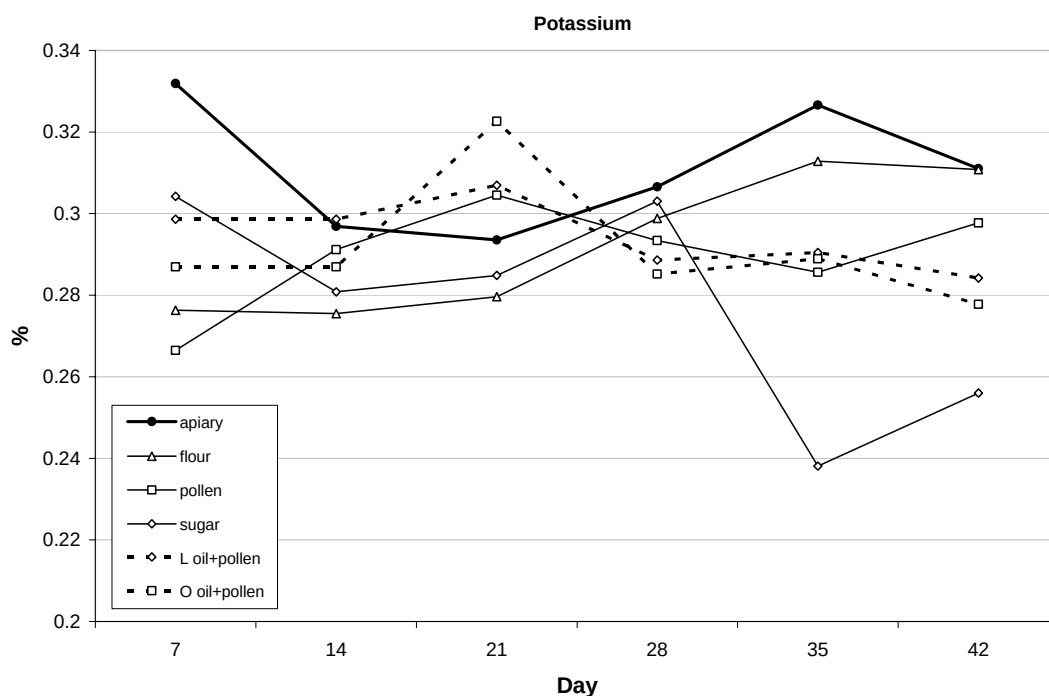


Figure 13-14. The predicted values of the concentration of body content of potassium (%) over 42 days between different diets (5% LSD = 0.04 %).

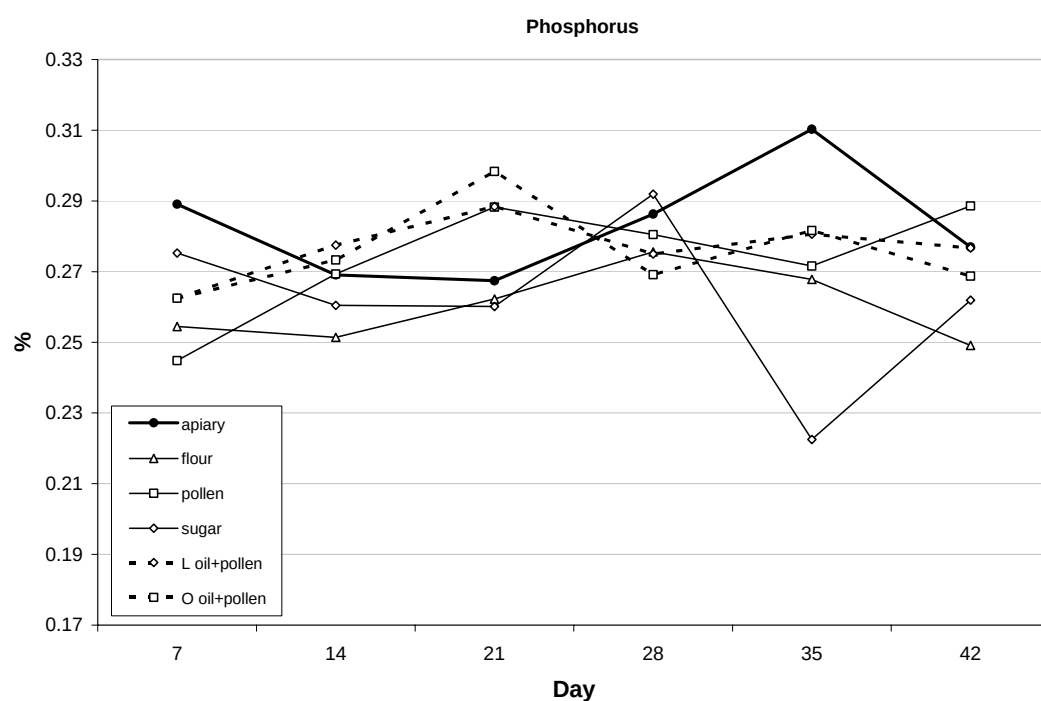


Figure 13-15. The predicted values of the concentration of body content of phosphorus (%) over 42 days between different diets (5% LSD = 0.04 %).

Sulphur

There were significant differences in sulphur levels with treatment ($P < 0.001$). Bees fed pollen and both oil+pollen diets increased sulphur concentration to day 21 (Fig. 13-16). Sugar and flour diets had peak concentrations at day 28 before declining. Bees fed the flour diet generally had the lowest uptake of sulphur. Bees from the apiary had an elevated concentration of sulphur from day 21 to 35 that subsequently declined.

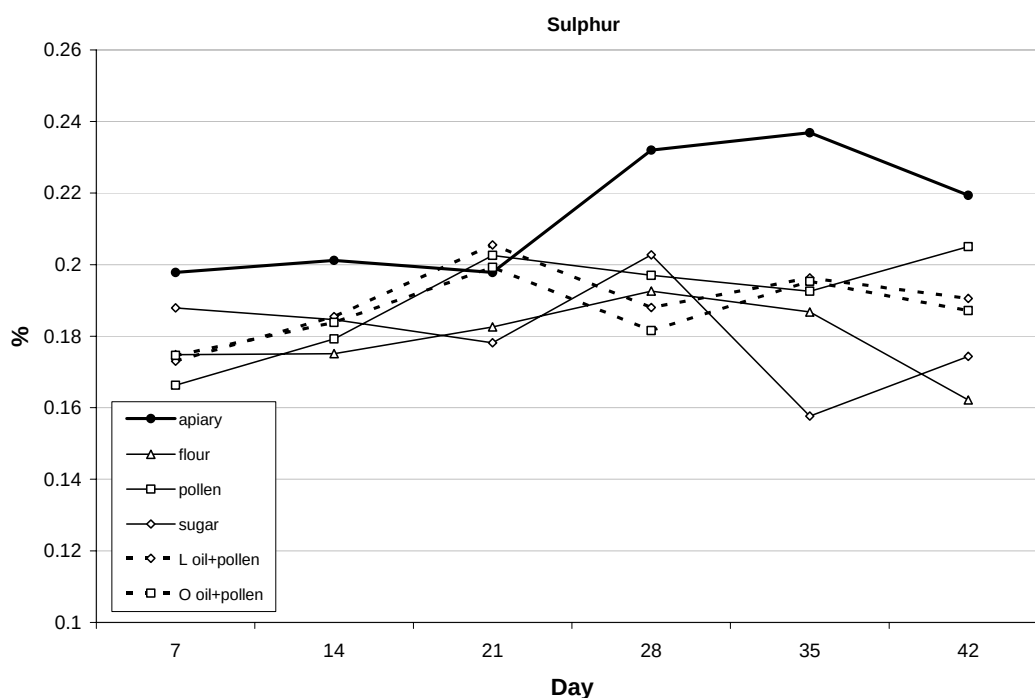


Figure 13-16. The predicted values of the concentration of body content of sulphur (%) over 42 days between different diets (5% LSD = 0.03 %).

