

**EFFECT OF FEEDS IN DEVELOPING THE
HYPOPHARYNGEAL GLAND OF
NOSEMA-FREE NURSE BEES FOR ESCORTING
QUEEN HONEY BEES (*Apis mellifera*) DURING
EXPORT**

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DECLARATION

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

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The experimental work was carried out at the Department of Agriculture and Food,
Government of Western Australia in South Perth

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'1 down, 276 to go...God give me strength...'

ABSTRACT

In 2006, Japan was one of the markets that contributed to the 4 million dollars live bee sales in Australia. In 2007, the export of queen honey bee from Australia was suspended when *Nosema apis* was detected in the nurse bees that were escorting the queens during a quarantine inspection off the Japanese ports. *Nosemosis* or nosema is a worldwide endemic disease caused by *N. apis*. Infected bees have reduced life span, energy, productivity, and develop deformed glands. As a result of this suspension, many Japanese fruit farmers had to hand pollinate many of their crops while Australian beekeepers suffered financial losses from the loss of trade. Thus, it will be extremely beneficial for both Japanese and Australian farmers if nosema-free nurse bees could be produced and used as escort nurse bees.

Two novel approaches of producing nosema-free bees are proposed: (1) treating infected nurse bees with heat therapy and (2) raising newly hatched nurse bees (HNB) in isolation of infected hives. These approaches may produce nosema-free escort nurse bees. Escort nurse bees feed royal jelly to queen bees in cages during the export journey, thus it is essential that HNB can synthesize and secrete royal jelly. This means that HNB need to develop functional hypopharyngeal glands (HG) in order to secrete royal jelly. Proteins found in pollen are said to be 100 % effective in stimulating full development of the HG. When bees have fully developed and functional HG, they should be able to feed and keep queens alive for the duration of the exporting journey (more than 8 days).

This project investigated whether nosema-free escort nurse bees could be produced by raising them in cages; whether feeding pollen and artificial feed could stimulate full development of the HG; whether there is a relationship between how much bees ate and the extent the HG development; whether the cage-raised escort bees are able to feed and keep 100 % of the queen bees alive during export for at least 8 days; and whether applying heat therapy could cure infection caused by *N. apis* without killing the bees.

Bees fed nutritionally balanced artificial feeds designated as Sub2, FB, Sub1 had significantly higher head weights (>10 mg) than those fed pollen feeds used in this experiment. Only Sub2 maintained a consistently higher head weight compared to hive-raised mature nurse bees that were fed icing sugar. Bees that consumed PrSub and Sub1 showed a significant ($P < 0.05$) positive correlation between feed consumption and the head weight of individual bees.

Despite carefully isolating combs from hives, newly emerged bees became contaminated with *N. apis* so nosema-free nurse bees could not be produced. The cage-raised nurse bees fed 16 different pollen and artificial feeds were not able to keep 100 % of the queen bees alive during export for 8 days. Bees that were fed pollen and artificial feed had median survival time of 4 days or less. Queens caged with nurse bees that fed on icing sugar survived much longer than queens caged with nurse bees that fed on pollen and artificial feed. In general, mated queens had significantly longer median survival time than virgin queens.

These findings suggest that protein consumption is not the key factor which regulates the development of the HG. Cage-raised nurse bees suffer abnormal behavioural and physiological developments because of possible lack of appropriate stimulations, hence their inability to properly nurture and feed queen bees. It is clear that cage-raised bees are fundamentally different from hive-raised bees, and so far, the only way to ensure bees develop properly is by raising them in hives. However *N. apis* spores are in virtually all hives, that means to truly produce nosema-free bees, research should focus on breeding naturally resistant nurse bees or genetically modify bees to become resistant to *N. apis*.

For decades, beekeepers and researchers have tried to formulate artificial feeds which can substitute pollen. Bees need pollen for protein to build and strengthen hives but pollen is expensive to buy and its availability is unpredictable. To prove that a nutritionally balanced artificial feed such as Sub2 can replace pollen, further research should be conducted in larger cage experiments measuring nutrition composition, longevity and brood rearing.

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ABBREVIATIONS

Bees developed for seven days on mix pollen feed	7DAY
Crude protein content	CPC
FeedBee®	FB
Hatched nurse bees	HNB
Hypopharyngeal gland	HG
Icing sugar	IS
Mature nurse bees	MNB
Mixed Pollen	MixP
Redgum (100%) as a pollen feed	Rg100%
Redgum and soy protein as a pollen feed	RgS
Pollen substitute feed	PrSub
Pollen supplement feed	Psup
Queen mandibular pheromone	QMP
Substitute artificial feed No. 1	Sub1
Substitute artificial feed No. 2	Sub2

CHAPTER 1: LITERATURE REVIEW

1.1. INTRODUCTION

In 2003, Animal Health authorities of Japan negotiated a set of quarantine health requirements for the entry of Australian honey bees. One of its clauses stipulated that honey bees to be exported must be free from *Nosemosis* or nosema, an intestinal protozoan infection of the honey bees caused by *Nosema apis* (pers. com. Hiroaki Yonai, Animal Quarantine Services from the Ministry of Agriculture, Forestry and Fisheries of Japan, May 15, 2009). An emergency survey revealed that suspension of queen bee exports to Japan in 2007 have forced fruit farmers to hand pollinate many of their crops (Kamiya 2009).

Colony Collapse Disorder has killed large numbers of European honey bees (*Apis mellifera*) in United States of America, Europe, Brazil, Vietnam and many other countries has caused large scale worldwide economic losses (Giersch *et al.* 2009, 117). *Nosema* species such as *N. apis* and *Nosema ceranae* are thought to be connected with Colony Collapse Disorder (Higes, Martín-Hernández and Meana 2006, 93). Bee health has become increasingly pivotal in the international trading standards, thus the production of nosema-free bees would be a competitive advantage for Australia over other countries.

Since the discovery of nosema by Zanders in 1907, researchers have tried countless attempts to eradicate nosema via chemotherapy. Of the many chemicals tested by researchers and farmers, fumagillin has been the most promising. However it is not a cure (Goetze and Zeuttschel 1959, 222). Like many chemicals, fumagillin only acts to suppress infection during certain seasons, and in some countries, its use is restricted, such as in Australia, because fumagillin residues persist in the honey (Benecke 2007).

The problems facing the production of nosema-free bees are that the disease is endemic, bees are easily infected, and the spores are very resilient and can remain viable for long periods (Furgala and Mussen 1978, 68; Gochnauer, Furgala and Shimanuki 1975, 639). All newly hatched nurse bees (HNB) are naturally free from nosema. However, if they are allowed to interact with other bees within the same infected hive, the HNB would inevitably contract the disease (Bailey and Ball 1991, 68; Higes *et al.* 2007, 212).

Currently, nosema-resistant bee populations do not exist, only that individual inborn colony hygienic behaviour influence the severity of the disease (pers. com. Dr. Rob Manning, Department of Agriculture and Food, Government of Western Australia, March 2, 2009). There are numerous studies on bee nutrition and how different feeds can be used to maximize bee performance in commercial beekeeping. The most prominently studied feeds involve the use of pollens (irradiated, freeze dried, fresh) and those artificially made. Haydak (1970, 143) and van der Steen (2007, 114) has shown that pollen play a significant role in the health and the development of hypopharyngeal gland (HG) in HNB which is responsible for producing royal jelly.

Traditionally, mature nurse bees (MNB) from beehives are used as escorts in cages to feed and keep queens alive during export. They are used because they have fully developed HG capable of producing and feeding royal jelly to the queens, however they are usually infected with nosema, thus they cannot be used as escorts in export markets such as Japan. Hatched nurse bees have undeveloped and non-functional HG so they cannot synthesize royal jelly, however, in the presence of good quality protein, found normally in fresh pollen, their HG can become fully developed in 6 to 12 days (Huang and Otis 1989, 265; Standifer 1967, 415). Studies have showed promising outcomes of pollen and artificial feeds in developing HG in HNB (Standifer 1967, 419), and thus they should technically substitute for MNB escorts. If MNB can be successfully substituted by HNB, it would overcome the quarantine barrier in

Japan, and possibly open new doors to unexploited countries hence giving Australia a competitive advantage of supplying healthier bees.

Heat therapy has been proven effective in curing microsporidian infection in various arthropods (Boohene, Geden and Becnel 2003, 1146-1152). Applying heat to hive equipments has also killed *N. apis* spores (Moeller 1978). It is also well known that in summer, infections are reduced dramatically, and the link between natural heat in summer and lower levels of nosema remains to be established (Hornitzky 2005). Subjecting infected live bees to variable degree of heat therapy would be a novel approach to killing the *N. apis* spores.

This review of literature consists of six sections. The first explores the queen bee industry in Australia, the quarantine situation in Japan and the impact of *Nosema* species on worldwide bee populations. The second elaborates on the biology of *N. apis*, its aetiology, symptoms, and epidemiology. The third discusses the different types of current chemotherapies. The fourth examines different types of pollen and artificial feed and how it influences the development of the HG. The fifth explores the environmental factors that impact nosema, with special emphasis on heat. And finally, the sixth concludes with the summary and an aim detailing a clear set of objectives for this project.

1.2. QUEEN BEE EXPORT INDUSTRY IN AUSTRALIA

In 2006, Australia exported 4 million dollars worth of live bees (New South Wales, New South Wales Department of Primary Industry 2007). Western Australia is one of the few places left on Earth to have European honey bees free from the highly destructive pathogens such as *Varroa destructor*, *Varroa jacobsoni*, *Tropilaelaps clareae*, *Acarapsis woodi*, European foulbrood (Australia Capital Territory. Animal Health Australia 2005), and *N. ceranae* (Giersch *et al.* 2009, 117; Hornitzky 2008a). The disease-free status and the fact that Western Australia has a well established quality queen breeding program make its queens highly sought after by buyers from all over the world. Canada, Middle East, and United States

of America are the biggest importers of queens from Australia. Other smaller markets include Japan, South Korea and Taiwan (Manning 1996).

According to Manning (1996), there are three main reasons for countries to import honey bees. Firstly, countries with severe winters such as Canada buy packaged bees to re-establish hives for honey production in spring. Secondly, package bees (1-2 kg bees plus a queen) can be added to existing hives to strengthen and build bee population so as to capitalize on honey production or pollination contracts and finally, countries such as Japan, South Korea and Taiwan import bees for crop and vegetable pollination.

1.2.1. Japan live bee export restriction

For decades, nosema was an infection that was solely attributed to *N. apis*, but recently, scientists found the cause was also associated with *N. ceranae* (Chen *et al.* 2009b, 204). Infections in bees have been known to reduce honey and royal jelly production, reduce life span by 50 %, and cause queen supersedure (Furgala and Mussen 1978, 66; Gochnauer, Furgala and Shimanuki 1975, 638-639; Hornitzky 2008a).

A complete suspension of Australian honey bee exports to Japan took effect in November 2007 due to the detection of nosema during a random quarantine sampling. Australia was the only country that had permission to export queens into Japan for pollination purposes. Since the ban of exports, many Japanese farmers had to hand-pollinate many of their crops. Japanese beekeepers believe that prolonged suspension of exports will drive up food prices because of lower crop yields (Kamiya 2009; Gutierrez 2009). There is no scientific evidence to suggest *A. mellifera* in Japan are infected by *N. apis*, but Chen *et al.* (2009b, 206) found both *N. apis* and *N. ceranae* in their native Asian honey bee.

1.2.2. *Nosema ceranae*: how it affects honey bee export from Australia

The term “Colony Collapse Disorder” was used to describe the sudden mass deaths of European honey bee populations in widespread colonies in the United States of America from late 2006 (Chen *et al.* 2008, 186). It took scientists a further two years to find a strong link that *N. ceranae* may have caused Colony Collapse Disorder, and when *A. mellifera* was artificially inoculated with this Microsporidia, 94.1 % of them died on the 7th day (Higes *et al.* 2008a, 2659-2661).

Little is known about the biology of *N. ceranae*, however it does share some similarity with *N. apis*. Like *N. apis*, it multiplies in the ventricular epithelial cells of bees before shedding into the ventriculus and bursting; leaking acidic digestive enzymes (Gochnauer, Furgala and Shimanuki 1975, 630; Higes *et al.* 2007, 211; Williams *et al.* 2007, 1). Higes *et al.* (2008a, 2659-2664) found that the percentage of infection by *N. ceranae* and the severity of it becomes lighter when infection occurred in late spring, compared to when it occurred in colder weather for *N. apis*. However Hornitzky (2008a) suggested that *N. ceranae* has no seasonality. Both studies by Chen *et al.* (2009b, 204-209), and Mart í-Hern ández *et al.* (2007, 6331) proved co-infection of *N. apis* and *N. ceranae* exists in both Asian and European honey bees (Table 1.1) and they speculated that *N. ceranae* is displacing *N. apis*.

Table 1.1: Prevalence of *N. apis*, *N. ceranae*, and co-infection in both Asian and European honey bees (¹ Chen *et al.* 2009b, 204 and ² Mart í-Hern ández *et al.* 2007, 6335-6336).

Infection	% infection	
	<i>A. ceranae</i> ¹	<i>A. mellifera</i> ²
No infection	No data	30.3
<i>N. ceranae</i> alone	31	53.8
<i>N. apis</i> alone	71	9.3
<i>N. ceranae</i> and <i>N. apis</i>	19	6.6

Fries *et al.* (1996, 356) first reported *N. ceranae* in *A. mellifera* in 1994, and more than a decade later, Colony Collapse Disorder became widespread in the United States of America, Europe, Brazil, Vietnam (Giersch *et al.* 2009, 117), Canada (Williams *et al.* 2008, 189), and Taiwan (Higes, Martín-Hernández and Meana 2006, 93).

According to Leung (2007), Australia is free from *N. ceranae*, however, more recent data from Hornitzky (2008a) showed that random bee samples from Queensland, New South Wales, Victoria, and South Australia were positive for *N. ceranae* while Western Australia and Tasmania remained negative. Although Hornitzky could not detect *N. ceranae* in honey samples from the six states, except for Queensland, Giersch *et al.* (2009, 117) did find traces of *N. ceranae* DNA in honey samples of all six states, except for Western Australia and Tasmania. The Asian honey bee *A. cerana* is the original host of *N. ceranae*, but it has jumped the species barrier to *A. mellifera* (Higes *et al.* 2008a, 2659; Williams *et al.* 2007, 189-190).

A recent PCR analysis of honey samples from all parts of United States of America confirmed the presence of *N. ceranae* prior to 1995 suggesting that *N. ceranae* is not a new emerging disease of *A. mellifera*, but instead it has been slowly spreading for more than a decade “under the radar” of beekeepers (Chen *et al.* 2008, 187). The situation is so severe that experts believe that by 2035, commercial bees would cease to exist if the continual decline of bees are not stopped (United States of America. The National Academies, 2007).

The honey bee pollinates more than 85 % of the major crops, 77 % of which are the world’s most economically important plant species, thus Colony Collapse Disorder could have a massive worldwide economic impact (Vergara 2008, 145-146). America was once a large queen export country but is now forced to import queens from other countries to replenish their diminishing supply since the start of 2005 (United States of America. The National Academies 2007). It seems it would be only a matter of time before other countries follow as their bee population continues to plunge.