Copper

There were significant differences in body copper levels with treatment ($P < 0.001$). From day seven to day 21, the copper levels in bees on sugar diets mirrored that of apiary bees but then diverged. Copper concentration of bees generally decreased over the duration of the experiment (Fig. 13-17). The bees on flour diets had a poor uptake of copper with levels generally lowest of all diets during the experiment.

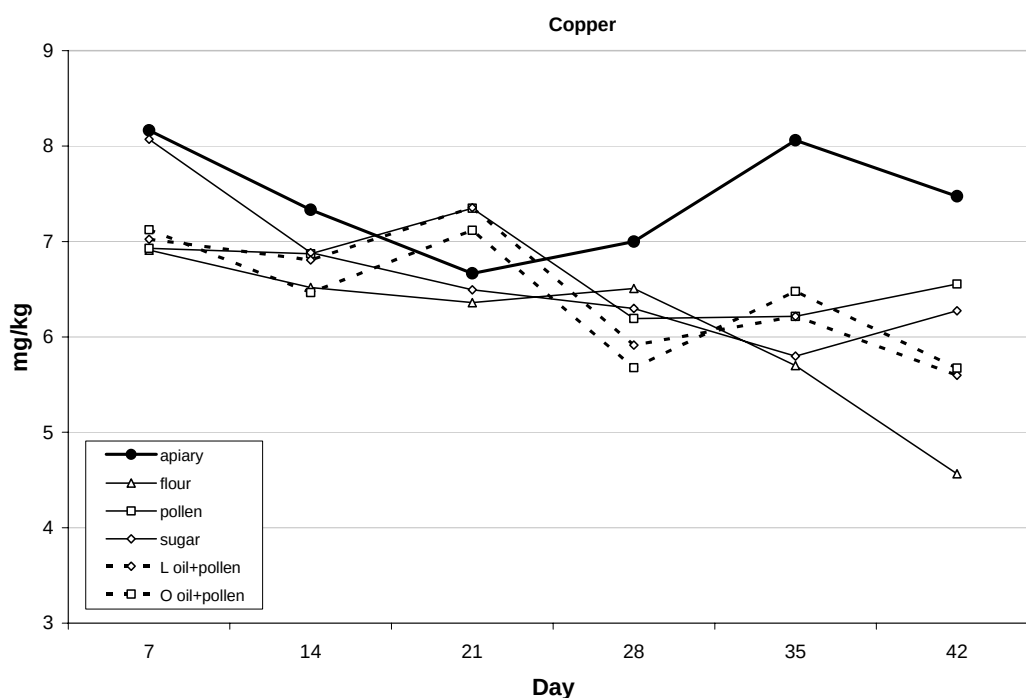


Figure 13-17. The predicted values of the concentration of body content of copper (mg/kg) over 42 days between different diets (5% LSD = 1.12 mg/kg).

Iron

There were significant differences in iron levels with treatment ($P < 0.001$). Body concentration of iron for all diets increased over the duration of the experiment (Fig. 13-18). The bees from the apiary, however showed a significantly higher cumulative increase in iron than bees that were caged.

Manganese

There were significant differences in manganese levels with treatment ($P < 0.001$). The concentration of manganese in the body of bees was similar for apiary bees and bees fed a diet of pollen in cages (Fig. 13-19). Both oil and pollen diets were similar in trend. The flour and sugar diets had the poorest uptake of manganese with sugar diets giving bees the lowest in the experiment.

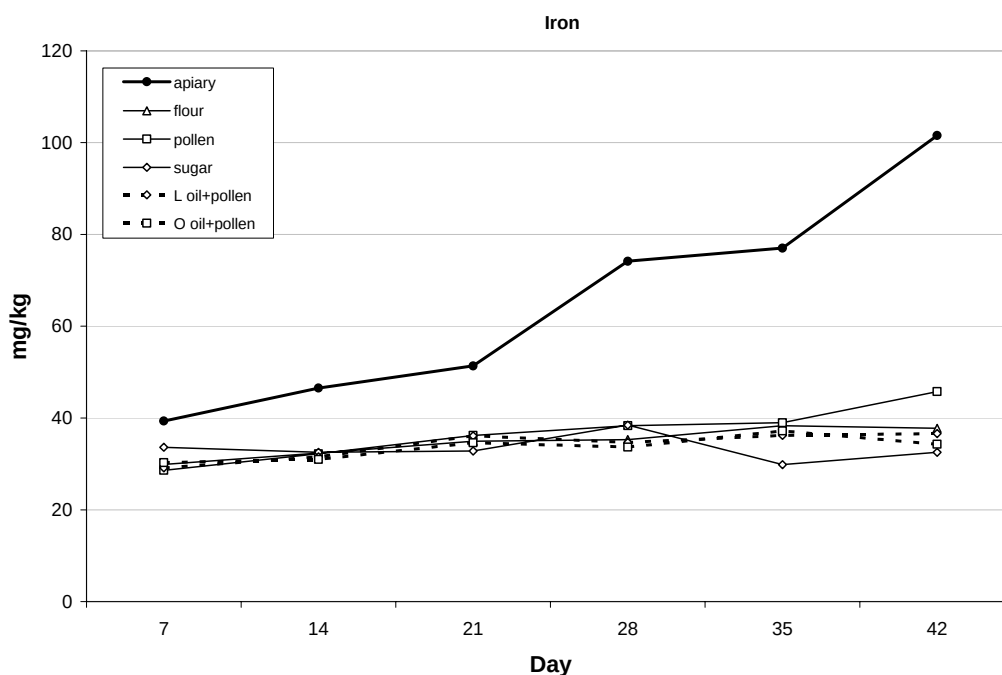


Figure 13-18. The predicted values of the concentration of body content of iron (mg/kg) over 42 days between different diets (5% LSD = 7.99 mg/kg).

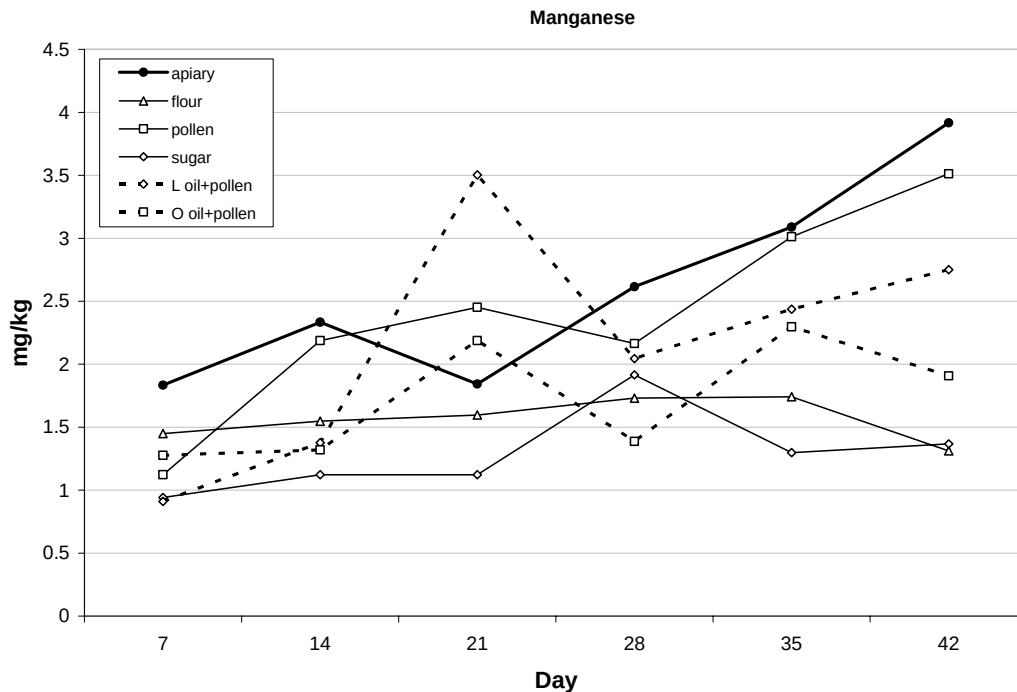


Figure 13-19. The predicted values of the concentration of body content of manganese (mg/kg) over 42 days between different diets (5% LSD = 1.35 mg/kg).

Zinc

There were significant differences in body zinc levels with treatment ($P < 0.035$). The zinc level in apiary bees gradually increased to reach a peak at day 35. At day 21, zinc levels peaked in bees fed pollen and the two oil+pollen diets (Fig. 13-20). The uptake of zinc from the flour and sugar diets decreased sharply after day 28.

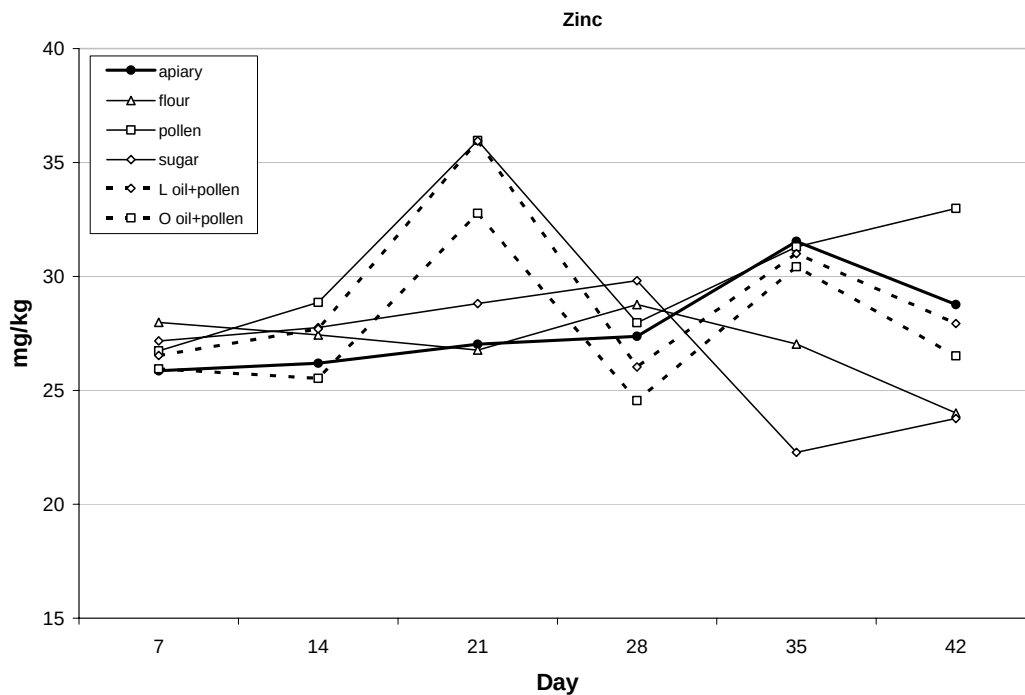


Figure 13-20. The predicted values of the concentration of body content of zinc (mg/kg) over 42 days between different diets (5% LSD = 7.45 mg/kg).

Discussion

Observation of “hairless” phenotype in a caged single cohort of honeybees as a potential indicator of behavioural status.

The data supported the hypothesis that the hairless bees observed in the experiment (see Fig. 1-1, Section 1) were of “forager status”. This indicates that a clear division of labour amongst bees of the same age had developed within the cages (Fig. 10-3, Section 10). The data supports Robinson *et al.* (1989) where single-cohort colonies establish a division of labour within a few days. This is in contrast to what normally occurs in a functional free-flying colony of honeybees. Young bees in these colonies have enlarged hypopharyngeal glands and foragers that have reduced hypopharyngeal gland acini are comparable in size to emerged bees (Lass and Crailsheim 1996). The forager bees identified from cages were likely to have entered the forager caste about day four to six (Schulz *et al.* 1998), day five (Robinson 2003) or day 13 (Robinson *et al.* 1989).

In 1940, Milojevic (1940) recorded a similar observation to that presented in Figure 10-3, albeit in different proportions. At the conclusion of his 70 day experiment involving emerged bees (single cohort), he found 70.6% of the remaining bees had well-developed hypopharyngeal glands (*viz.* heavy head weights); 18.1% had partially developed glands and 11.3% had entirely reduced glands (light head weights). The difference in proportions is perhaps largely due to the different tasks the caged bees were confronted. Milojevic's student used combs of young brood in cages whereas in the experiment (in this chapter) the emerged bees had initially no brood because the bees had to build their combs from supplied waxed plastic foundation. At the time, Milojevic noted that the appearance of social differentiation of individual bees was in sharp contrast to normal bee activity, which is linked to definite age classes. Milojevic's 'forager' caste percentage (11.3%) is similar to levels (5-10%) shown by Giray and Robinson (1994) [cited in Elekonich *et al.* 2001] from single cohort colonies in cages. In this experiment, the percentage of bees with light head weights and in hairless condition, suggesting forager status was 20.8%, a percentage within the range of Milojevic's data.

Since the publication of Milojevic's paper, it has been well established that manipulation of the colony age demography does cause accelerated, retarded or reversed behavioural development of honeybees (Huang and Robinson 1996). In the case where a single cohort of emerged bees are used in cage experiments, behavioural development is retarded because the ageing population structure results in the appearance of 'overaged nurse' bees (see Fig. 10-3). These over-aged nurse bees continue to care for larvae and the queen rather than follow the normal caste-age related development (polyethism) to forager (Robinson 1992).

The ability of the honeybee to perform tasks outside of or switching within their normal (generalised) caste repertoire: nurse bee>house bee>field bee is a response to changes in population structure. Some examples are: establishment of a colony after swarming (bees are ageing and are not being replaced immediately with nurse bees); time of year (e.g. after winter colonies could be broodless, i.e. no nurse bees), food availability (e.g. poor pollen or nectar flows where the queen stops laying), predation (e.g. rainbow birds [*Merops* spp.] which can severely reduce the number of foraging bees) and climate (e.g. drought/rain, which affect food supply).

The method of randomly collecting bee samples from cages could result in the data being too variable due to the very fact that some of the collected bees, could be by the nature of their altered behavioural development, belong to different castes. For example, there are significant differences between nurse bees and foragers for juvenile hormone titres (Huang and Robinson 1996, Robinson *et al.* 1989), hypopharyngeal gland development [responding to protein] (Maurizio 1950, Maurizio 1954), haemolymph proteins such as vitellogenin (Fluri *et al.* 1982), protein synthesis (Knecht and Kaatz 1990), iron accumulation (Locke and Nichol 1992), abdominal lipid (Toth and Robinson 2005) and diet (Crailsheim *et al.* 1992). The effect, considering a couple of these scenarios, is that samples could contain bees with either well-developed glands or poorly developed glands or low and high concentrations of a mineral such as iron.

By noting the physical state of at least one of the age castes in cage experiments, i.e. the hairless bees, then one source of variability to the data could be eliminated and deliver a better outcome of statistical significance. However, the hairy bees alone can also contribute some variability to results. Brouwers (1982) showed that the activity of full-grown hypopharyngeal glands of queens and, same-aged summer bees (young nurses) showed conspicuous variation.

The present results also suggest that oleic acid caused an increase in hairless bee numbers in those cages fed redgum pollen enhanced with this fatty acid. Pollen with normally high concentrations of oleic acid may have a significant influence in terms of ageing/longevity on bee populations in the field. A survey by Manning (2001) showed considerable variation in the percent of oleic acid present in the pollen from a range of plant species.