This could be a big opportunity for WA to substantially boost queen exports within the coming years. Although on price, queens in WA cannot compete with other major exporters such as New Zealand, Egypt and Indonesia, they are some of the healthiest honey bees in the world, and if nosema-free bees were commercially available, it would give WA another competitive advantage (Manning 1996).

1.3. NOSEMA APIS BIOLOGY

Nosema apis belong to a large group of obligated intracellular parasites omnipresent in all animal phyla called microsporidia (Didier 1998, 1; Higes *et al.* 2007b, 211; Higes *et al.* 2008b, 374). Balbiani created a genus for *Nosema* under fungi in 1882, because the infection strategies, life history and diagnosis of the spore are typical characteristics of fungi (Keeling and Fast 2002, 93-95). Although *N. apis* spores are only 3 to 7 μm long and 3 to 4 μm wide, they are compact and sophisticated with many components that are reflected in Figure 1.1 (The World Organisation for Animal Health 2009).

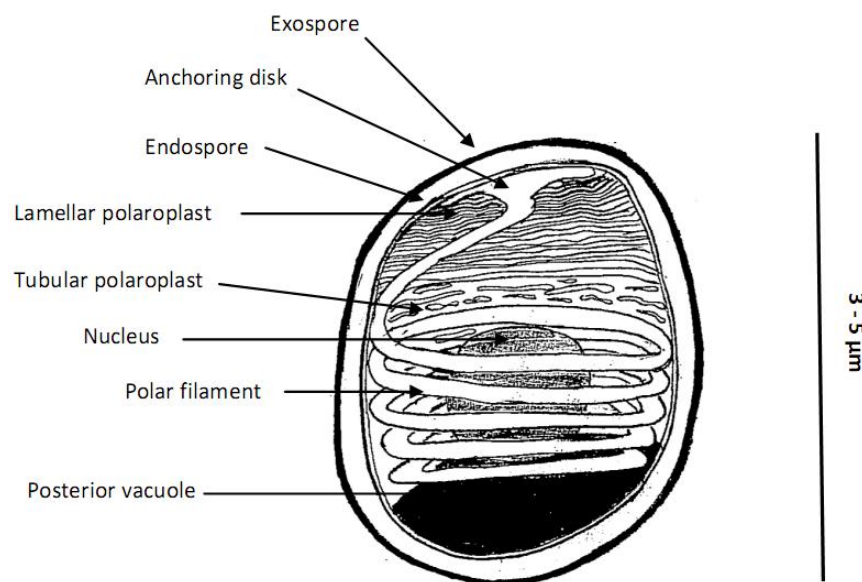


Figure 1.1: Diagram of the fundamental structures of a microsporidian spore.

The spore itself contains all the structures necessary for successful infection of its host, but the three most significant features associated with infection are the posterior vacuole, polar filament and the polaroplast (Keeling and Fast 2002, 93-95).

In *N. ceranae* endemic areas, standard microscopy is no longer the common protocol in the identification of nosema, instead it is increasingly replaced by the use of PCR as *N. ceranae* and *N. apis* are indistinguishable under the optical microscope (Martín-Hernández *et al.* 2007, 6331, Chen *et al.* 2009a, 145). Figure 1.2 shows *N. apis* under a optical microscope or see Steche and Held (1981) for pictures of spore under an electron microscope.

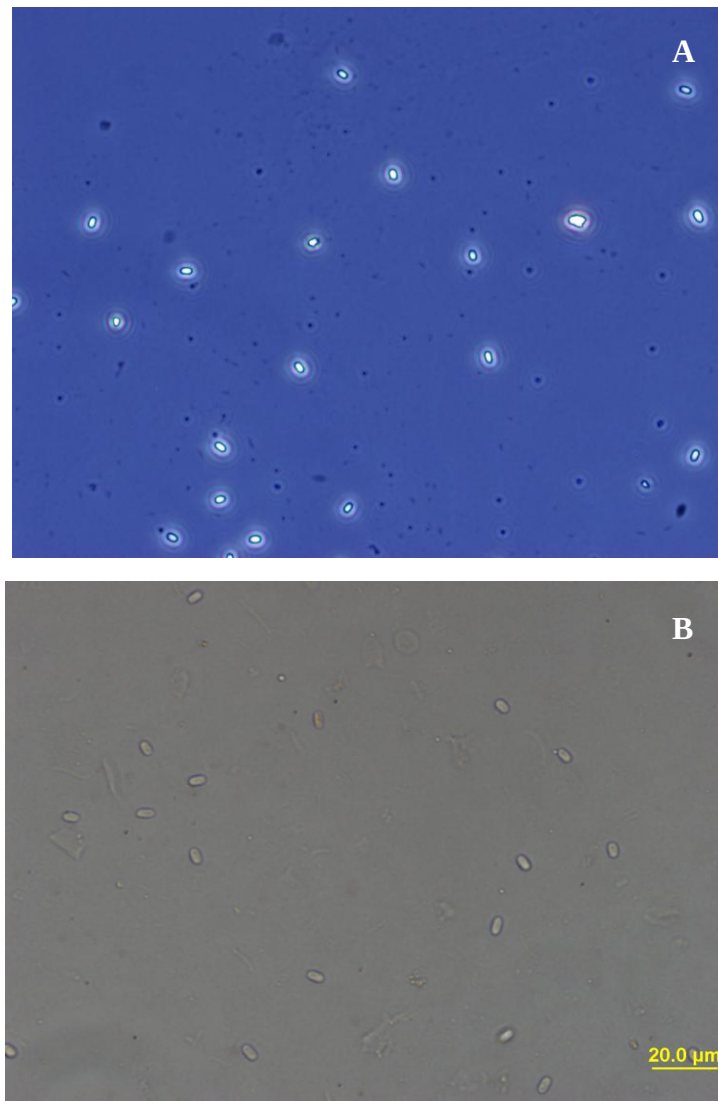


Figure 1.2: Normal microscope images of *N. apis* spores. A= phase contrast; and B= bright field. Spores are elongated and are 3 to 5 μm in length.

1.3.1. Life cycle of *Nosema apis*

Outside its host, *N. apis* spores are dormant, as it is in a vegetative form. In this form, they are non-infectious; it is only when they are ingested by its host that they become active. One spore is sufficient to cause an infection and within 7 to 9 days the spores become mature and can replicate to 50 million spores in a short time (Bailey and Ball 1991, 64-72; Hornitzky 2005; Malone and Gatehouse 1998, 172). Figure 1.3 shows the life cycle of *N. apis* spores in a bee intestinal epithelial cell (also see *Nosema apis* (2004) and Appendix 1 for more detailed information).

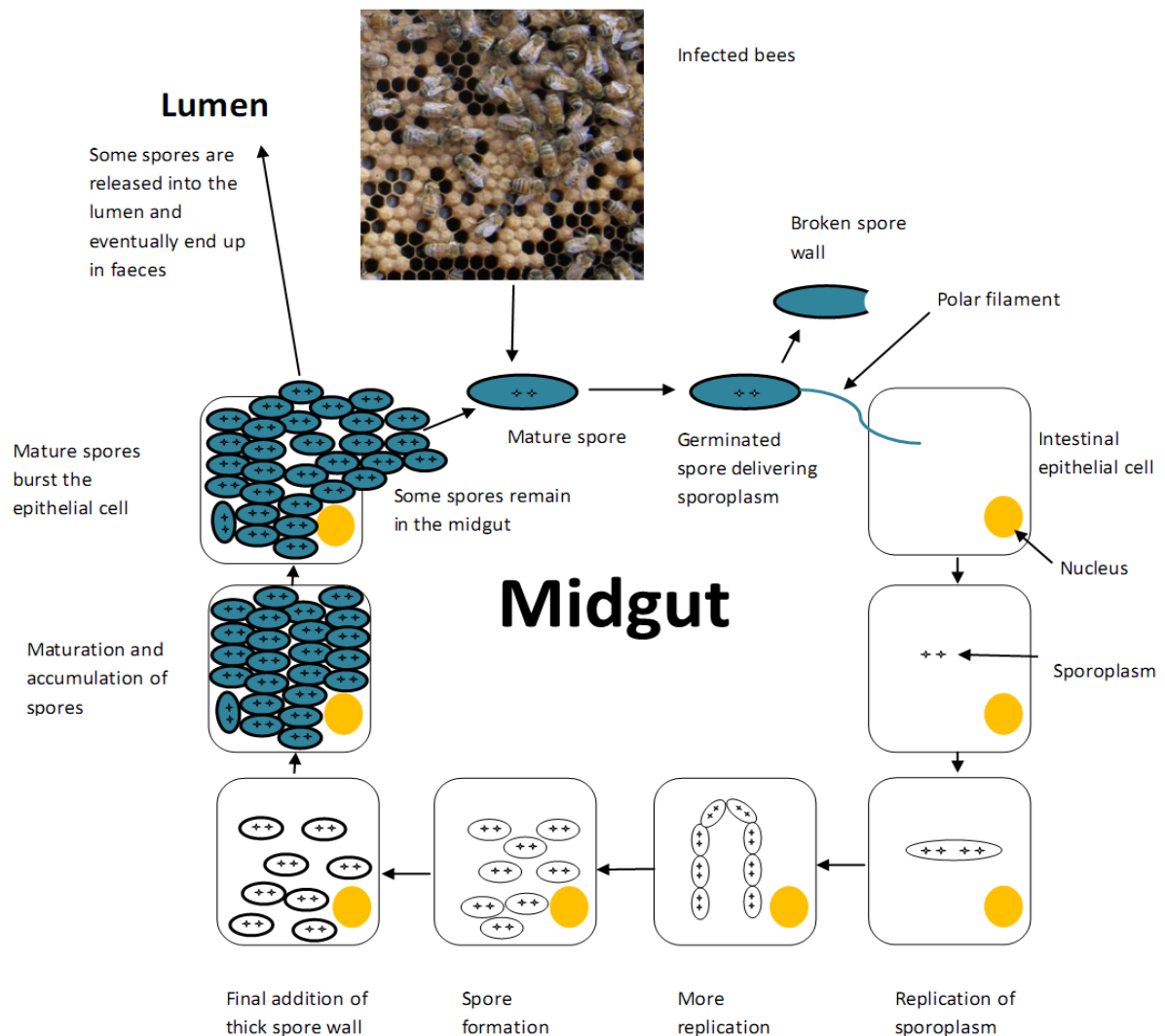


Figure 1.3: Diagram of the life cycle stages of *Nosema apis* in mid-gut epithelial cells of bees.

1.3.2. How *Nosema apis* spreads

Honey bee colonies typically consist of 15000 to 50000 bees that are genetically related. This makes them highly susceptible to rapid epidemic transmission of a pathogen (Fries and Camazine 2001, 199-202). The intimate contact between one another in this highly dense population offers diverse opportunities for the transmission of spores (Table 1.2 show the list of ways *N. apis* can spread).

Table 1.2: List of way *Nosema apis* can be spread (¹ Bailey and Ball 1991, 64-72; ² Fantham and Porter 1912, 163-189; ³ Fries and Camazine 2001, 199-202; ⁴ Hornitzky 2005; ⁵ Webster *et al.* 2004, 49).

Method of spread	Reference
Consuming contaminated food or water: brought in by other bees or water source that have been contaminated with wind-borne spores	1, 2, 4
Grooming or allogrooming while soiled with contaminants	1, 4
Removing drops of infective excrement (faeces of infected bees or foreign invaders such as wax moth and ants) via ingestion. Most predominantly during winter confinement (raining and or cold days)	1, 2, 4, 5
Introduction of spores by robber bees from other hives when they steal the honey (from weak or dead hives)	2
Cleaning infected and dead cadavers in hives	2, 4, 5
Spores are present on plants and plant material picked up by forager bees	2
Visit to several hives by infected drones (bees that drift between hives)	2
Human spread by using contaminated hive equipment when beekeepers are manipulating the hive.	2
Trophallactic interchange of food and glandular substances	3

1.3.3. Effect of *Nosema apis* on honey bees

Severe infections cause heavy swelling of the ventriculus which becomes so constricted that bees would be unable to digest any food (Shimanuki and Knox 2000, 17). This form of malnutrition leads to a chain of reaction that ultimately reduces worker life span, productivity and cause premature death. Table 1.3 gives a list of effects of nosema on honey bees.

Table 1.3: Effects of nosema on honey bees. ¹ Bailey and Ball 1991, 65-66; ² Furgala and Mussen 1978, 66; ³ Gochnauer, Furgala and Shimanuki 1975, 638-639; ⁴ Mart ín-Hern ández *et al.* 2007, 6331-6338; ⁵ Higes *et al.* 2008a, 2663; ⁶ Hornitzky 2008a.

Variations	Bees with Nosema	Reference
Physiological changes	Decrease royal jelly	3
	Deformed hypopharyngeal gland	2
	Decrease honey production	2
	Decreased life span by half	2, 3, 6
Seasonal variations	Highest spore count in spring	2
	Lowest spore count in summer	4
Clinical signs	Crawling bees	5
	Dysentery on hive (high water content)	1, 3, 4
	Supersedure of queen	2, 3, 6
	Higher failure to produce larva from egg	5
	Disjoint wings	2
	Distended abdomens	2
	Absence of the stinging	2
Molecular observation	Reduced RNA synthesis	1
	No cytoplasmic granules of $\text{Ca}_3(\text{PO}_4)_2$	5
	Reduced nitrogen in fat bodies	5
	Reduced hemolymphs	5

Hornitzky (2005) found that healthy colonies produced 24.5 kg more honey on average compared to infected populations. In a severely infected colony, the queens can be superseded, and that would double the loss of honey production. Infected queens produced 12 % less brood than healthy queens and so in some cases, the death rate exceeds the birth rate and that causes gradual depopulation of the colony.

Experimental evidence revealed that bees digestive proteolytic enzymes decrease dramatically three days post infection, hence the bee's ability to digest proteins is compromised. In effect, protein digestion and absorption is reduced which explains why nosema can lead HG deformities in HNB (Malone and Gatehouse 1998, 171-173).

Infected bees often escape the notice of beekeepers as they do not consistently display any outward symptoms or clinical signs, and when they do, the signs are non-specific, and are not limited to this disease (Bailey and Ball 1991, 64-72; Gochnauer, Furgala and Shimanuki 1975, 638-639; Hornitzky 2005). One of the marked changes to honey bee physiology is the swelling of the ventriculus which usually leads to distension of the abdomens (see Figure 1.4), however this is not always indicative of nosema disease.



Figure 1.4: Bee ventriculus. A= diseased bee ventriculus: swollen and soft, and B= healthy bee ventriculus: clear and transparent.