As far as a viral pathogen is concerned, Gochnauer's (1978) review mentioned that infected adult bees seem to age more rapidly than healthy bees (in relation to sacbrood virus). Rinderer (1974) found that mortality from viral infection to be tempered by the feeding of sugar, which he considered contained ribonuclease that could destroy infectious RNA, as most viruses are RNA viruses (Gochnauer 1978).

Gochnauer (1978) noted that the presence of paralysis viruses in bees did not cause a readily identifiable natural infection and in bumblebees for example, paralysis virus seemed to occur naturally in different species again without any evidence of symptoms. Burnside (1933) noted sick bees may retain their hair or become partially or nearly hairless. In cage experiments by Burnside (1933), some bees inoculated with extract prepared from macerated sick bees showed no difference in death rate or symptoms of paralysis to bees not inoculated, and that this had some relationship to the temperature of the room where the experiment was conducted. I did not notice any of the symptoms Burnside described: *'trembling of the body and wings, sprawled legs and wings and greasy-looking abdomens'*. Kulincevic *et al.* (1973) noticed in their cage experiments (using about 100 bees in each) the disease (characterized by bees attacking sick bees) developed in all cages without any intentional introduction of the pathogen.

There appears to be some inconsistency whether the virus described has an associated connection to the hairless phenotype observed in cage experiments. Our examination supports the view that there is no connection and that the phenotype is a phenomena resulting from cage experiments in which single cohort honeybees rapidly undergo behavioural change. Head weights strongly associate hairlessness with bees being forager status or "older bees" in a same-age colony.

The phenomenon of hairless bees was revealed in the final experiment of a series that determined the longevity of bees fed pollen that was enhanced with fatty acids and the last experiment conducted for this project. The differences in head weight measured from the two phenotypes of bee were significantly different but as a proportion of the population under experiment, the influence of the hairless bees on the data was relatively minor. I would propose that the observation would have had little or no bearing on the outcomes presented. Nevertheless, in future work of a similar nature, it may be worth recording the phenotype differences alongside the other data recorded for statistical purposes.

Longevity of honeybees fed linoleic and oleic acid enhanced redgum (*Corymbia calophylla*) pollen diets; high and low fat soya bean flour diets and lupin and flour diets mixed with redgum pollen.

Discussion

Measuring life-span of emerged bees fed pollen or a substitute feedstuff is one of several ways of evaluating the effectiveness of diets (Schmidt *et al.* 1987). This method worked well in our experiments in determining the value to honeybees of twenty-two different diets.

The addition of different fatty acids to low lipid content pollen such as redgum pollen showed honeybees were far more sensitive to pollen diets containing added oleic acid than those diets enhanced with linoleic acid. Diets containing 2% oleic acid had similar mortality to the pure pollen diets, however at concentrations of 6% oleic acid or greater, mortality significantly increased.

The results suggest that pollen enhanced with linoleic acid up to 6% more than that occurring naturally in redgum pollen was able to support longevity at a level only slightly worse than diets of fresh redgum or crushed and irradiated redgum pollen. However, when used at 10% and 16% in pollen, mortality began to increase. The oleic acid concentration of the 6% linoleic acid+pollen diet was 11.2 mg/g but for the 10% linoleic acid+pollen diet, where mortality increased, the oleic acid concentration was 18.2 mg/g and therefore the level at which the concentration of oleic acid could begin to be a problem for bees (e.g. in substitute feedstuffs) might be within the range: 13.9 mg/g (2%O diet) to 18.2 mg/g. Oleic acid is the predominant fatty acid of adult bees, constituting more than 60% of the total lipid but lower concentrations exist from larval to pupal stages of 40.4% to 45.2%, respectively (Robinson and Nation 1970).

The honeybees have an obvious tolerance of linoleic acid compared with oleic acid which is fortunate as linoleic is the dominant fatty acid (2.77 - 5.81 mg/g) in eucalypt pollen (Manning and Harvey, 2002) or the second most dominant at an average 5.13 mg/g for 577 plants tested (see RIRDC DAW-100A) and in some European plants (Battaglini and Bosi 1968) that beekeepers use. Gilby (1965) noted a few species of butterfly also require linoleic (and linolenic acids) where a deficiency of these fatty acids in insects causes a dramatic failure of pupal or adult ecdysis (Nation 2002).

Of the flour diets, the low-fat Promine™ soya bean flour had the lowest oleic acid concentration of approximately 2.6 mg/g and was the best of the soya bean flours tested in giving bees greater longevity but still less favourable than the pollen diets. The result indicated that the lipid composition of the defatted and full fat flours could be associated with reduced longevity where palatability could have been affected. Of all the diets fed over 42 days, the only feeds to foster brood production were RG, cRG, 2%L, 6%L, 2%O and PRO10, however, other researchers have reported brood production from soya bean flour diets (see Haydak and Tanquary 1942, Standifer *et al.* 1973). There are problems with using soya bean flour as a feedstuff. Haydak (1949) said it caused an 'abrupt impairment of brood rearing' due to niacin being a limiting factor in the soya bean flour.

Generally, adding progressively more pollen to flours reduced the risk of bees dying from soya bean flour diets. An exception was with the lupin flour where the addition of 10% pollen caused a significant shift in the hazard model towards making a lupin+pollen diet a riskier diet. Further testing of lupin as an ingredient is required before any general use in the beekeeping industry can be recommended.

The five diets which had the highest consumption: RG, 2%L, 6%L, cRG and 2%O consumed (Fig. 11-7, Section 11) corresponded to the five diets with the lowest hazard ratio (Fig. 11-6, Section 11). The other diets where low consumption caused an increased risk of bees dying then palatability of the diet could be an issue and an important consideration when adding or using different feedstuffs in diets.

The pollen (cRG) used in conjunction with the flour diets was over six years old and earlier research e.g. (Haydak 1961) showed that pollen of this age was almost worthless for the development of young bees and should not be considered in feed diets for bees. However, it seems that the sterilising process

by gamma-radiation has enabled its 'quality' to be preserved or retained. The crushing of the pollen into a fine powder caused an increase in the concentration of linoleic acid from 2.12 to 15.7 mg/g. It is worth noting that linoleic acid is an antimicrobial lipid (Feldlaufer *et al.* 1993b).

Beekeepers should continue to use cane sugar as a carbohydrate source when artificially stimulating their beehives prior to honey flows or pollination contracts. Invert sugar-fed bees had the shortest life-span (14 days) compared to cane sugar (17 days) which incidentally was lower than the 20 day life-span recorded from sugar diets in experiments by Schmidt *et al.* (1987).

In experiment two, the inside walls of cages showed spectacular evidence of faecal streaking from bees fed fungal-contaminated cane sugar syrup, a characteristic usually associated with honeybees infected with the parasite *Nosema apis*. No nosema spores were found in bee samples in our experiments and our finding is supported by El-Shemy and Pickard (1989) who never found *Nosema apis* spores in worker bees less than eight days old. As the bees in this experiment were all newly hatched and had no contact with older bees, the faecal streaking observed was certainly linked to fungal elements in the sugar syrup.

Fungi can cause mortality in honeybees either as a result of nectar fermentation or from mycotoxins. In Brazil, *Candida guilliermondii* was identified as the cause of high mortality among larvae and pupae in beehives (Portugal *et al.* 1995). *Candida* species have been associated with honeybees from other parts of the world. *Candida mogii* (the imperfect form of *Zygosaccharomyces rouxii*) has been identified from fermented German honeys (Schneider *et al.* 2003).

Excess moisture (60-90%) in most honeys commonly leads to fermentation by yeasts (Smith *et al.* 1969), which infer they are commonly present in honey. In the field, nectar in flowers, especially over wetter (winter) months when the humidity is high, would also ferment. Under these circumstances, and based on the rapid death of bees observed in this experiment, some degree of mortality in feral and managed colonies of honeybees must occur as fermented nectar is consumed. Bees often give the appearance of being 'drunk' on the ground under flowering trees at a certain time of the year (Birtchnell *et al.* 2005). Yeasts and *Penicillium* spp. are dominant mycobiota on pollen (Gonzales *et al.* 2005, Gilliam and Prest 1972) and some isolates of *Aspergillus niger* (identified contaminant in the cane sugar diet in experiment 2) can produce a mycotoxin known as ochratoxin (Gonzales *et al.* 2005), which may have contributed to the high mortality measured.

Beekeepers use descriptive terms such as 'dysentery' or 'spring dwindling' to describe the unexplained rapid loss of bees from their hives. Coincidentally, winter flowering species on which Australian beekeepers usually place apiaries are known for these phenomena. For example, karri (*Eucalyptus diversicolor*) after winter, 'a few colonies had an attack of dysentery during winter' (Hall 1929); bees were 'lacking in stamina' (Hall 1932). Lang (1908) experienced 'dysentery' after 'flowers were coming out in bloom (but) it kept on raining' and Armstrong (1985) noted that '(NSW) we wintered bees on (mugga) ironbark which was a risky thing to do because of winter mortality is often very high on this tree.' A similar phenomenon is experienced by beekeepers with winter wandoo (*Eucalyptus wandoo*) in Western Australia. In South Australia, excess moisture inside the hive in winter was associated with 'spring dwindling' (Willoughby Lance 1936).

All these observations, which are only a few of the many reported and published over the years, could in fact be records of the impact yeasts have had on honeybees or indeed on native insects. The yeast-honeybee relationship is little studied and further research in this area is likely to give some of these anecdotes some scientific base.

The longevity experiments demonstrated that honeybees are more tolerant of linoleic than oleic acid as concentration increased in diets. As lipid concentration increased in diets their consumption decreased. The decreased consumption would restrict the amount of protein available to the bees that would be required to stimulate the development of the hypopharyngeal gland and hence the ability of the larvae to be fed. Head weight measurements were taken of bees sampled weekly during the longevity experiment and are presented in Section 12 (above) together with the effect of the 22 different diets fed.

The effect of diet on head weights (hypopharyngeal gland) of honeybees

- A. LINOLEIC AND OLEIC ACID ENHANCED REDGUM (*CORYMBIA CALOPHYLLA*) POLLEN DIETS**
- B. SOYA BEAN AND LUPIN FLOUR DIETS AND MIXES CONTAINING REDGUM POLLEN**

Discussion

The peak in head weights at seven to 14 days of emerged bees, marked and returned to a hive in an apiary corresponded to the period when the hypopharyngeal gland reaches maximum size (Dade 1962) and maximum weight (Fluri *et al.* 1982), where acini diameter peaks (Crailsheim and Stolberg 1989) and where gland activity is elevated (Huang and Otis 1989). The gland is the largest in the head cavity (Wang and Moeller 1969) averaging 8.50 mg to 9.94 mg in weight (Pickard and Kither 1983) therefore the use of head weights to monitor change in hypopharyngeal gland should be a legitimate way of assessing the probable impact of feedstuffs on honeybees. Certainly, in experiments conducted so far, some feedstuffs did show an increase in head weight where some concomitant development of the hypopharyngeal gland occurred.

Measuring head weights would be a practical way of assessing artificial diets and different pollens other than measuring honeybee longevity. If a diet did not cause an increase in head weight from day seven in cage experiments then the feedstuff was not suitable despite each of the diets capability of varying life-span. The head weight of emerged bees is an important measurement prior to any experimentation and should be included in subsequent statistical analyses. De Groot (1953) noted there was considerable variation in newly emerged bees and this variation was an important factor if homogenous material is required for experiments.

Enhancing redgum, a high protein pollen with two different fatty acids showed for the first time that particular fatty acids, at certain concentrations can illicit a response from the hypopharyngeal gland, either beneficially or deleteriously. Those diets that elevate head weight sufficiently could lead to the brood being adequately fed to allow larvae to develop and, for some of the diets, for larvae to pupate and later emerge.

Linoleic acid was more benign than oleic acid. The concentration of oleic acid that limited head weight increase and brood production was confined to about 2% (13.9 mg/g) in a diet. For linoleic acid enhanced diets (2% and 6%), the concentration of linoleic acid could be as high as 28 mg/g, which also contained oleic acid at 11.2 mg/g (see Table 7-1, Section 7) and where brood developed (Table 12-4, Section 12). The data suggest that oleic acid concentrations greater than 14 mg/g could be detrimental to the activation of the hypopharyngeal gland.

Of the flour diets, the only diet where eggs were produced was from the Promine™ plus 10% cRG pollen which was low in oleic acid (0.5%). It may not mean that the low concentration of oleic was responsible for egg production because the queen may have responded to the high protein content of the feedstuff. However, it does not explain why the defatted soya bean flour (DF), a diet also low in oleic acid (and high in protein), caused the queen to lay no eggs. For full-fat soya bean and lupin flour, the oleic acid concentration exceeded the proposed 14 mg/g limit and may be the reason why the hypopharyngeal gland did not develop sufficiently to allow feeding of the larvae or illicit a reproductive response from the queen.

Soya bean products contain a relatively large number of antinutritional factors such as compounds classified as protease inhibitors, phyto-hemagglutins (lectins), urease, lipoxygenases and antivitamin factors which are normally destroyed by heat or by more elaborate processing (van Eys *et al.* 2004). A protease inhibitor, soya bean trypsin inhibitor when feed to bees (0.1%-1%) reduced hypopharyngeal gland protein content, midgut proteolytic enzyme activity and caused low survival of bees (Sagili *et al.* 2005). Soya bean trypsin inhibitor fed to newly emerged bees for 10 days significantly reduced the

mean weights of the hypopharyngeal gland and the mean diameter of the glands' acini (Babendreier *et al.* 2005). Of the two soya bean flours used in the experiment, full fat was processed from pure dehulled beans whilst the defatted flour was processed by expeller and solvent extraction. Only the defatted flour would have been subjected to a heat process during the desolventizer process and this would have inactivated any anti-nutritional factors. Whether there was any underlying influence of trypsin inhibitor on bees fed the diets in these experiments is not clear.

However, the use of both soya bean flours, whether defatted or full-fat, or lupin flour did not offer any benefit by way of increasing head weights, even when pollen of up to 30% was added to the diets. Only the flour with the lowest fat concentration, Promine™ showed some promise. It is a protein concentrate from soya bean and the high protein concentration (62.4%) is no surprise but with a high protein content there is a low lipid concentration, and in processing, the least amount of trypsin inhibitor is retained (van Eys *et al.* 2004) (Table 7-2, Section 7).

The general lack of response of head weight growth to flour diets was similar to that found by Herbert and Shimanuki (1977) and it could be related to the lack of vitamins. Haydak (1949) found soya bean flour diets improved brood rearing by the addition of two vitamins, niacin and riboflavin, indicating some associated hypopharyngeal gland development. The soya bean flour process might also cause vitamin loss.

Beekeepers have observed that bee colonies perform better on one source of pollen but not another and the same pollen source may not have the same effect on bees each time it becomes available (Somerville 2000b). Beekeepers have also gained knowledge of poor-quality pollen by watching the performance of their bees which was later borne out in experiments of longevity (Schmidt *et al.* 1987). Pollen analyses in these cases showed pollen to be low in protein because of a large amount of oily external pollenkitt. Pollen quality (good quality = high protein) and quantity affect bee longevity and development of the hypopharyngeal gland.

In experiments by Standifer *et al.* (1970), diets containing 34% dandelion (*Taraxacum officinale*) pollen were no more effective in mean gland development ($P < 0.05$) than diets containing 10% saguaro pollen (*Carnegie gigantea*) or 10% gumweed (*Grindelia squarrosa*) pollen despite there being differences in dietary protein of 5.13%, 2.02% and 1.38%, respectively. But even if the diets were fed in such a way as to have the same nitrogen (protein) value in the diet, they may not develop hypopharyngeal glands of similar size (Standifer *et al.* 1970).