1.3.4. Epidemiology and incidence of disease

The health status of *A. mellifera* around the world was thoroughly reviewed by Matheson (1993, 176-201), and found that *N. apis* is universally distributed where data was available. There were countries where data could not be obtained, thus the status of nosema in those countries is ambiguous. Nepal, Pakistan and Central African Republic are suspected to be free from nosema, but it is not for sure (Matheson 1993, 176-201 and 1996, 45-50). The incidence of nosema caused by *N. apis* is conspicuously seasonal (Doull 1961, 228-235). During early winter, the infection is usually low, as it reaches spring through to early summer, the infection would be at its peak. In the hot summer months, infection drops dramatically hence the colonies generally carry small numbers of infected bees (see Figure 1.5). This is thought to be due to the improved flight conditions, such that bees are able to defecate outside the hive to reduce spread (Goodman 2007).

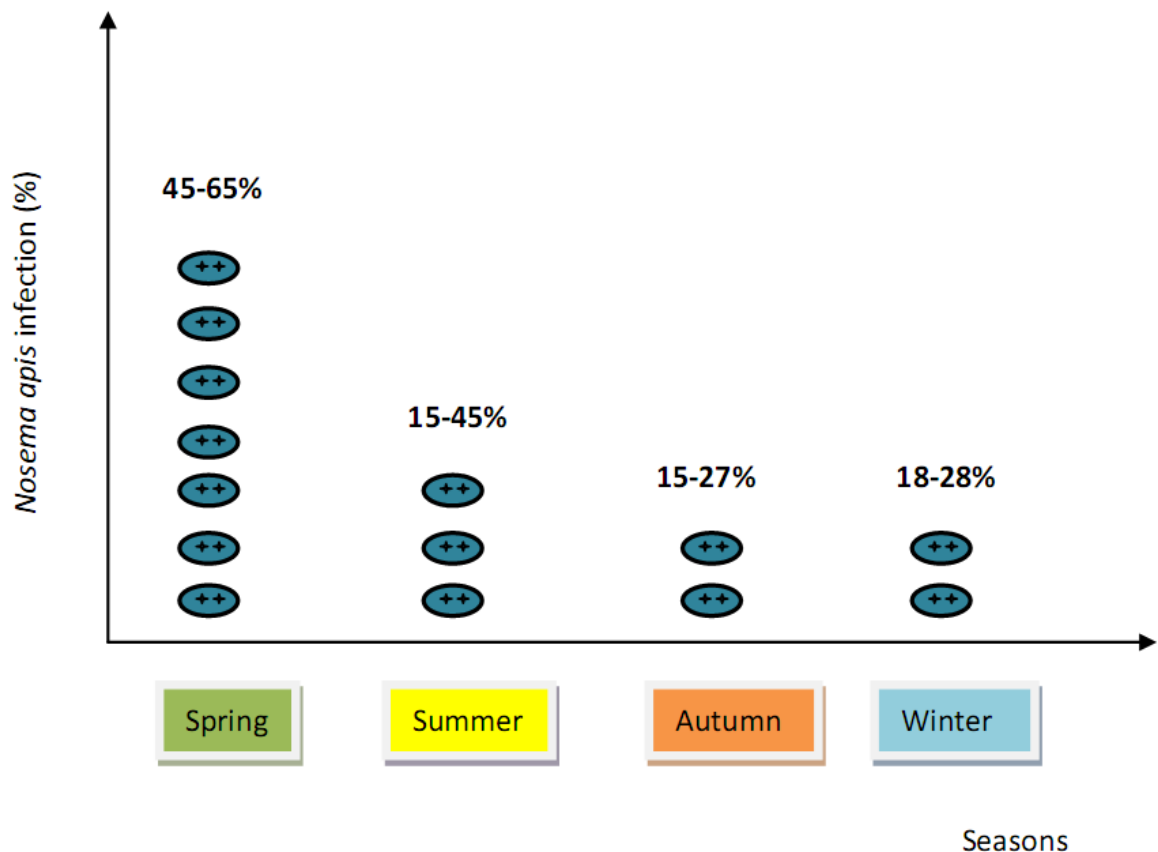


Figure 1.5: Estimated *Nosema apis* infection in bees (percentage) for different seasons in South Australia during 1958. Information was adopted from Doull (1961).

In autumn, a small peak of infection transpires followed by a steady rise during winter (Doull, and Cellier 1961, 280-287; Hornitzky 2005 and 2008b). Goodman (2007, 1) explained that serious autumn outbreak can occur where there are heavy summer rainfalls, early autumn during fine weather, and if the bees are working on grey box (*Eucalyptus macrocarpa*), ironbark (*Eucalyptus sideroxylon*) and white box (*Eucalyptus albens*).

The peak in infection during spring was thought to be related to an increase in the transmission of spores between infected and uninfected bees, and increase foraging and exposure to the environment outside the hive (Mattila and Otis 2006, 709).

Gochnauer, Furgala and Shimanuki (1975, 615-662) and Webster *et al.* (2004, 53) found that queens and nurse bees are both equally susceptible to nosema, but Steche (1977, 33) reported that queen bees are extremely difficult to infect unless they were caged. The queens' lack of involvement in chores of the hive, such as cleaning, removing contaminated faecal material via ingestion and feeding limits her exposure to spores thus also offer an explanation as to why most queens are nosema-free (Bailey and Ball 1991, 70; Loskotova, Peroutka and Vesely 1980, 154). The addition of the peritrophic membrane in queens was hypothesized to have been responsible for less infection (Steche 1977, 33).

Nosema spores are resilient, so they are resistant to a range of treatments, such as refrigeration, lypophylization, and exposure to microwaves (Gochnauer, Furgala and Shimanuki 1975, 635-643; Revell 1960, 1132-1133). For this reason, HNB that are initially nosema-free would contract the disease if they are not isolated from the colony as they hatch (Bailey and Ball 1991, 68; Higes *et al.* 2007b, 212).

1.4. CONTROL OF NOSEMA IN GENERAL AND VIA CHEMOTHERAPY

There are numerous elements which contribute to the severity of nosema infection, including apicultural practices (hive manipulation, positioning of hives in the landscape and hygiene of equipment), feed (availability of natural resources [pollen] and supplementary feeding),

season, and application of antibiotics (Fries and Camazine 2001, 199-202). Various chemicals have become increasingly popular for controlling nosema, but none have been found to cure the disease. Table 1.4 shows the common factors that increase nosema infection and the remedial measurements beekeepers can take to minimize nosema.

Table 1.4: Factors that increase nosema infection. ¹ Bailey 1995, 388; ² Bailey and Ball 1991, 641-642; ³ Hornitzky 2005; ⁴ Kaya 1977, 192; ⁵ Manning 2006, 172; ⁶ Moeller 1978; ⁷ Somerville 2007.

Factors that increase nosema infection	Remedies
Reuse of contaminated combs ²	Heat equipment for 24 hours at 49 °C ⁶ ; fumigate equipment with glacial acetic acid for 24 hours and air it for a week prior use ⁶ ; and direct sunlight on equipment for 15 to 32 hours ⁴
Colony disturbance ³	Opening and closing the lid can kill many bees. Avoid disturbing hive during winter and early spring ³
Relocation ^{1,3}	Avoid transportation of bees for long distances ⁷
Inadequate ventilation for hive ^{2,3}	Position in sunny area protected from prevailing wind ^{2,3}
Feed sugar solution as supplementary feed ⁷	Supply a mixture of protein rich food, such as irradiated pollen, artificial pollen or protein ^{5,7}

The combined use of the remedies described in Table 1.4 as well as chemotherapy or antibiotics would be the most promising amalgamation to ensure the maximum health of honey bees, regardless of whether they are infected. For over a century, scientists have endeavoured to find a cure for nosema with no success. Efforts are not completely futile, because they were able to find good chemotherapies that can effectively reduce severity of nosema infection, such as fumagillin and thymol (Goetze and Zeuttschel 1959, 222.).

1.4.1. Current chemotherapy

The most accepted substances still being used today is fumagillin, thymol and essential oils. Fumagillin is a naturally occurring secondary metabolite secreted by the saprotrophic fungus *Aspergillus fumigatus* typically found in soils and decaying organic matter (Latgé 1999, 310-311). It was the first substance to be reported to work against *N. apis* (Stanimirović 2007a, 1). It was an active ingredient in the first pharmaceutical drugs developed and sold commercially for the treatment of nosema, called Fumidil B (Kochansky and Nasr 2004, 301). Fumidil effectively reduce nosema caused by *N. apis* (Kochansky and Nasr 2004, 301; Moffett, Lockett and Hitchcock 1969, 886-889; Yücel and Doğaroğlu 2005, 1142-1144) and *N. ceranae* (Higes *et al.* 2008a, 2659).

In Australia, the use of fumagillin is restricted to commercial queen breeders (Benecke 2007) and in Germany, both Fumidil B, and Nosemack are prohibited, though thymol is not (Mutanelli 2003, 164-167). This is due to growing concerns about fumagillin posing a genotoxic risk. Recently, Stanimirović, Kulišić and Milanka (2007b, 257) showed that exposure of human lymphocytes to fumagillin at low concentrations caused significant reduction in lymphocyte proliferation, thus concluding it to be a genotoxic risk to beekeepers and consumers of the honey.

Fumagillin is also an anti-angiogenic compound, meaning that it suppresses the formation of new blood vessels. If used in cancer patients, it would be advantageous (Liu *et al.* 1998, 1324; Sin *et al.* 1997, 6099) but if used on healthy individual, it could be deleterious. It is for these reasons why better alternatives should be sought for suppressing or curing nosema that present minimal risk to bees and humans.

An increasingly popular alternative is the use of thymol; it is natural and relatively safe to handle. Research has shown that winter fed bees with sugar syrup and thymol remains nosema-free but not in spring (Rice 2001). Products such as Protofil (thymol based) claim to

penetrate all spore membranes hence preventing subsequent development of the parasite (refer to Appendix 2 for comparison of the fumagillin and thymol).

The performance of two apiaries fed with Protofil were monitored for the period from 1998 to 2002, where one apiary achieved 45-74 % reduction in nosema, while the other showed 44-83 % reduction (Chioveanu, Ionescu and Mardare 2004, 31-38). A three year study has unveiled that long term usage of thymol as a preventative agent for nosema significantly increased the brood surface area and honey production. Thymol is economical, practical and non-toxic in hives for the production of organic honey (Yücel and Doğaroğlu 2005, 1142-1144).

Another promising new product called Nozevit® has been applied to hives to determine its capacity of curing and preventing infection. Nozevit® cannot cure or prevent an infection, however, results showed a significant drop in spore count of treated colonies versus the untreated colonies (Gajger *et al.* 2009, 485-489). These findings were based on mini-scale research that involved two hives, and thus further comprehensive research should be carried out to confirm the results.

Overall, chemotherapy only acts to curb infection, it is not a cure; nevertheless combined use of them with good apicultural practices should reduce economic losses caused by the disease.

1.5. POLLEN, ARTIFICIAL PROTEIN AND ROYAL JELLY AND HOW THEY RELATE TO THE HYPOPHARYNGEAL GLAND

Royal jelly is a type of specialised proteinaceous food secreted by a highly complex food organ in nurse bees called the HG. Larvae in general and queens are fed exclusively with royal jelly. Fresh royal jelly consist of 60-70 % water, however, it is rich in protein (9-18 %), lipids (3-8 %), sugar, vitamins, hormones, enzymes, mineral substance and other vital components (Olimpia, Mărghițaș and Dezmirean 2008, 331).

During the export of queens, several escorts (MNB) nurture and feed the royal jelly to the queen to keep her alive for the whole journey. In a hive, a queen lives for 2-5 years (Haddad, Kelbert and Hulbert 2007, 601), however the fluctuation in temperature, the possible lack of fresh water and food, and unfavourable conditions associated with being caged (and exported) can drastically reduce her life span to just days (author's preliminary finding). The export process which includes quarantine can sometimes take more than a week, and this could pose a serious risk on the queens' vulnerable life outside their hives (pers. com. Dr. Rob Manning, Department of Agriculture and Food, Government of Western Australia, March 2, 2009).

Isolated queens are said to self-feed for up to 68 days, but this only happens when environmental conditions are optimal and fresh food and water is available to them *ad libitum* (Dietz 1975, 132-133). However, in the small export cage, queens may need to share with thousands of other caged bees bundled into packages and placed in cold airplane cargo and then perhaps hot delivery trucks with only icing sugar candy (IS) for the MNB to feed on. Hence the queen relies heavily on the royal jelly that is synthesized and fed to her via stomodeal trophallaxis by her MNB. The survival of MNB and their ability to synthesize sufficient amounts of royal jelly may dictate the survival of their queen. Hive-raised nurse bees such as MNB have fully developed and functional HG (about 6-day-old).

Pollen was proven to be 100 % effective in stimulating the growth and development of the HG of bees under normal hive conditions (Haydak 1970, 143; Hrassnigg and Crailsheim 1998, 929-937; van der Steen 2007, 114). By using feeds with one or more pollen or vegetable protein, it may help stimulate full development of the HG in caged HNB.

1.5.1. Hypopharyngeal gland development

Between the ages of 0 to 22 days, the size of a young bee's HG is positively correlated to their fresh head weight (Deseyn and Billen 2005, 53; Hrassnigg and Crailsheim 1998, 929-937). Normally, the first two days of life for a newly emerged bee is revolved around being fed

royal jelly and self-feed on pollen (fresh and stored), honey and nectar to stimulate the development of their food glands, fat bodies and other organs (Dietz 1975, 132).

Newly hatched nurse bees commence pollen consumption as early as 2 hours after hatching and it reaches a maximum on the 5th day where it gradually declines to an end as they change their role to become foragers at about the 21st day (Painter and Bieseke 1966, 1414-1415). Normally, nurse bees begin secreting royal jelly on their 5th or the 6th day after hatching through to about their 11th day (Painter and Bieseke 1966, 1425-1416), but Cruz-Landim and Hadek (1969, 121) suggested it continues through to their 17th day.

Huang and Otis (1989, 264-275) established that from day 0 to 3, nurse bees from broodless and broodright colonies shared a striking analogous increase in HG activity, though not to make food proteins, but rather to make structural proteins for developing the gland. After the 3rd day, broodless nurse bees displayed consistently lower HG activity which corresponded to the basal level of activity. At the basal level, the HG was deemed incapable of secreting royal jelly.

As they change their role to become a forager around the 20th day, their HG shrinks and they begin to secrete enzymatic proteins to accommodate their foraging activities (Huang and Otis 1989, 264-275). In an experiment, nurse bees were exposed to different brood status combs, and their HG activity were recorded in descending order of having larvae only > mixture of larva, egg and pupae > egg and pupae only > egg only, or pupae only or broodless. In that study only the presence of larva stimulated significant HG activity and Huang, Otis and Teal (1989, 455-456) linked this to nurse bees having direct larval contact.

Under normal hive conditions where a mixture of brood is present in the colony, HNB can fully develop their HG upon consumption of pollen. The quantity and the quality of protein secreted by the HG are governed by the brood status, the bees' age, hormones, balance of nurse and forager bees (Hrassnigg and Crailsheim 1998, 929-937; Kubo *et al.* 1996, 291), and their lifespan (Moroń and Woyciechowski 2009, 193-200).

Juvenile hormone plays a significant role in determining honey bee castes in the larval stages (Wirtz and Beetsma 1972, 519), however it also exerts great influence on the development of the HG, in fact the increase level of it in older bees has been associated with premature HG hypotrophy. Environmental stress such as increase CO₂ and temperature increases juvenile hormone secretion (Ohashi *et al.* 2000, 1089-1093). Parasitic stress such as nosema infection directly shorten a bee's life expectancy, and when this threshold is lowered, young bees are forced upon precocious foraging (Moroń and Woyciechowski 2009, 193-200) which will reduce HG activity (Webster *et al.* 2004, 49).

The normality of a hive is maintained by a fine balance of castes (division of labour), and any imbalance would cause dramatic changes to a hive's dynamic. The removal of a queen from a hive changes the division of labour. Pheromones that suppress ovarian development in worker bees are lost (Huang and Otis 1989, 264-275) and as a directly consequence, portions of workers will commence laying unfertile eggs, while another portion revert back to being nurse bees to secrete royal jelly for the drone larva. In contrast, the removal of nurse bees would result in foragers reversing back to the role of nurse bees while the removal of foragers will prompt young nurse bees to adapt to precocious foraging (Ohashi *et al.* 2000, 1089-1093).

1.5.2. Pollen, artificial pollen and royal jelly

High protein body weight is strongly correlated with bee longevity and productivity, while low protein body weight usually means short lived bees that are less productive. Bees which suffer from protein stress can collapse following heavy flow of nectar (Stace 1996, 5-12). Researchers and beekeepers have been using pollen supplement and artificial feed to maintain healthy bees, increase bee longevity, build up colonies, and boost brood rearing and pollination (Standifer *et al.* 1978, 1-3).

The minimal crude protein content (CPC) needed in any pollen supplementary feed is 20 % and in it should contain a minimal level of all the essential amino acids for bees. Most of the

pollen from Australian native flora meets these standards except for some plants where iso-leucine and valine is sub-optimal. In nature, bees consume a wide range of pollens, thus the iso-leucine requirements are met. Spotted gum pollen has 25 % to 33 % CPC thus it is said to be very good for hive build up, whilst pollen from pine tree which contains 5-7 % CPC is less effective (Stace 1996, 5-12). However, pollens with higher CPC does not always represent a better quality pollen, for example balsamroot (*Balsamorhiza sagittate*) has 18.3 % CPC promoted good development of the HG, whilst lombardy poplar (*Populus nigra* L.) with 36.5 % CPC promoted little development. The common dandelion (*Taraxacum officinale*) has 15.1 % CPC yet, when compared with 17 other CPC superior pollens in a longevity trial where 50 % of the bees lived up to four times longer (Standifer 1967, 417-422).

Pollen that promotes good health in bees is determined by the balance of essential amino acids rather than a total CPC and that deficiency or complete lack of one or more amino acids will result in health consequences. A mixture of pollen would be the best feed as different pollen can compensate for the lack of certain amino acids (Standifer 1967, 417-422). There are individual pollens which exceed the minimal amino acid requirement, three of which are found in WA including karri (*Eucalyptus diversicolor*), forest blackbutt (*Eucalyptus patens*) and redgum (*Corymbia calophylla*) (Manning 2001).

Over the years, copious research has been performed on honey bee nutrition and the effect of single or combined feeds of pollen, artificial, carbohydrates, amino acids and lipids. Although sugar solution sustains bees for a relatively long time, it does not have any protein and in theory, this would not enable HNB to synthesize any royal jelly as amino acids must be consumed in order for it to be used in its synthesis (Doull 1974, 17; Haydak 1937, 261).

Standifer (1967, 417-422) tested pollen supplements at 2.5, 5, 7.5 and 10 % CPC of 25 culinary pollens found in the United States of America for their ability to stimulate HG development. All the feeds consisted of variable levels of pollen (to desired concentration), Nulomoline (dextrose and levulose at 1:1 ratio), cellulose, and water. These were fed to caged HNB kept in optimum living conditions. The degree of HG development was ranked based on

a scale of 1 (no development) to 4 (full development respectively). Balsamroot, saguaro cactus (*Carnegiea gigantea*) and red clover was ranked 3.4-3.5 whilst the lowest was alfalfa (*Sorghum bicolor*) at 1.5.

Manning (2006, 85-130) researched extensively on the use of redgum pollen with flour, fatty acids, and different sugars. He found (in descending order) that redgum pollen with 2 % linoleic acid, redgum alone, or with 6 % linoleic acid, or with 2 % oleic acid and 6-years-old crushed irradiated redgum pollen stimulated the highest HG growth rate out of the many combined pollen substitute feeds. These feeds also corresponded to being most consumed and longest lived (in different order). Schmidt, Thoenes and Levin (1987, 176-183) also used 100 % pollen, and he showed that nurse bees fed only one type of pollen (desert flora origin) lived 35 to about 65 days (the same for sugar syrup), whereas those fed with a 5 pollen mix lived 90 days.

Artificial feeds (food fortified with protein rich substance to replace pollen) commonly contain high protein mixture which may include soyflour, brewer's yeast, dried skim milk (lactose-free), and egg yolk powder. Soyflour has been used for decades as a substitute, as it contains high CPC, however research have found it to be of little use stimulating honey production and the growth of HG (Manning 2006, 85-130; Somerville and Collins 2007).

Brewer's yeast provides a good source of water soluble vitamins such as vitamin B complex which is why they are added to most artificial feeds (Black 2006; Somerville 2005). To make these feeds more desirable, sucrose and olfactory stimulating additives can be added (i.e. rum). Rogala and Szymaś (2004, 29-34) formulated a complex homemade feed which consisted of potato protein 32 %, soy cake 18 %, rapeseed cake 6 %, yeast (*Candida utilis*) 6 %, wheat flour 14.8 %, maize grits 17.5 %, soy oil 3.3 %, lecithin 0.5 %, Polfamix 1.4 %, Vitazol AD3EC 0.2 %, glucose and vitamin C 0.1 %, and various amino acids. Results showed a promising 62 % of the caged HNB fed with this feed had 3rd and 4th degree (maximum) HG development.

Regardless of the type of feed, it is unambiguous that pure mixtures of fresh or irradiated pollen provide the most success in HG development of HNB (Black 2006; Rogala and Szymaś 2004, 29-34; Schmidt, Thoenes and Levin 1987, 176-183; Standifer 1967, 417-422). Having said that, substitute feeds are more economic when compared with pure pollen feeds. A thorough review of literature showed that much focus has been placed on how man-made artificial feeds can benefit bees in the apiaries or under laboratory conditions, however, no combined research has been done to assess whether the use of different feeds can enable HNB to simultaneously develop their HG and secrete royal jelly for their virgin queens in confinement prior to and during exporting conditions.

1.6. ENVIRONMENTAL INFLUENCE ON NOSEMA

Nosema infection is a fairly predictable disease in that it follows a structured seasonal trend in most countries, the highest incident and most severe infections occur in spring, and in summer the infection becomes drastically reduced (Furgala and Mussen 1978, 70; Doull and Cellier 1961, 280-287; Hornitzky 2005 and 2008b) with the exception of some Mediterranean regions (Lotfi *et al.* 2009, 257). There are many controversial theories for these trends, Mattila and Otis (2006, 709) believe that the increased prevalence of the disease in spring is due to the emergence of many new bees, and that the amplification in the interaction between the worker bees and the outside environment increases the spread.

Bailey and Ball (1991, 70) argued that the spring peak is associated with the massive spring clean up of the accumulation of infectious excreta (as a result of winter enclosure from very cold condition). In summer, bees defecate outdoors, therefore infections are reduced. Others suggested that in summer, bees are exposed to high temperatures therefore the multiplication of *N. apis* becomes inhibited (Bailey and Ball 1991, 69). Burnside and Revell (1933, 603) and Manning (1993, 288) observed that under warmer environmental conditions the prevalence of nosema spores is significantly reduced.

It's well known that the temperature in hives are well regulated and rarely exceeds 35 °C for long periods as required for inhibition of *N. apis* spores in their experiment. Lotfi *et al.* (2009, 255-257) believe the causes for the hike in infections are connected with humidity, such as spring in Northwestern Iran and summer in Mediterranean regions where infections are most abundant. Sadly, the relationship between season and bees susceptibility to infection has been neglected and the explanations are just mere assumptions.

The unarguable fact is that when infected bees are subject to elevated temperatures, the *N. apis* experience retarded multiplication, thus it is reasonable to assume that *N. apis* is vulnerable to the external temperatures (Burnside and Revell 1933, 603).

Heat therapy is an effective decades old practice commonly applied to microsporidian infections in arthropods. Boohene, Geden and Becnel (2003, 1146-1152) investigated the effects of heat therapy on *Nosema muscidurafacis* which causes nosema disease in wasps (*Muscidifurax raptor*). They found that parasitoid pupae heated for 45 minutes at 50 °C offered a 100 % cure (18 % relative survival), while at 45 °C for 60 minutes, 75 minutes, 3 hours, and 5 hours offered an 85-100 % cure.

It has been shown that *N. apis* spores remain relatively infective for long periods when they are frozen, dried on hive equipments or preserved in honey (Furgala and Mussen 1978, 68; Revell 1960, 1132). At room temperature and in darkness, spores remain viable for 2-4 months, but when exposed to direct sunlight (crushed gut or in aqueous suspension), they lose viability within 15-51 hours. Direct heat applied to spores in water for 10 minutes at 57-60 °C (Moeller 1978) or air dried spores for 3 or 5 days at 40, 45 or 49 °C also appears to kill the spores (Malone, Gatehouse and Tregidga 2001, 258). Heat therapy will be an original method to investigate whether incubating MNB in elevated temperature can inactivate *N. apis* spores while keeping the bees alive.

1.7. CONCLUSION

International bee decline is strongly linked to nosema and has reached an economically damaging proportions, thus the need for a supply of healthy bees has never been so desperate. The change in the quarantine laws in Japan currently poses a financial loss for queen breeders in Australia. If nosema-free bees can be produced, Western Australia would be able to resume queen export to Japan. It would also mean that Western Australia bees would be more competitive because they would be healthier and this may open new doors for trading.

Although pollen and artificial feed have been researched and used for decades, the main focus of this research has been on boosting the potential of honey production, pollination and brood rearing, but little research has focused on the ability of pollen and artificial feed to stimulate HG in HNB to synthesize royal jelly for queens during export. This poses greater challenges, because not only do the queen and her nurse bees have to be nosema-free, but nurse bees need to keep the queen alive for more than a week.

If heat therapy is proven to be effective at eliminating nosema in live bees, this would widen the export opportunity and would have significant worldwide implications as this could be the cheaper and environmentally safer alternative to controlling or eradicating nosema.

1.8. PROJECT AIM

This project will attempt to answer questions relating to the effect of raising HNB in cages instead of their normal environment and the behavioural and physiological impact on them. Also, it will assess whether pollen and artificial feeds can stimulate full development of HG in caged HNB and finally relating that to their ability to escort and keep virgin queens alive in export cages.

The aims of this project is to investigate (A) whether pollen and artificial feeds can properly develop the HG of caged nurse bees, (B) if there is a relationship between how much a bee

consumes and how much her HG will develop, (C) if nosema-free HNB or escorts can be produced, (D) see whether these escort bees can feed and keep 100% of the queens alive for more than 8 days during export, and (E) whether applying heat therapy on bees can cure nosema infection.

CHAPTER 2: EFFECTS OF POLLEN AND ARTIFICIAL FEEDS ON THE HYPOPHARYNGEAL GLAND OF NEWLY HATCHED NURSE BEES

2.1. INTRODUCTION

In order for Japan to accept live queen exports from Australia, it is a condition of entry that bees are free from *Nosemosis* or nosema which is caused by an intestinal protozoa called *Nosema apis* (Kamiya 2009). This disease is currently endemic around the world and has no cure (Goetze and Zeuttschel 1959, 222); however a novel technique involving the use of newly hatched nurse bees (HNB) that are free from this disease may be the answer to this problem. All bees are born nosema-free, and it is only upon contact of contaminated material or interaction with infected bees would then contract the disease (Bailey and Ball 1991, 68).

Theoretically, isolating HNB and raising them outside the hive in a nosema-free environment should produce nosema-free bees. The challenges of raising young bees outside the hive is stimulating normal behavioural and physiological growth. Behavioural traits include, developing bees' ability to recognise and feed different aged larva and accept and feed introduced queens. Physiological traits include fat body and hypopharyngeal gland (HG) development and function. Nurse bees need fully developed and functional HG in order to secrete royal jelly (Huang and Otis 1989, 264-275). During export, queens are vulnerable and need to be continuously fed royal jelly that is synthesised by the escort nurse bees (Dietz 1975, 132), thus it is important that escort nurse bees have a fully developed and functional HG.

Pollen is the main source of protein in honey bee feed, and the amino acids derived from the protein are used to build structural elements of muscles, tissue and gland, including the HG (Dietz 1975, 131-146). Under the normal hive condition, pollen was proven to be 100 %

effective in stimulating the growth and development of the HG of bees (Haydak 1970, 143; van der Steen 2007, 114). The quantity of protein in pollen is defined by the crude protein content (CPC) where 20 % is the bare minimum for honey bee nutrition, the CPC has a vast parameter ranging from 2.9 % for pistillate kiwifruit (*Actinidia deliciosa*) to as high as 53.4 % in Solonaceae plant (*Solanum douglassii*) pollen. The quality of the protein is defined by a balanced of the ten essential amino acids; theonine, valine, methionine, leucine, iso-leucine, phenylalanine, lysine, histidine, arginine and tryptopan (Black 2006).

Many native floral pollens in Australia are deficient in iso-leucine and or valine, thus to ensure all amino acid requirements are met, a mixture of pollen would be provide most success in developing the HG in honey bees (Black 2006; Rogala and Szymaś 2004, 29-34; Standifer 1967, 417-422). One of the few native species which meets the minimum requirement is redgum. Manning (2001) proved that both fresh and 6-year-old irradiated redgum are equally effective in stimulating the development of the HG.

Although fresh pollen is the most desirable protein source for honey bees, artificial feeds remain more economical. Rogala and Szymaś (2004, 29-34) showed that a nutritionally well balanced homemade artificial feed is also capable of stimulating full development of the HG. There are many studies into what individual artificial feeds can do for honey bee, however no combined research has been done to assess whether pollen and artificial feed can stimulate full growth of the HG in HNB and whether they can secrete royal jelly when caged with virgin queens during export conditions.

There are three aims to this experiment. First, to see whether these feeds will be able to stimulate full development of the HG by measuring the head weight. Second, to see how much bees eat and how this relates to the HG development (head weight). Lastly, to see whether isolating HNB from the hive (cage) will produce nosema-free bees.

2.2. METHODS

2.2.1. Experimental design

This experiment was divided into three parts.

Part 1: Cage-raised HNB were fed a mix pollen feed (MixP) to encourage the development of their HG. After 7 days, they became 7DAY bees and were fed three other pollen feeds and four other artificial feeds for 9 days. The first 7 days was enough time to allow them to properly develop the HG.