Perhaps the only component of pollen that could be causing variability in the development of the hypopharyngeal gland are the fatty acids. Any effect from minerals has been discounted (Herbert and Shimanuki 1978b). Singh *et al.* (1999) showed clear evidence of inhibitory effects of sunflower (*Helianthus annuus*) pollen lipids to the gustatory (feeding) response of bees when compared to an identical lipid concentration from rape (*Brassica campestris*). The suggestion was that it was the individual fatty acids in the pollen (like oleic acid for example) that could be responsible for differential stimulatory or inhibitory effects in bee gestation, which would ultimately impact upon hypopharyngeal gland development (and therefore head weight).

The sensitivity of fatty acid concentration in pollens shown in the experiments conducted here reveal that as well as concentration, composition of fatty acids in diets will be an important consideration. Standifer (1966b) found that dandelion pollen is highly attractive to bees, and is excellent for brood rearing despite its unusually high percent of total lipid (7.3%). From Table 7-1 (Section 7), this 7.3% total lipid is close to the limits that coincide with a nil response of head weight gain when oil was added to pollen. For example, the total lipid content of the pollen + linoleic acid diet appears to have a physiological limitation at about 11.1% (i.e. the 10%L diet) which still enabled some head weight increase whilst the limit for the pollen + oleic acid diet is somewhere greater than 1.1% but less than 7.2% (the 6%O diet). Standifer's observation that 'excellent brood rearing' occurred from a diet of dandelion pollen is in stark contrast to research by Herbert *et al.* (1970) who found bees fed dandelion pollen diets were unable to rear brood. Illustrating how important lipids are in diets was an experiment by Haydak and Tanquary (1942) who fed bees a solvent-extracted soya bean flour diet containing skim

milk powder (total lipid 1.5%). They found the diet caused a higher mortality than when it was restored and retested with a crude oil extract to 5.5%. Herbert *et al.* (1970) discovered why the dandelion pollen in their experiments was unable to rear brood. When fortified with L-arginine, the diet did result in complete brood rearing. This indicates that besides fatty acid composition, a diet high in lipid concentration can adversely affect the protein concentration and therefore amino acid composition.

Interestingly, and without apparently conducting any research, Stace (2005) recommended the addition of 6% by weight of vegetable oil (cottonseed or soya bean) to artificial bee feeds when apiaries were on Eastern Australian eucalypt pollen flows. This was probably a calculated guess after reviewing the lipid averages of pollen from various parts of the world which appear to be about this level. As vegetable oils have fatty acid compositions that vary considerably, caution should prevail until further research is conducted.

Herbert and Shimanuki (1977) found the same diets they tested reared brood to the sealed stage one year and not the next. The year to year variability could be due to the significant seasonal effect on the head weight (viz. hypopharyngeal gland) of emerged bees. De Groot (1953) reviewed a number of German language scientific papers that stated life-span and physiological condition (nitrogen content and dry weight) of bees were influenced by season. Kunert and Crailsheim (1988) found seasonal differences in fresh weight, protein, triglycerides and fats of emerged bees. After standardizing our data to remove the effect of season and experiment on the head weights of emerged bees, *season* and *experiment within season* was still found to have an effect on both growth rates of apiary bees and those bees treated with one of the diets. The effect of diet on the head weights was found to be dependent on the season, making comparisons between diets a complex matter.

Head weights were only measured from surviving honeybees and as diet was also found to have an effect on the survival of bees, it is possible that the mean head weights of the surviving bees may not be representative of the whole sample (dead and alive bees). The probability of death may be related to head weight and this also needs to be further explored.

The use of soya bean flour in honeybee diets has been promoted for many decades. The results of measuring head weight has found that as a feedstuff, it had no beneficial influence over the hypopharyngeal gland development despite being useful in increasing honeybee longevity when there was no other source of protein. There is an underlying problem with soya bean flour as a dietary food for honeybees and this was explored in Section 13 (above) by measuring 20 elements such as protein, lipid, seven minerals and 11 fatty acids contained in the body of honeybees. The effect on body nutrition of enhancing pollen with fatty acids and feeding honeybees only sugars was also studied. Bees from a hived colony that foraged in the natural environment were also examined and used as a comparison to gauge the effect on bees of being 'force-fed' diets in cages.

Nutrient composition of honeybees.

- A. RESPONSE TO DIETS OF LINOLEIC AND OLEIC FATTY ACID ENHANCED RED GUM (*CORYMBIA CALOPHYLLA*) POLLEN
- B. RESPONSE TO PURE DIETS OF SOYFLOUR (HIGH, MEDIUM AND LOW FAT) AND LUPIN FLOUR

Discussion

Body protein in the caged honeybees rapidly increased in the first seven to 14 days after emergence as bees become nurse bees, comb building bees or bees processing nectar. A similar trend was observed for lipid, copper, iron, manganese and sulphur. In this early period of development, emerged bees undergo physical change characterized by a rapidly developing hypopharyngeal gland which, when fully developed, occupies a substantial proportion of the head cavity.

The hypopharyngeal gland produces the food for the worker bees and the queen and therefore a suite of nutrients must be available as precursor chemicals to the manufacture of worker and royal jelly that is fed to larvae. Haydak (1970) found that at 12 ± 2 days of age, nurse bees produced a milky-opaque secretion and at 17 ± 2 days of age a watery-clear secretion which may indicate a change in requirement of some nutrients.

During this early period of adult life, wax is also produced. Wax from the ectodermal glands builds the comb structure of the colony and was found to be dominated by palmitic acid at a high concentration of 106 to 132 mg/g (Table 13-1, Section 13). Gilby (1965) states beeswax consists, mainly of mixtures of long-chain alcohols (C24 to C34) and paraffins (C25 to C31) and esters of these two structures. Palmitic acid is a much shorter chain carbon, C16:0.

Zinc and phosphorus accumulated more slowly in the body of the bee than all other nutrients measured. Potassium levels, in general, declined in the two week period after hatching and these elements are probably not intrinsically linked to the processes involved in the development of the hypopharyngeal gland.

A number of the fatty acids accumulated rapidly in the bees' body over the same period as the hypopharyngeal glands develop and these included palmitic, palmitoleic, linoleic, linolenic, Unk5, Unk6 and lignoceric acids. Stearic acid accumulated less rapidly.

There is a substantial amount of lipid depletion from honeybees before bees commence foraging. The lipids are mainly metabolized during nest work activities such as nursing, feeding nestmates or producing wax. Foraging honeybees rely on carbohydrates and not stored lipid for energy when flying (Toth and Robinson, 2005). Therefore, a variety of fatty acids are likely to be important components in any of the nurse bee's activities.

A few fatty acids are probably not involved in hypopharyngeal gland growth and function. Myristic acid is a candidate, as the highest concentration was in emerged bees, which declined rapidly after hatching and remained low as bees aged in both cage and apiary treatments, indicating that this is a normal trend. The Unk3 fatty acid also declined in apiary bees after hatching but then increased after day 14. In the cage experiments, the accumulation of Unk3 was opposite to apiary bees and may be as a result of confinement in a cage.

Honeybees in cages showed some similarities in nutrient concentration over the duration of the experiment to bees raised in an apiary. However, some fatty acids behaved differently. The concentration of the stearic acid collapsed within seven days of hatching in cages which was in contrast to bees from the apiary. Similarly, linolenic acid accumulation in body tissue of caged bees was lower than that acquired by bees from the apiary.

In the apiary, the newly emerged bees that were placed in a colony in the apiary had access to other young bees with fully developed hypopharyngeal glands and had access to older bees where through

trophallaxis (the transfer of food by mouth from one individual to another) there may be a critical chemical imparted to the newly hatched bees that enabled stearic acid to be accumulated from the supplied diet. Nurse bees, when nine days old, preferentially donate jelly to their younger nest mates (Crailsheim 1998) and the proteinaceous jelly produced by 100 nurse bees within one night could be found in 15.6% of the colony population (Crailsheim 1992).