Part 2: 7DAY bee were fed four pollen feed, including MixP, 100 % irradiated redgum (Rg100%), redgum and soy protein isolate (RgS), and Psup (equal blend of MixP: Rg100%: RgS). All MixP feeds were replaced with three other pollen feeds, while one cage continued to be fed MixP.

Part 3: 7DAY bees were fed four artificial feed, including Sub1 (slightly modified homemade feed derived from van der Steen 2007, 115), Sub2 (nutritionally balanced feed), FeedBee® or FB, and PrSub (equal blend of Sub1: Sub2: FB). Figure 2.1 shows the experimental design.

Everyday the consumption and death rates were measured and 50 bees were sampled from each treatment. As soon as bees were sampled, they were placed in a freezer -4 °C to await processing. The bees were fed the different feeds for 7 days in Part 1 and 9 days in Part 2 and 3 (see Figure 2.1). HNB and MNB fed on icing sugar (IS) served as controls. HNB (fed IS) was used as a control for HNB fed on MixP because they are the same age and that because IS is a pure carbohydrate, it can be used to compare the effects of pollen on the development of the HG. MNB (fed IS) was used as a control for 7DAY bees fed on eight pollen and artificial feed because they are the same age and that because IS is the standard feed given to bees during export.

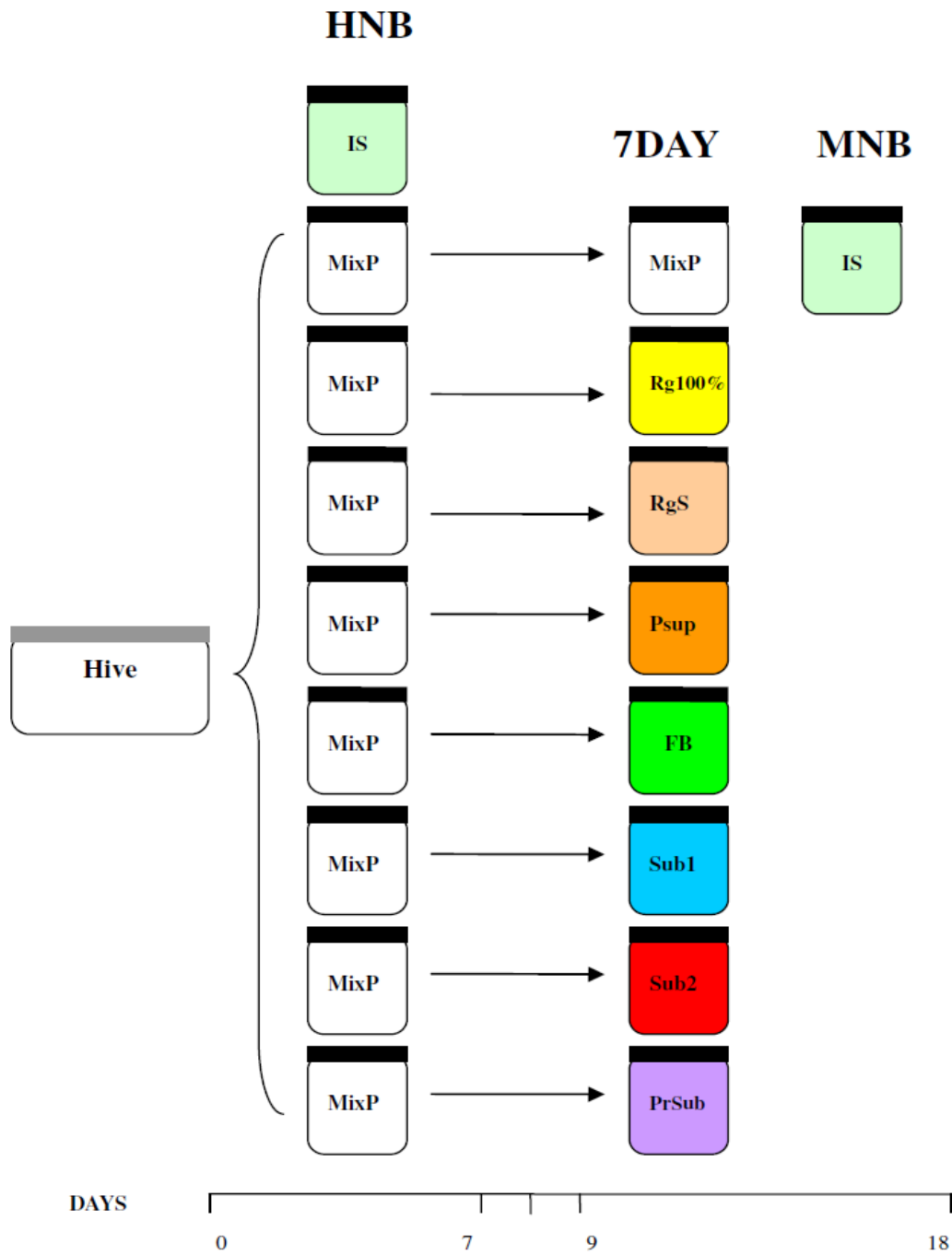


Figure 2.1: Experimental design for HG development. *HNB*= hatched nurse bee, *7DAY*= 7-day-old bees, *MNB*= mature nurse bees, *FB*= FeedBee®, *IS*= icing sugar, *MixP*= mix pollen, *PrSub*= pollen substitute, *Rg100%*= redgum pollen, *RgS*= redgum and soya isolate, *Sub1*= Substitute No. 1, and *Sub2*= substitute No. 2

2.2.2. Ingredients used for the feed

Sugar solution or syrup (50 % w/w) was made by measuring out the ingredients and boiled for 5 minutes (the solution was cooled prior use). All ingredients were roughly combined before they were each transferred into a Kenwood KM250 (serial: 0450428) Electronic Dough Mixer (made by Britain Kenwood Ltd.) and was mixed thoroughly for 5 minutes on minimum speed and another 5 minutes on level 2 speed to make soft patties. All weighing was done on an ACB300 Weighing Machine (Crown Scientific Pty. Ltd. of New South Wales).

Pollen feed ingredient: MixP feed is a concoction of three freshly collected and mixed pollens from Ningham Station during August 2009, Kununurra during October 2008, and South Perth during August 2007 (Western Australia). Irradiated redgum pollen used for Rg100% and RgS were from Beez Neez Apiaries, the soy isolate is from MyoPure™ in Australia (Batch: 026210), and the rum is from Bundaberg (37 % Alc/Vol) in Queensland. The grape seed extract is from Vinlife (batch: N05010, South Australia), supplied by Dr. Soressa Kitessa from CSIRO in Floreat.

Prior to use, all the three batches of mix pollens were placed in plastic vials and sterilized by exposing it to 30,800 and 32,500 Gy (gamma-irradiation) in a Gammacell-200® Cobalt-60 at the dosage rate of 2100 R/min. To make MixP, 500 g mixture of the three pollen batches were blended into powdery consistency for 2 minutes in a meat grinder cup using Ouke Food Processor ZHJ-308K3 (made in China). To make it into a soft patty, 200 mL of syrup was added into the pollen mix. This process was repeated with the irradiated redgum except that 260 g of redgum was mixed with 49 mL of syrup to make Rg100%. To make RgS, 316.7 g of powdery irradiated redgum pollen, 158.3 g soy isolate, 25 mL (5 %) rum, 0.02 % w/w grape seed extract, and 200 mL of syrup was mix thoroughly. To make Psup, equal proportions of MixP, Rg100% and RgS were blended thoroughly.

Artificial feed: Soy protein isolate, micellar milk protein and whey protein concentrate are all from MyoPure™ in Australia (batch: 026210, 1233116822, and 1238116822 respectively). Also purchased in Australia: Brewer's yeast from Kakula's Brother; white cane sugar from Bundaberg; pure icing sugar from CSR™; Bee premix from DSM Australia Pty. Ltd.; and fibre or cellulose powder (CP118) from Chem-Supply of South Australia (batch no: 002509). Jarrah honey was from Wescobee of Western Australia. Cold pressed flaxseed or linseed oil (batch: 618280) was from Nature's Own of New Zealand. Almond oil was from Laboratories Pty. Ltd. of Victoria. And Evening Primrose oil (batch: 92067) was from Blackmores Ltd. of New Zealand. FeedBee® is a commercial honey bee food mix made by Bee Processing Enterprises Limited and distributed by Apiary Services Australia Pty. Ltd. in Australia.

To make Sub1 the following was used: 79 g soy protein isolate, 52.6 g brewer's yeast, 84.2 g micellar milk protein, 21 g whey protein concentrate, 263.2 g syrup, and 5 drops of flaxseed oil. To make Sub2 the following was used: 345 g soy protein isolate, 150 g fibre, 13 g Bee Premix, 50 g blend of almond oil, evening primrose, and linseed oil blend (1: 1: 1), 246 g Jarrah honey, 196 g pure icing sugar and 384 mL syrup. To make FB, 400 g FeedBee® and 174 mL syrup were used. PrSub contained equal proportions of Sub1, Sub2 and FB. The ingredients were combined and blended to make a soft patty.

All the different feeds were moulded into pre-weighed small Petri-dishes and pressed into standard wooden export cages (this was for the longevity experiment in Chapter 3). They were all sealed and frozen at -4 °C until the day of the experiments.

2.2.3. Bees

Frames of capped brood that were about to hatch were removed from their hives (any bees that were on the frames were shaken or brushed off) and placed in a clean wooden hive box inside a thermal regulated room. Brood frames were kept at 32-35 °C in the room for several days to allow for them to hatch. When sufficient numbers of bees have hatched (progress checked daily), 500-700 HNB were gently brushed into a funnel into a specially designed

cage placed on top of a two decimal Sartorius weighing balance. Approximately 58 g of HNB were brushed into each large cage.

Initially six cells containing different aged larvae were placed into each cage in attempt to allow the HNB to interact with the larvae as research have found this interaction to be important in stimulating the growth of the HG (Huang, Otis and Teal 1989, 460). However, instead of caring for the larvae, all the HNB cannibalised them. As a result, queens were introduced into the cages instead. The HNB in each cage were fed MixP for 7 days (after bees become 7-day-old, they are referred to as 7DAY bees) before their feeds were changed to the other seven feeds for another 9 days. Caged bees were kept in a Day-night thermostat controlled incubator from Lindner & May Pty. Ltd., Brisbane (serial: 521).

2.2.4. Cages

Large cages: these were made from plastic 760 mL honey tub (Silverlock WA packaging T30603) with a snap-on lid (Silverlock WA packaging T30404). For good ventilation, the base of the tub was cut out and an aluminium mesh screen floor was inserted (90 mm diameter).

To increase the ventilation, several 2 mm (diameter) holes were drilled on the lid, and a dozen narrow slots were cut into the sides of the container. The lid had two screws (on smaller lids) and two holes cut to size to fit water and sugar feeders which was finally made (after several prototypes were trialled) from a 25 mL syringe that contained sugar syrup. Two strips of screen mesh were attached to the screws on the underside of the lid to support the small cluster of bees. A small door was made at the base of the container to remove and insert feed and remove dead bees (see Figure 2.2).

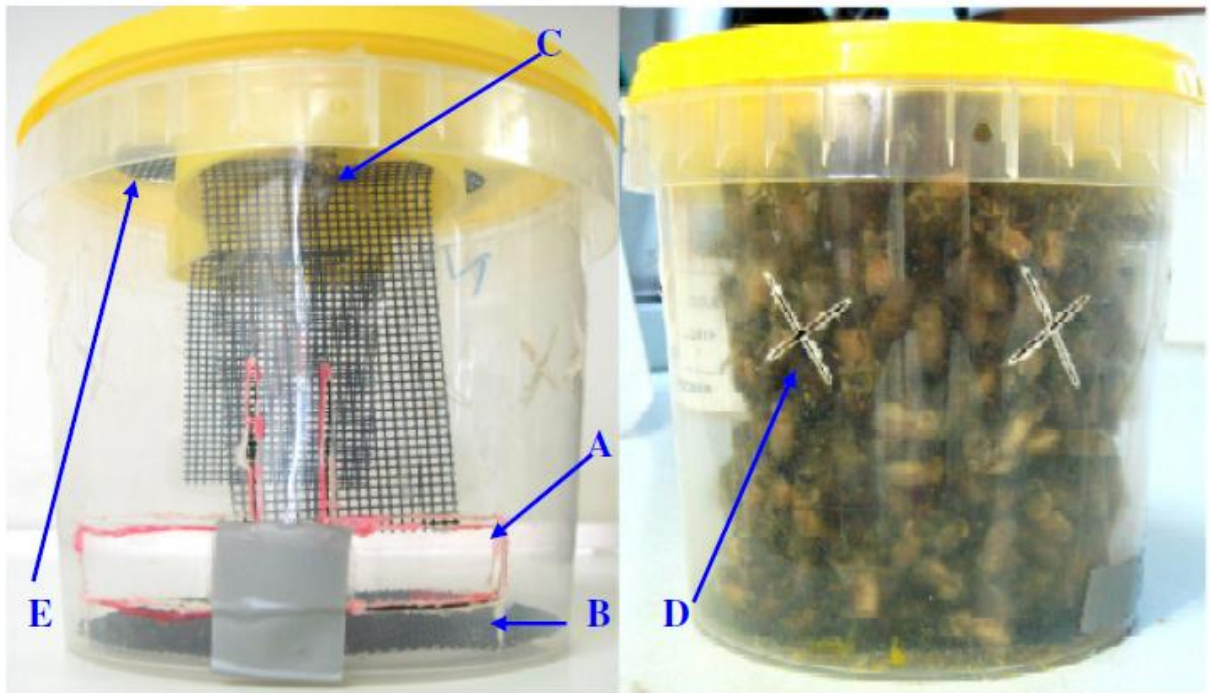


Figure 2.2: Left: picture is an empty large cage, and Right: shows the same cage filled with bees hanging onto mesh. A= door, B= aluminium mesh for ventilation, C= screw and mesh for bees to climb, D= narrow slits, and E= a hole for the water and sugar supply in the lid.



Figure 2.3: Bees in large cages inside incubator. The syringes used were 5 mL (earlier version).

2.2.5. Nosema count

The procedure for examining *N. apis* spores followed Cantwell (1970, 222-223) where 30 bee abdomens were used. An Improved Neubauer Counting Chamber was used to count the *N. apis* spores. Samples where spores were not identified were re-concentrated and view under the microscope again. To re-concentrate the solutions, the crushed bee abdomens were filtered through a 90 µm and 30 µm sieve into a beaker. The solutions were drawn into a standard 25 mL plastic syringe and pumped through a Millipore Swinnex® 25 Filter Holder (SX0002500 made in Massachusetts) containing a 11 µm Millipore Hydrophilic Nylon Net Filters (NY1102500 made in Ireland) into a 50 mL Axygen Conical-Bottom Centrifuge Tube (BD Bioscience SCT-50mL-500). The filtered solution was centrifuged for 30 minutes at 3000 g force in a Thermo Electronic Jouan CR4i Centrifuge (SN: 307050085, Waltham, Massachusetts). The supernatant was removed, and 1 mL of distilled water was added to resuspend the pellet. The resuspended pellet was then view under a microscope.