Palmitoleic acid concentration was higher in caged bees than emerged bees reared in the apiary. Bees fed diets of flours or just sugar accumulated high levels of palmitoleic acid in body tissue. Diets of sugar were capable of increasing lipid levels in body tissue and bees were able to do this at similar concentrations to bees fed diets of pollen or flours. Linoleic acid was not involved in the suite of fatty acids that made up the overall increase in body lipid from sugar diets, nor was it available from flour diets despite its widely varied but high concentrations in the flours tested.

The lack of accumulation of linoleic acid by bees from flour diets is a new discovery and is perhaps the critical factor that has been missed in artificial diets that have been based on soya bean flours. For example, soya bean flour has been a widely used substitute for pollen and has been mixed with dried brewers yeast and dry skim milk (Haydak 1970). By itself, soya bean flour is noted to have some deleterious effects as shortening life-span and only a slight effect on activating the hypopharyngeal gland and fat body (Maurizio 1950). Haydak (1949) had proposed that a deficiency of the vitamin, niacin was the main problem with soya bean flours, nevertheless, at the time Maurizio (1950) said her observations agreed with observations in other countries of the 'occasional failure of soya-bean flour feeding'. DeGroot (1953) found there was a nutritional surplus of the essential amino acids except methionine in soya bean flour which 'seemed somewhat deficient'. Despite numerous attempts at supplementing flours with all sorts of additives over the years, diets of pure soya bean flour are still being used by Australian beekeepers (Somerville 2005a).

The data (Fig. 13-9A) clearly showed that perhaps either internal mechanisms within the bee are blocking the uptake of this essential fatty acid from flour diets or there is still a component in the flour that is deleterious to membrane transport of linoleic acid. The fatty acid source in the flour may be in a form that is nutritionally unavailable. Lipid uptake/conversion from sugar excluded linoleic acid. A carbohydrate source may not be a precursor for this fatty acid even though it is capable of being able to allow bees to increase body lipid (Fig. 13-2).

Despite being totally different diets in terms of protein and lipid content, the similarity of the response to the flour and sugar diets (Fig. 13-9A) is scientifically important and warrants further research.

Remarkably, protein levels were also increased when bees were fed sugar diets and levels remained above 20% of body weight for the six week experiment. Haydak (1937) showed a similar result and surmised that his increase in percent body nitrogen on a protein-free diet was due to the depletion of stores of fat and glycogen acquired prior to hatching. Lipid concentration rapidly fell after day 28 from 4.63% to 1.45% but protein levels only fell from 22.1% to 20.4% which tends to support Haydak's research that bees were 'mining' their own bodies for any short fall in nitrogen from the fat bodies in the abdomen.

Herbert *et al.* (1977) noted that honeybees seemed unable to utilize high protein diets, but the highest protein (low lipid) diet (Promine™; 62.4% protein) tested was able to give bees greater longevity than soya bean flour which contained less protein and higher amounts of lipid. Promine mixed with 10% pollen was the only flour that encouraged the queen to lay eggs. This was perhaps due to the much smaller negative change in head weight (relative to pure pollen diet) associated with the Promine diet when compared to the other flour diets. The use of Promine by bees is perhaps due to the massive requirement of nitrogen, which increases by 93% in the heads of bees during the first 5 days from emergence (Haydak 1934). Interestingly, in contrast to Promine's high protein content, Herbert *et al.* (1977) found that optimal brood rearing was achieved in diets that contained between 23 and 30% protein.

Several research papers note that high protein diets are detrimental to honeybees. De Groot (1953) coined the term 'protein poisoning' to describe observed reductions in longevity. However, the protein sources were added to liquid sugar, generally at levels less than 10% and not fed as dry ingredients. Nevertheless, it is a problem for other bee species. High protein (66%) soya bean powder used in a pollen diet, when fed to sweat bees (*Lasioglossum zephyrum*) did cause high mortality and provisioning of the nest decreased as both dietary soya bean powder in the diet and dietary protein increased (Roulston and Cane 2002).

Caged bees accumulated most minerals at similar rates to apiary bees with the exception of iron and manganese. Both these elements showed significant differences. Iron accumulation in caged bees was poor when compared to bees from the apiary. As the bees aged, iron accumulated in body tissue in bees from the apiary. The difference may be due to apiary bees having access to the outside environment and sunlight. Their associated requirement of iron for orientation and directional flight is well known (Herbert 1997).

Manganese produced a similar response to linoleic acid in that bees fed on flour and sugar diets failed to accumulate this mineral to levels in bees fed pollen. The similarity of linoleic acid and manganese in poor uptake, may mean there is some connecting relationship between these two elements within a chemical pathway of the bee. These experiments indicate that Manganese and linoleic acid need to be added to artificial diets in a form as yet unknown and is perhaps why the flour diets performed poorly with respect to life-span, longevity, hypopharyngeal gland development (head weight increase) and brood development.

The analysis of minerals of bees and their nutritional requirement is poorly understood (Herbert 1997). It appears that the addition of minerals to artificial feedstuffs can improve the ability of bees to rear brood (Nation and Robinson 1968). Herbert and Shimanuki (1978b) recommended a diet formulation for feeding bees should contain 1000 mg/kg (0.1%¹) potassium; 500 mg/kg (0.05%) calcium; 300 mg/kg (0.03%) magnesium and less than 50 mg/kg (0.005%) each of sodium, zinc, manganese, iron and copper. Any diet that contained mineral concentrations of four or eight times greater than these recommended levels suffered high mortality although bees still reared brood. The data presented in this chapter provides evidence that mineral uptake in bees can be significantly affected by diet components and it is recommended that this area of nutrition should be further studied.

Single-cohort colonies such as those used in this experiment establish a division of labour within a few days producing bees of the same age performing different tasks (Robinson *et al.* 1989). Toth and Robinson (2005) showed that nutritional differences are more closely related to behavioural state than to age. Thus the way the experiment was conducted, using same aged bees could have influenced any result of measured nutritional change because of pronounced behavioural change. For example, Toth and Robinson (2005) found there are significant differences between lipid stores of nurse bees (mean values >1.4 and <2.3 mg) and foraging bees (mean value <1 mg) though they found no significant lipid differences between honeybee foragers and reverted nurses (bees of foraging age placed in cage experiments that revert to being nurse bees).

Examination of the nutrient components of honeybees fed diet mixtures was able to identify two possible components that may have affected head weights of bees. Bees fed flour diets had head weights that should have increased, as the hypopharyngeal gland developed. The poor uptake of linoleic acid and/or manganese from flour diets needs to be corrected by supplementing flour diets with these elements. Further cage tests would be able to verify this finding.

In cage experiments, pollen diets provided the high concentrations of protein, Unk3 acid, stearic acid, linoleic acid, linolenic acid, Unk5 acid, Unk6 acid, manganese and zinc in bodies of bees which may have improved their longevity and life-span and increased head weight response.

Some fatty acids were identified to be available from pollen only and again the importance of these acids such as Unk5 and Unk6 is not known and needs further research.

¹ % concentration was used in this thesis for the macro-nutrients.

Beekeepers that extensively feed sugar to honeybees with no additional supplements of pollen, for example, are not providing any source of linoleic acid to the colony. Again this needs further research and testing to determine a method of mixing a fat and a sugar solution (both being incompatible in solution) that can be fed to honeybees.

General Discussion

Enhancing low lipid pollen with fatty acids was hypothesized to improve honeybee longevity. A range of concentrations of linoleic and oleic acid were added to redgum pollen and these diets were trialled in small cages each containing c. 1400 bees. A range of soya bean flour diets with low, medium and high lipid levels and flour milled from lupin were also trialled.