2.2.6. Measuring the development of the hypopharyngeal gland

There are many ways of measuring the size of the HG including dissection, volumetric estimation, weighing dry mass of the head, and histology. However such methods are time consuming and difficult. A relatively effective and novel way of measuring the impact of feed upon the HG is measuring the head weight of honey bees as developed by Manning (2006, 125). This method measures the effectiveness of the feed upon increasing the weight or stimulating the development of the HG. Each day, 50 bees were sampled randomly from each treatment, and their heads were removed using a sharp scissors and weighed individually on a Delta Range PB303 (Mettler Toledo Ltd. of Western Australia).

2.2.7. Statistical analysis

Using GenStat (D'Antuono 2009; GenStat Committee 2009; R Statistical System 2009) the analysis of variance was used to analyse and compare the mean head weights (mg) for the

bees on individual feeds over time (days) of sampling and the mean daily consumption of bees. The least significant difference or LSD was used to compare means for significance levels at $P < 0.05$. The plot of the means have error bars of 1.5 times the standard error of the mean. From the boxplots of the head weights (Appendix 4), we have assumed that the variability across times was approximately equal and hence the average variability was used to compute the standard errors. Therefore error bars look similar, and where there is no overlap, the differences are regarded to be significant.

Other graphs were produced using Microsoft Excel 2007. The correlation coefficient for feed consumption and head weight was analysed using Excel based on the Pearson Correlation Coefficient model. From standard statistical tables, the cut-off for an absolute value of the correlation coefficient to be significant ($P < 0.05$) with $N=9$ is 0.67 (D'Antuono 2009). Any correlation outside this range is regarded as not significant. The protein compositions for each of the feeds are estimated according to the protein content of the relevant ingredients. The full list of essential amino acids is listed in Appendix 3.

2.3. RESULTS

2.3.1. Head weight

Part 1: daily comparison of head weight between HNB fed on MixP and IS show that HNB that were fed MixP had significant higher head weight than HNB fed on IS on days two, six and seven, however it the other four days were not significantly different (Figure 2.4).

Head weight comparisons of the HNB fed MixP and IS were made with hive-raised bees head weight data from Dr. Rob Manning (summer 2003). The head weight trend of these hive-raised bees agreed with the size of the HG measure by Deseyn and Billen (2005, 52-56). The hive-raised bee head weights can give a rough guide to what might be expected of bees raised in their natural environment and allow comparison to cage-raised bees. Hive-raised bees showed a 14 % increase in head weight at 7 days whereas caged HNB fed MixP showed

a 15.6 % decrease and HNB fed IS showed a 24.5 % decrease. Hive-raised nurse bees maintained an mean head weight that was higher than 11.7 mg from 7-21 days which was significantly higher than MixP, IS and other fed feeds ($P < 0.05$).

The hive-raised nurse bees displayed an increase in head weight from 0 to 7 days and then slowly decreased to the head weight of newly hatched 0-day-old bees (undeveloped or non-functional gland) on the 28th day. Results indicated that good selections of pollen (protein) such as found in MixP could not develop the HG to the extent seen in hive-raised nurse bees. There was not enough head weight data of HNB fed MixP and IS to be compared with hive-raised bees which extended to 28 days. From 0 to 14 days, there seemed to be a distinct and constant decrease in the head weight of caged HNB fed MixP and IS (see Figure 2.5).

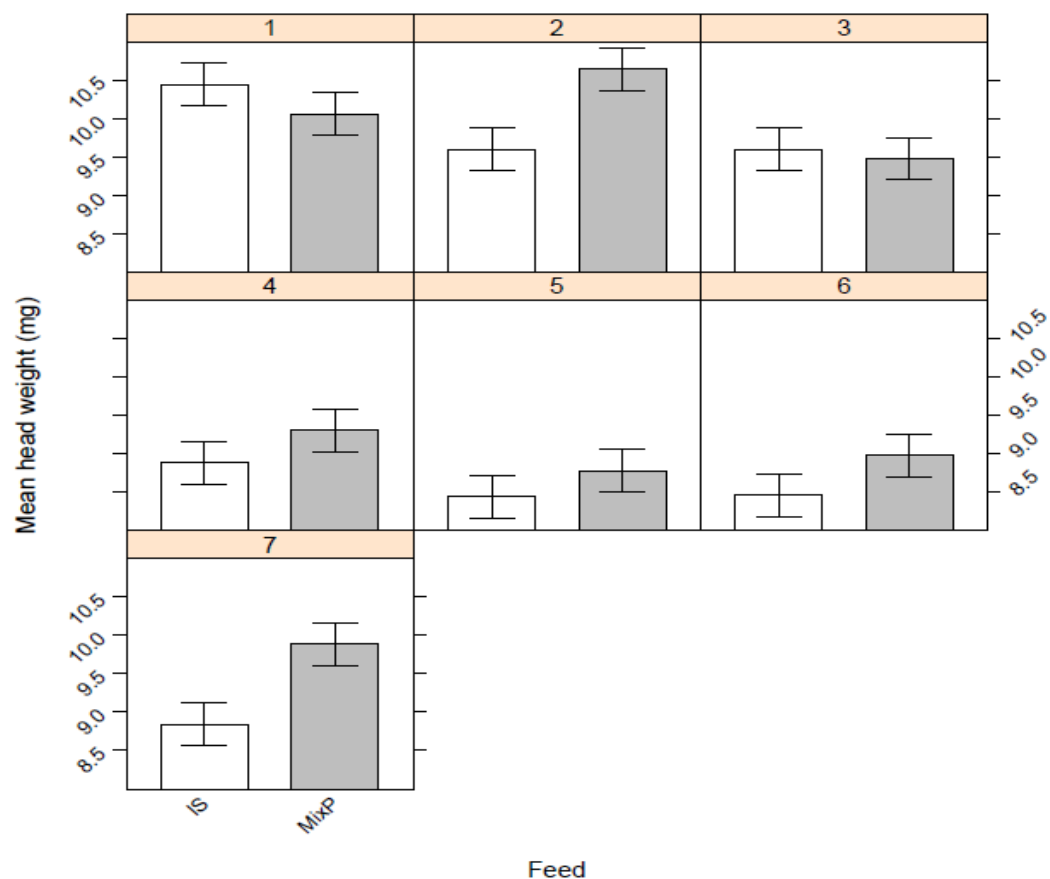


Figure 2.4: Daily mean (1-7 marked boxes) head weight (mg) comparison between hatched nurse bees fed on icing sugar (IS) as control and mix pollen (MixP) for 7 days. The error bars are 1.5 times the standard error of the means, where the bars do not overlap, it means they are significantly different to each other ($N=50/day$ per treatment).

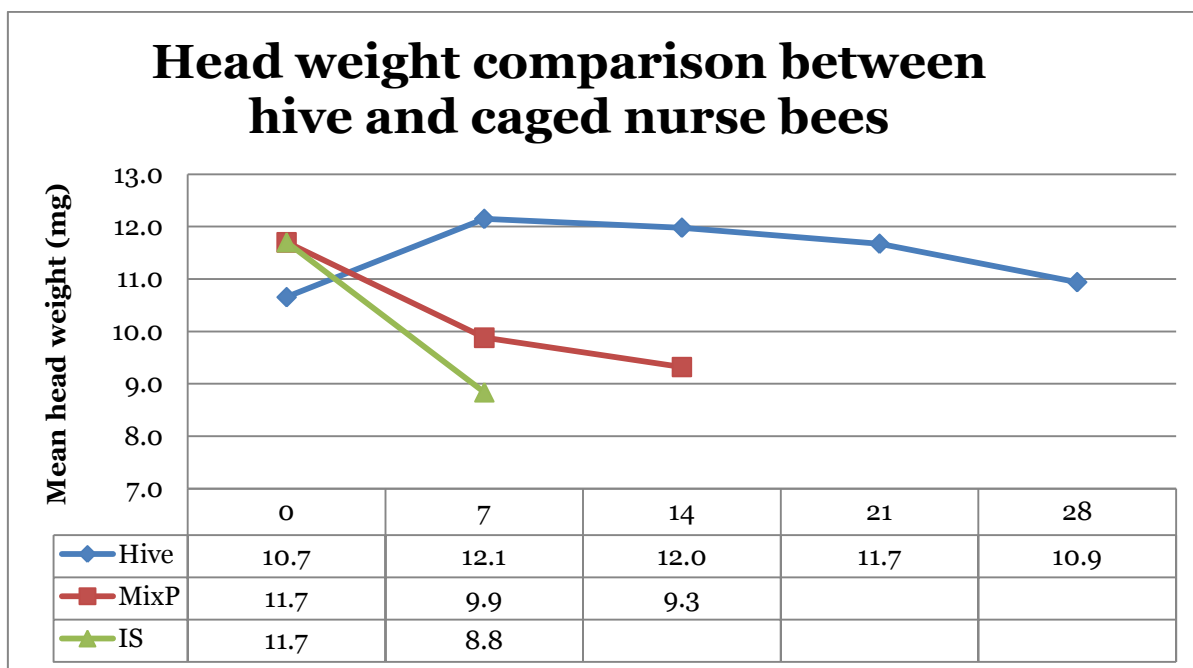


Figure 2.5: Mean head weight comparison between hive and caged nurse bees (0 to 28-day-old). *Hive*= bees that were raised in a healthy normal hive, *MixP*= caged hatched nurse bee (HNB) fed on MixP feed, and *IS*= caged HNB control fed IS (*N*=50/day per treatment).

Part 2 and 3: cage-raised HNB that were fed for 7 days on the MixP feed are now referred to as 7DAY bees, and mature nurse bees (MNB) will serve as the control fed on IS. These 7DAY bees were fed eight different feeds and their mean head weight are shown in Table 2.1. The 7DAY bees that consumed Sub2 had the highest head weight, followed by FB and Sub1 which were not significantly different to each other for days 9 to 11. The 7DAY bees fed Rg100% had the lowest head weights followed by, MixP, RgS and Psup. It was surprising to note that 7DAY bees that consumed Sub2 had significantly higher head weight compared to the MNB (IS) control on all the days except for days 12 to 14 and 16. Both 7DAY bees fed Sub1 and FB had significantly higher than MNB control, but only on days 12 to 14 and the remainder were either similar or significantly lower than the control. When PrSub was compared to Psup it was better at developing the head weight. The artificial feeds seem to have a better effect on the weight of the HG than feeds fortified with fresh or irradiated pollen.

Table 2.1: Mean head weight (mg) of 7-day-old (7DAY) bees fed on different pollen and artificial feeds for experimental days 9-17. MNB fed on IS was the control. Different colour circles represent significant difference ($P \leq 0.05$) while same coloured circles represent non-significant difference (LSD= 0.4819). *FB*= FeedBee®, *IS*= icing sugar, *MixP*= mix pollen, *PrSub*= pollen substitute, *Rg100%*= redgum pollen, *RgS*= redgum and soya isolate, *Sub1*= Substitute No. 1, and *Sub2*= substitute No. 2. On the chosen experimental days, *H*= highest head weight, *M*= median head weight, and *L*= lowest head weight ($N=50/\text{day per treatment}$).

Days	FB	IS	MixP	PrSub	Psup	Rg100	RgS	Sub1	Sub2
%									
9	11.64 ●	10.00 ● M	9.16 ● L	10.98 ○	9.96 ●	9.76 ●●	9.34 ●●	11.76 ●	11.90 ● H
10	11.08 ●	10.08 ● M	9.02 ●	10.12 ●	9.64 ●	8.70 ● L	9.02 ●	10.86 ●	11.28 ● H
11	11.06 ●○	10.04 ● M	9.34 ● L	11.34 ○ H	9.94 ●	9.48 ●	9.42 ●	10.72 ●	11.10 ●○
12	10.94 ● H	10.60 ●●	9.52 ●	10.74 ●●	9.68 ●	8.86 ○	7.90 ● L	10.44 ●	10.30 ● M
13	10.00 ●●	10.28 ●	8.64 ●	10.34 ●	8.16 ● L	8.36 ●	8.64 ●	9.58 ● M	10.36 ● H
14	10.82 ● H	10.44 ●●	9.32 ●	10.42 ●●	9.38 ●	8.62 ○ L	9.18 ●	10.26 ● M	10.78 ●
15	10.14 ●	10.28 ●	8.88 ●	10.38 ●	8.98 ●	8.50 ● L	8.76 ●	10.02 ● M	10.96 ● H
16	9.82 ●●	10.14 ● H	8.58 ●●	9.56 ●○	8.94 ●●	8.10 ●● L	8.58 ●●	9.54 ●○	9.28 ○● M
17	11.42 ●●	11.14 ●●	7.94 ○ L	11.58 ●	8.46 ●	8.18 ○●	8.20 ○●	10.88 ● M	11.70 ● H

Figure 2.6 shows a comparative overview of the head weight of bees fed different feeds over time (see Appendix 4 for bee head weight on individual feed over experimental days 9 to 18).

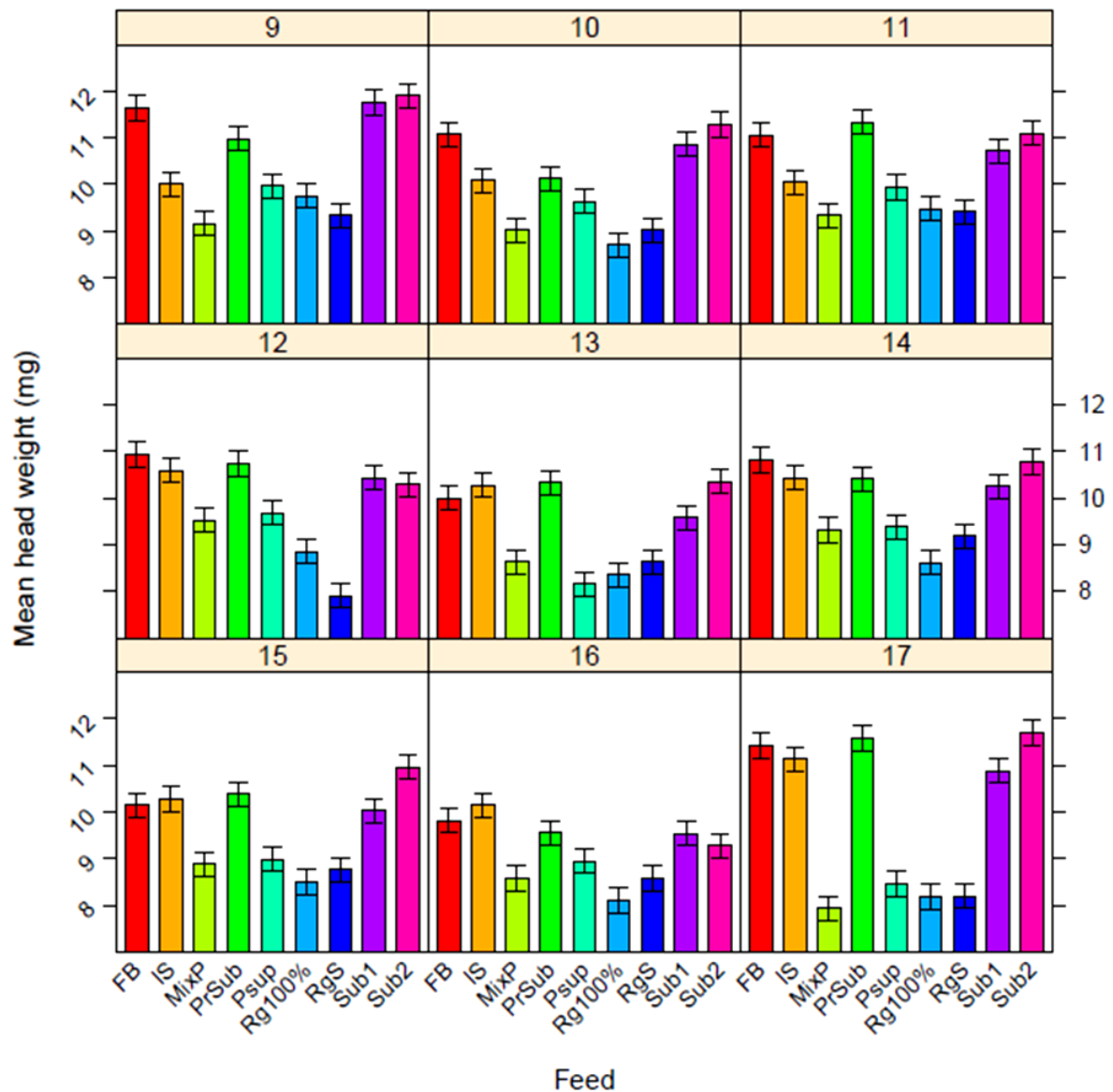


Figure 2.6: Daily mean head weight (mg) comparison between mature nurse bee (MNB) fed IS and 7-day-old (7DAY) bees fed four pollen and four artificial feeds over experimental days 9-17 (9 days). The error bars are 1.5 times the standard error of the means, where the bars do not overlap, they are significantly different to each other. *FB*= FeedBee®, *IS*= icing sugar, *MixP*= mix pollen, *PrSub*= pollen substitute, *Psub*= pollen supplement, *Rg100%*= redgum pollen, *RgS*= redgum and soya isolate, *Sub1*= Substitute No. 1, and *Sub2*= substitute No. 2 ($N=50/\text{day per treatment}$).