Honeybees fed diets of redgum pollen had the lowest mortality of twenty-two diets tested for six weeks and had a life-span greater than 42 days. The lowest life-span (50% mortality) of 14 days was from bees fed invert sugar. A diet of cane sugar enabled 50% of the population to survive 17 days. Linoleic acid mixed with a redgum pollen diet in concentrations of greater than 6%, limited life-span to 24-25 days, whereas bees were more sensitive to oleic acid where life-spans decreased to 15-21 days when pollen diets had concentrations >2%.

Of the flours, the soya bean flour protein concentrate with the lowest fat content (0.6% lipid) gave bees a greater life-span of 26 days whereas bees fed a defatted (solvent extracted) and full-fat soya bean flour had lesser life-spans of 19 and 20 days respectively. Bees fed on lupin flour had a slightly better life-span (23 days). Adding pollen to lupin flour caused an increase in mortality but for the other flours, an addition of pollen to soyflour was beneficial.

Overall, the results showed the deleterious effect of oleic acid was greater on honeybees when used in high concentrations in pollen diets whereas honeybees were more tolerant of high concentrations of linoleic acid in diets. In terms of longevity and life-span, pollen that had been crushed and irradiated and several years old was as good as fresh pollen in tests conducted.

Honeybees with greater life-spans were those bees that consumed more of the diet presented to them (Table 18-1). As the oil content of the pollen or soya bean flour increased, less of the diet was consumed which may have compromised the bee's dietary protein requirement and issues with sensory attractiveness and palatability of the feedstuff arise.

Increasing concentrations of individual fatty acids influenced hypopharyngeal gland development but the overall effect is that when total lipid concentration is increased, a reduction in protein concentration occurs which also affects gland development. Fatty acid enhanced pollen diets that contained up to 2% oleic acid increased head weight greater than diets containing 6, 10 or 16% oleic acid, whilst the head weight response to added linoleic acid was greater from diets with concentrations up to 6% which had a relationship to diet consumption (Table 18-1). Bees fed those diets had an increased head weight and were the only colonies able to feed larvae hatched from eggs that the queen had laid on the comb. This head weight response to test diets could be developed as a measure of hypopharyngeal activity and in this way artificial diets can be further refinement.

None of the fatty acids added to pollen caused head weights to exceed those of bees fed pure pollen diets. Therefore, there is no advantage, in respect of hypopharyngeal gland development, to adding oleic and linoleic acid to pollen in an effort to improve the nutritional value of redgum pollen. In the parameters measured (longevity, head weight, brood), diets enhanced with 2% linoleic acid showed no difference in rankings to pure pollen diets (Table 18-1).

Diets based on flours of soya bean and lupin, when compared to pollen diets, corresponded to honeybees having lighter head weights. Soya bean flour diets, whether low, medium or high fat, showed differences in head weight at day seven and in growth rate during the six week experiment. But in terms of head weight growth rate, the defatted soya bean flour was the only flour significantly different to the

protein soy concentrate Promine™ which had an overlapping similarity matrix for growth rate and diet with the full-fat soya bean flour diet despite large differences in fat content. For diet alone, the similarity matrix showed a significant difference between full-fat and defatted soya bean flour on head weight.

Honeybees readily consumed the defatted soya bean flour which subsequently decreased life-span and head weight growth indicating that as a feedstuff ingredient it is a poor choice. However, the exception was when 30% pollen was incorporated into the flour diet which improved life-span and head weight growth (Table 18-1).

The effect of diet on head weight and on the growth rate of the head weight was found to be dependent on *season* and *experiment within season* which meant that a diet that may have a certain effect over summer, for example, may have a different effect during a different season, making comparisons between the diets a complex matter.

Table 18-1. Summarised ranked data from Section 11. (diet consumption, diet hazard) and Section 12. (head weight growth, number of cells with eggs and larva).

Diet	Consumption of diet	Diet hazard-life-span	Head weight growth	Breeding (eggs/larva)
RG	1	2	2	2
2%L	2	3	1	3
6%L	3	4	3	4
CRG	4	1	5	1
2%O	5	5	4	5
DFSOY	6	16	21	
DFSOY10	7	15	12	
DFSOY30	8	10	7	
DFSOY20	9	13	18	
10%L	10	7	10	
PRO	11	8	8	
6%O	12	11	6	
PRO10	13	9	9	6
LUP	14	14	15	
16%L	15	6	14	
FFSOY	16	17	11	
FFSOY10	17	12	19	
LUP10	18	19	16	
10%O	19	18	20	
16%O	20	21	17	
CS	21	20	13	
INV	22	22	22	

Same aged bees with characteristics of 'hairlessness' and 'hairy' were observed after conducting a series of single cohort cage experiments. These hairless bees were phenotypically similar to forager honeybees in apiaries. Measurements showed hairless bees had significantly lighter head weights ($P < 0.001$) than hairy bees (normal looking nurse bees). A hairless-black bee viral syndrome was described in the literature in 1978 but following an examination by a veterinary pathologist at a viral laboratory, no viral particles were seen associated with the phenotype described in this experiment. Despite differing diets fed to honeybees containing various concentrations of fatty acids, the head weights of the two types of bees responded similarly. A trend was found of increasing head weight following a pollen diet containing 6% linoleic acid enhanced pollen diet but a reversed trend in head weight after the addition of oleic acid at the same concentration. There were significant differences ($P < 0.001$) in head weight between diets containing linoleic or oleic acid and to fatty acid concentration in the diet.

The phenotype phenomenon was assumed to have had no influence on the outcome of the experiments conducted in this project. However, oleic acid-enhanced pollen caused bees to behave differently to those bees fed linoleic-enhanced diets. Increased numbers of hairless bees in small caged colonies resulted and may have implications in colonies when bees collect pollens containing high concentrations of oleic fatty acid.

In Western Australia, emerged bees raised in winter (June-August) for experiments had the highest mean head weight with bees born in summer (December-February) having the next highest weight, followed by bees born in spring.

Accidental fungal contamination of sugar diets in one experiment caused high mortality of bees and this may have implications in the context of fungal contamination of nectar in flowers where bees might be subjected to the same effect. During the onset of winter, high levels of moisture in the air may dilute nectar sugars from flowering plants and cause fermentation.

Of the twenty nutritional components measured, sixteen within the bees' body were significantly influenced by diet ($P < 0.04$). Those influential were protein, fatty acids (palmitic, palmitoleic, Unk3, stearic oleic, linoleic, linolenic, Unk5, Unk6, lignoceric) and minerals (sulphur, copper, iron, manganese and zinc). Palmitic acid was found to be the dominant fatty acid found in wax scales produced by bees in cages. Those elements unaffected by diet diversity were: lipid, myristic acid, potassium and phosphorus. Three unidentified fatty acids [those exceeding 0.10 mg/g in body concentration] were found after pollen and flour diets were fed.

Both linoleic acid and manganese may need to be added separately to artificial feedstuffs for honeybees and may need to be supplied in a dietary form, yet to be determined. Artificial diets that used flours (soya bean/lupin) failed to impart linoleic acid to honeybees, even though the diets themselves were high in concentrations of linoleic acid. Bees fed sugar diets also failed to accumulate linoleic acid even though the diet increased total body fat content. Manganese was also identified as being poorly absorbed in the bees' body after consumption of the flour diets.

There were differences between some of the nutrient components of emerged bees placed in cages and those returned to a normal functional hive in an apiary. The components that showed differences between caged and non-caged bees were the fatty acids: Unk3, linolenic and lignoceric acid and iron.

There is an obvious need to further research the effects of dietary substances on honeybees in relationship to their actual nutritional requirements. Despite decades of use, soya bean flour as an ingredient, has clearly not been researched thoroughly. Soya bean flour is an example of one ingredient of the many used by the beekeeping industry and I suggest that far more research is required to be conducted on the basic parameters, such as longevity and hypopharyngeal gland development, that would give an indication of any ingredient's nutritional value to honeybees.

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