Majority of the head weights of 7DAY bees fed artificial feed were above 10 mg, whereas bees fed pollen were all below 10 mg. The head weights of the MNB control were significantly higher than head weight of the pollen fed bees.

2.3.2. Feed consumption in relation to head weight

During the seven day developmental period, all bees were fed MixP and the indexed daily consumption per bee is shown in Figure 2.7. Each bee consumed 5.05 mg on their 1st day whilst diminished to 0.15 mg by the 7th day. After replacing the MixP with a fresh dish of MixP the consumption increased to 2.72 mg by the 9th day before slowly decreasing again to as low as 0.02 mg by day 17 (see Figure 2.8). This trend was also observed for other feeds where fresh feed was readily consumed, but declined over time (see Figure 2.8). Within the 9 day experiment, MixP and Sub1 was most consumed averaging 7.08 mg/bee followed by 6.14 mg/bee for FB, 4.51 mg/bee for Sub2, 4.25 mg/bee for PrSub, 3.58 mg/bee for RgS, 3.20 mg/bee for Psup and 1.86 mg/bee for Rg100% (see Figure 2.9).

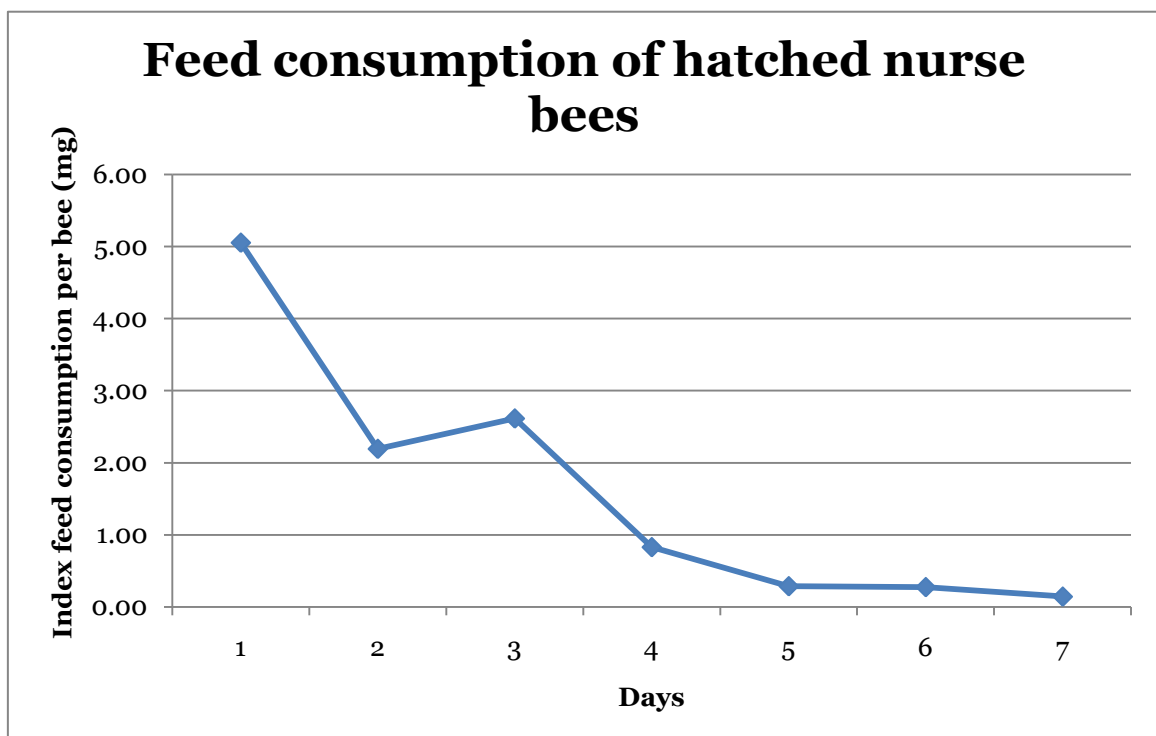


Figure 2.7: Mean mix pollen (MixP) consumption per bee (mg) for experimental days 1-7.

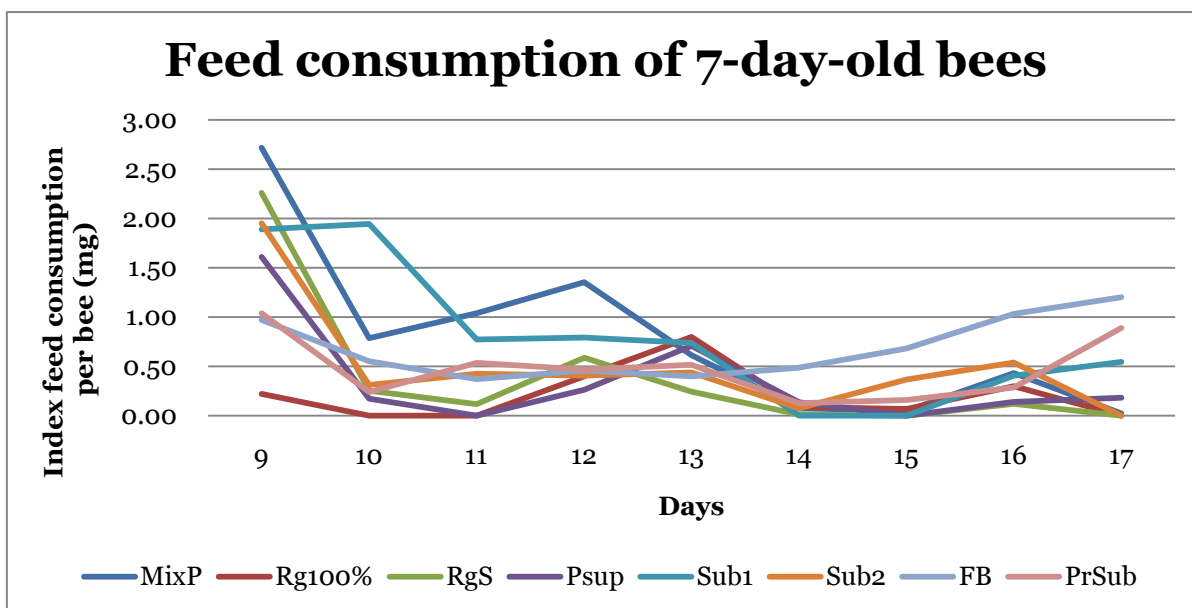


Figure 2.8: Daily index feed consumption per bee in mg. *FB*= FeedBee®, *Psup*= pollen supplement, *MixP*= mix pollen, *PrSub*= pollen substitute, *Rg100%*= redgum pollen, *RgS*= redgum and soya isolate, *Sub1*= Substitute No. 1, and *Sub2*= substitute No. 2 (*N*=50/day per treatment).

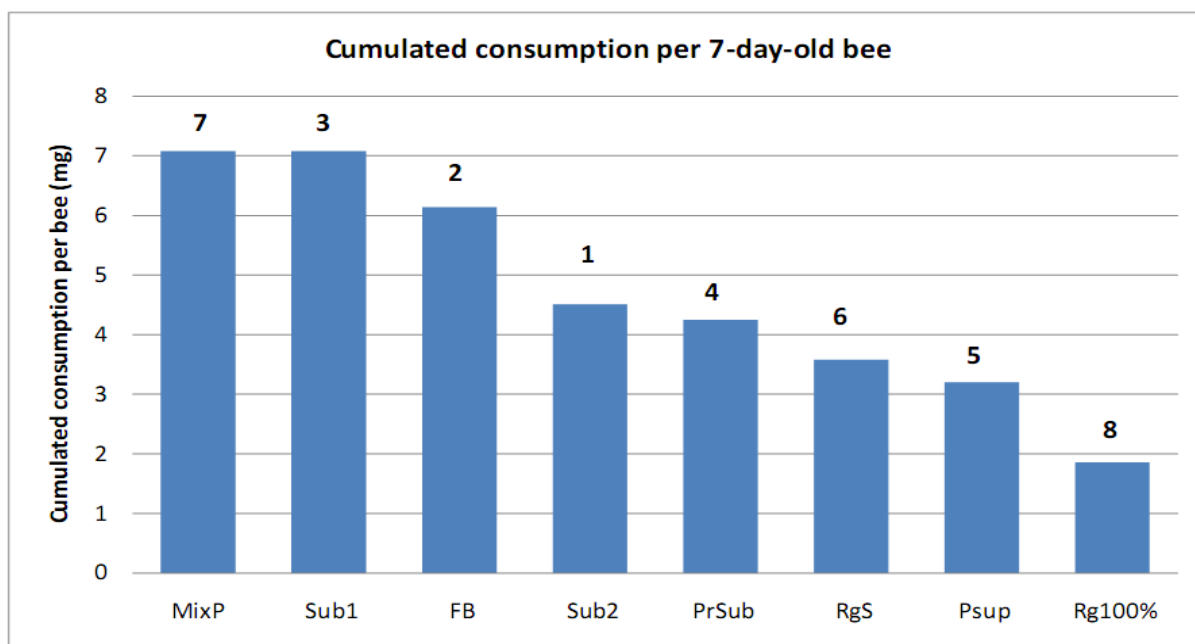


Figure 2.9: Total cumulative feed consumed (mg) by each bee from days 9-17. 1= highest, 8= lowest ranking for head weight associated with being fed the different feeds. *FB*= FeedBee®, *Psup*= pollen supplement, *MixP*= mix pollen, *PrSub*= pollen substitute, *Rg100%*= redgum pollen, *RgS*= redgum and soya isolate, *Sub1*= Substitute No. 1, and *Sub2*= substitute No. 2.

Table 2.2 showed the Person Correlation Coefficient of feed consumption per bee and mean head weight per bee. All the feeds had a positive correlation except for Rg100%, however only PrSub and Sub1 were significant (above 0.67) and the remainders were weak (refer to Appendix 5 for more details).

Table 2.2: Correlation between head weight per bee and feed consumption per bee where 0.67 is the significant cut-off value ($P < 0.05$) for all feeds ($N=9$). *= $P < 0.05$, FB= FeedBee®, *Psup*= pollen supplement, *MixP*= mix pollen, *PrSub*= pollen substitute, *Rg100%*= redgum pollen, *RgS*= redgum and soya isolate, *Sub1*= Substitute No. 1, and *Sub2*= substitute No. 2.

Artificial feeds	Correlation coefficient	Pollen feeds	Correlation coefficient
PrSub	0.69*	MixP	0.47
Sub1	0.68*	RgS	0.28
FB	0.19	Rg100%	-0.19
Sub2	0.18	Psup	0.16

One of the most consumed feed was MixP, but it stimulated the second lowest head weight. Sub1 and FB was also consumed at high levels, but they stimulated the second and third highest head weight. Bees fed on Rg100% had the lowest feed consumption and head weight. Sub2 was consumed on a moderate level, but interestingly it promoted the heaviest head weight.

Both PrSub and Sub1 feeds had a strong positive correlation between consumption and head weight, meaning it is very likely that if bees consume more of the food, their head weight will increase. All the pollen based feeds had non-significant correlation as the values were all below 0.67. The protein composition of all the feeds was estimated and they are shown in Table 2.3.

Table 2.3: Estimated protein composition in feeds. * Ideal consumption of feed to achieve 1 mg/bee per day of protein. ^ The pollen source is unknown, but many native floral species of Australia have CPC of 25% (Stace, 1996) thus this was used to calculate the approximate protein content in MixP feed. *FB*= FeedBee®, *Psup*= pollen supplement, *MixP*= mix pollen, *PrSub*= pollen substitute, *Rg100%*= redgum pollen, *RgS*= redgum and soya isolate, *Sub1*= Substitute No. 1, and *Sub2*= substitute No. 2.

Feed	Crude protein content in feed (%)	Consumption per day		
		Ideal feed consumption (mg/bee)*	Actual feed consumption (mg/bee)	Estimated protein consumed (mg/bee)
MixP	17.9 [^]	5.59	0.79	0.14
Rg100%	22.5	4.45	0.21	0.05
RgS	34.1	2.93	0.40	0.14
Psup	24.8	3.88	0.36	0.09
Sub1	41.0	2.44	0.79	0.32
Sub2	25.9	3.86	0.50	0.13
FB	25.0	4.00	0.68	0.17
PrSub	30.6	3.27	0.47	0.14

Black (2006) estimated that the daily protein requirement of a bee is 0.5 mg, but based on 70 % digestibility of pollen and 70 % biological value, 1 mg/bee per day is required (0.5/0.7/0.7). To obtain the daily protein requirement of 1 mg/bee from pollen containing 25 % Crude Protein Content (CPC), a bee must consume 4 mg of the feed (1/0.25). The ideal protein intake varies in correspondence to the percentage CPC found in pollen or artificial feeds. According to calculations all the bees in the experiment did not eat the ideal amount of protein required for healthy development.

On average, bees fed Rg100%, and Psup received less than 10 % of their daily protein requirement for healthy development of the HG. Bees fed on MixP, RgS, PrSub, and Sub2 received less than 15 %, and only bees fed on FB and Sub1 received more than 15 % of the daily protein requirement. Results suggested that the amount of protein consumed is not related to the head weight, for example, bees fed Sub2 had the heaviest head weight but each bee only consumed about 13 % of the daily protein requirement, while bees fed MixP had the second lowest head weight but each bee consumed 14 % of the daily protein requirement.

2.3.3. Nosema infection

Nosema spores were present in caged bees of all feed treatment. Figure 2.10 show the spore counts for the 9 days that bees were fed the experimental feeds.

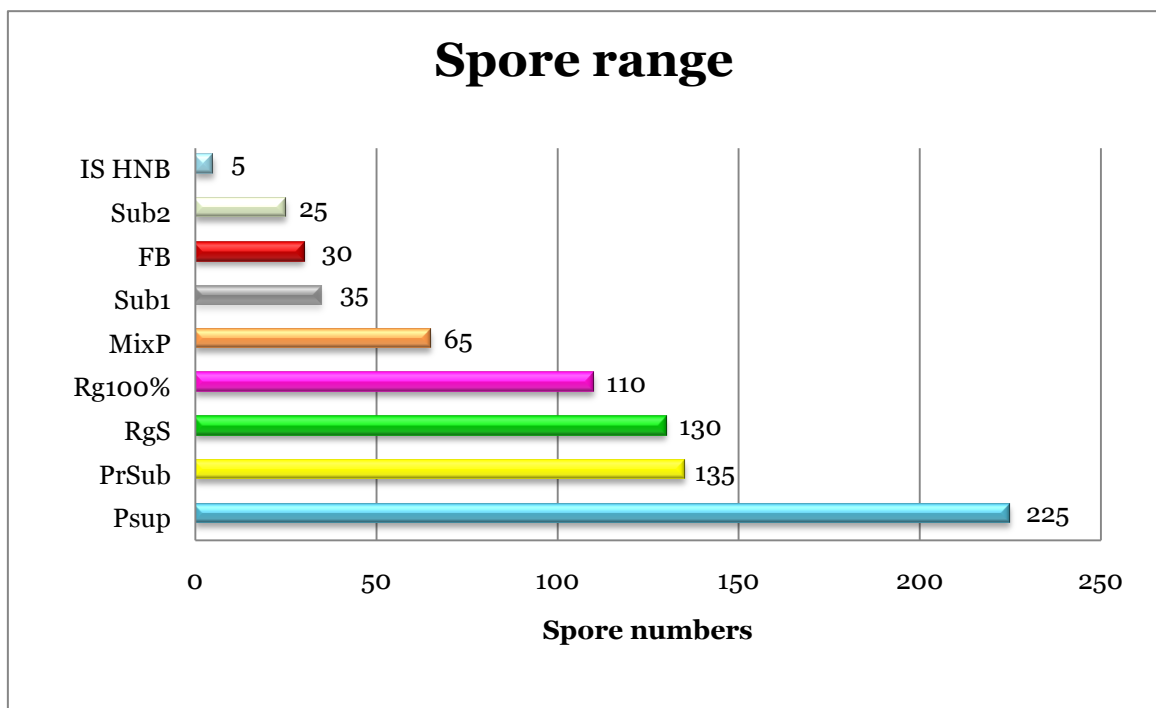


Figure 2.10: Spore range (in 10,000s). *IS HNB*= hatched nurse bees fed icing sugar, *FB*= FeedBee®, *Psup*= pollen supplement, *MixP*= mix pollen, *PrSub*= pollen substitute, *Rg100%*= redgum pollen, *RgS*= redgum and soya isolate, *Sub1*= Substitute No. 1, and *Sub2*= substitute No. 2 (*N*= 300/feed).

All the bees had a spore range of 0 to 2.25×10^6 except for MNB fed on IS which had a minimum of 4.75×10^6 . HNB (on IS) had the lowest overall count, and Psup had the highest count. Figure 2.17 does not show MNB spore counts as they exceeded 3.0×10^7 . The minimum spore numbers found in MNB compared to the maximum spore numbers found in HNB (IS) was higher by 95 fold and in 7DAY bees feed on Sub2 was 19, FB was 15, Sub1 was 13, and MixP was 7 folds higher.

Despite careful isolation of the bees from the hive, various plant pollen as well as *N. apis* spores were observed in 0-day-old HNB (see Figure 2.18). This indicated that nurse bees have had contact with pollen and *N. apis* spores prior to the commencement of the experiment.

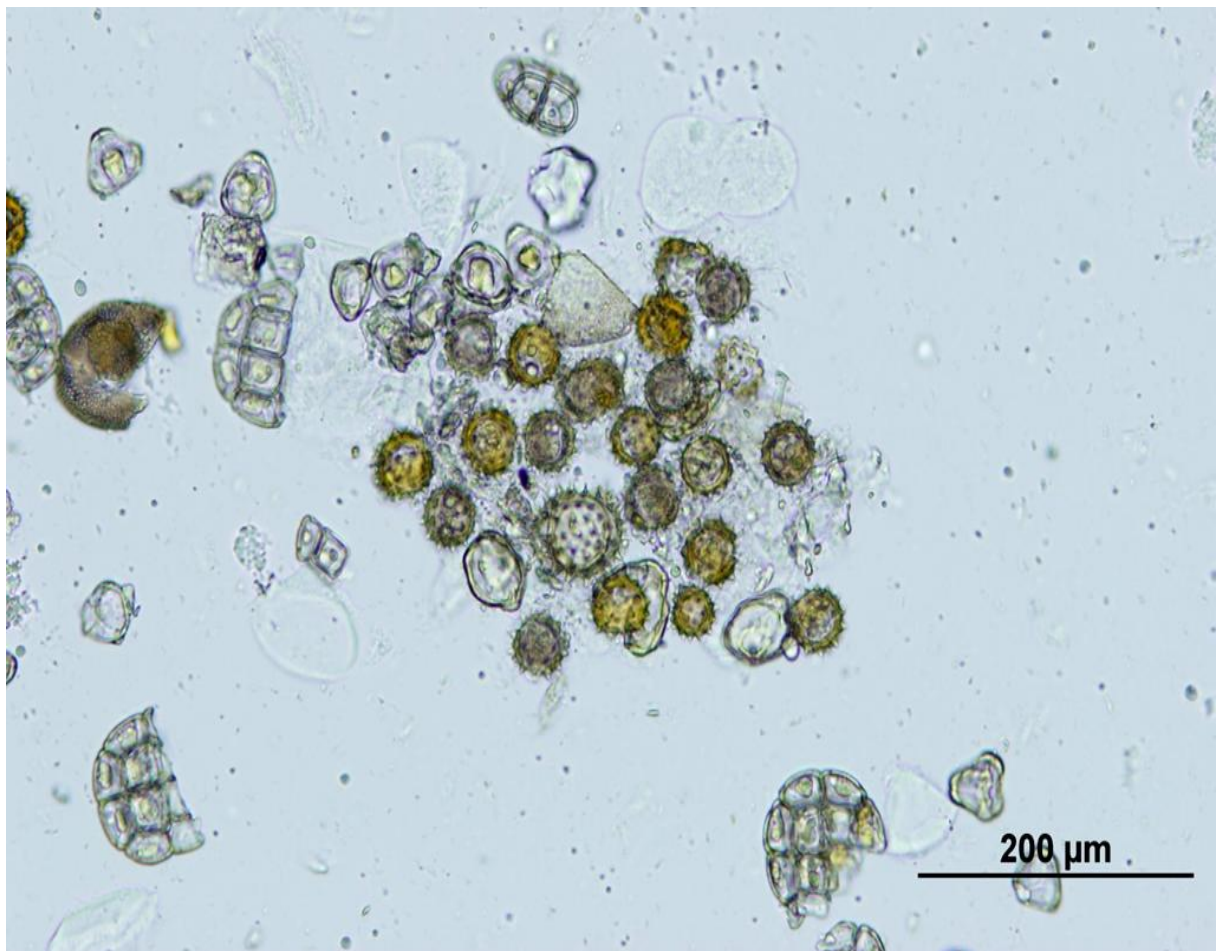


Figure 2.18: Various types of pollen found in the gut of 0-day-old hatched nurse bees (200 x magnifications).

2.4. DISCUSSION

2.4.1. Head weight

The first aim of this experiment was to see whether cage-raised HNB can fully develop their HG when fed different pollen and artificial feeds. To this day, no one has been able to formulate a feed that can replace or indeed exceed the quality of pollen. This is the first experiment to effectively measure the HG development in response to different pollen and artificial feeds and showed that two nutritionally formulated feed and one commercial feed were able to stimulate significantly higher head weight compared to all other pollen feeds tested.

To give the best nutrition to caged HNB to develop their HG, they were fed MixP, fresh water and sugar syrup. In attempt to stimulate and activate the HG, cells of royal jelly contained six different staged larvae were placed in queen cells inside each cage. However, all the larvae were cannibalized the following day, and a mated queen was introduced instead. In nature, during periods of high demand where protein supply can be low, bees commonly cannibalize larvae for their rich protein source or when the larvae are ill (Schimickl and Crailsheim 2002, 423; Webster, Peng and Duffey 1987, 225). In this case there was plenty of pollen (protein); however the rich protein from the royal jelly (from older nurse bees or cannibalized larva) could be essential ingredients which shape the development of bees.

Many studies have been performed on the diameter of acini, size and chemical activity of the HG to understand their typical developmental phases. The head weight positively correlates to the acini size and development of the HG and many researchers have used the HG development as a criterion to judge the dietary protein adequacy of pollen or artificial feeds (McCaughey, Gilliam and Standifer 1980, 76). The head weights for hive-raised bees suggested that after hatching, the HG continues to grow up to the 7th day then slowly shrink to the weight of an a newly hatched bee on the 28th days. In Deseyn and Billen's (2005, 52-56) experiment, the HG of the bees shrunk to the size of an undeveloped or non-functional gland

15 days after hatching. All the HNB that were fed experimental feeds could not develop comparable head weights to the hive-raised bees.

The 7DAY bees fed artificial feeds stimulated mean head weights greater than 10 mg, whereas bees fed pollen feeds were less than 10 mg. Manning (2006, 112-120) observed that fully developed glands are more than 10 mg, but interestingly showed that HNB with semi developed HG were still capable of producing sufficient royal jelly to sustain low levels of reproduction within a small colony. This could mean that bees fed Sub2, FB and Sub1 feed were able to stimulate full growth of the HG and also was able to secrete royal jelly. MixP represented a nutritionally balanced feed, because it contained more than 10 different pollens. The fact that Sub2, FB, Sub1 and PrSub stimulated heavier head weight than MixP clearly indicated that those feeds had superior nutrition.

One could argue that because some of the pollen which made up the MixP feed was more than 1-year-old that it would have lost 75 % of their ability to stimulate the HG as suggested by Haydak (1961, 345). All the pollens used were freshly collected and stored at -20 °C and were only defrosted when they were irradiated. Hagedorn and Moeller (1968, 89) illustrated that feeds fortified with either 1-year-old pollen or fresh pollen stimulated the same HG development. Similarly, Pernal and Currie (2000, 402) found that the HG development between nurse bees fed with freeze stored 1-year-old pollen and fresh pollen were identical. Dietz and Stevenson (1980, 143) recorded that dried frozen pollen was able to support brood rearing even after 11 years of storage. The irradiation has little affect in the nutritional composition according to a study conducted by Manning (2006, 135 and 173) where he found that the fresh and the irradiated six year old redgum had similar protein, fatty acid and mineral profile and that both were equally effective in the longevity experiment.

Despite the availability of a good protein source (MixP) for HNB in cages, their head weight continued to decline after emergence. It seemed unarguable that there are other important physiological stimuli lacking which prevented further growth of the HG, such as the absence of uncapped egg and larvae (Huang, Otis and Teal 1989, 460), trophallactic interaction

(Huang and Otis 1989, 264), and different aged bees (Huang and Robinson 1992, 11727). Lass and Crailsheim (1996, 347) observed that the trophallactic donation by older bees to younger nurse bees were not the key regulatory factor in HG development and functionality but this could not be ruled out for sure. Contrary to this theory, Manning (2006, 112-120) fed irradiated redgum pollen to caged HNB which lasted more than a month, and some queens laid eggs that reached the larva stage and some pupae stages and some actually hatched. In his experiment, he proved that HNB did not need to be exposed to older nurse bees to develop and activate the HG. However it remains unclear whether secretion of royal jelly was a result of egg laying by the queen or if the queen began egg laying because she knew her nurse bees were capable of secreting royal jelly. In the hive, Crailsheim saw that 1 to 3-day-old bees received most of their food from 7 to 14-day-old bees which indicated that the main food source for those bees was royal jelly (1990, 418).

Fifty percent of the dry weight of royal jelly is made up of protein (Li *et al.* 2007, 8411), 46-89 % of which is water soluble (Chen and Chen 1995, 195). This means that protein from royal jelly can be readily ingested and absorbed by bees, therefore young bees can absorb a high amount of protein very quickly. In this experiment, only small amounts of royal jelly were provided, and thus HNB had to rely heavily on eating pollen to get protein and low digestibility would unquestionably hinder protein absorption.

Difference in pollen digestion and absorption is dependent on the age of the bee, type of pollen and seasonality of pollen, and in general, only 77 % of pollen is digested (Crailsheim 1990, 419) but ranges from 30-90 % (Black 2006). The time required for pollen digestion range from few hours to a day because of its tough exterior wall (Crailsheim 1990, 419). Another factor which reduces digestibility is the absence of microorganisms in the pollen. Microorganisms found in beebread are believed to be responsible for pre-digestion (fermentation) and secreting digestive enzymes which enhance pollen digestibility in bees (Fernandes-Da-Silva and Serrão 2000, 42). Because all the pollens were sterilized using gamma irradiation, it was highly probable that the beneficial microflora was also inactivated. Poor pollen digestibility could have contributed to slow and low protein absorptions for the

caged HNB when quick and high protein absorptions were needed hence the suppressed HG development.

The second aim was to see whether there was a relationship between how much bees eat and how well their HG develop. In this experiment, only the consumption of PrSub and Sub1 had a strong positive relationship with the head weight.

Honey bees can be fastidious eaters, even known to discard older pollens when there is abundance of fresh pollen (pers. com. Dr. Rob Manning of Department of Agriculture and Food, Government of Western Australia, September 2, 2009), and chooses food based on complex visual, olfactory and taste cues. Bees cannot distinguish protein composition and the nutritional value of food which make studying the effect of good nutritional food particularly difficult (Pernal and Currie 2002, 369).

Black (2006) estimated that honey bees need on average 1 mg of ideal protein per day (essential and non-essential amino acid). In this experiment, MixP contained only 17.9 % CPC and bees consumed 0.79 mg per day compared to RgS which contained twice the CPC but bees consumed only half as much. Bees only consume 5-32 % of the ideal protein needed for healthy development. This could be the key reason why the development of the HG of caged bees was so different to with the development of hive-raised bees.

Sub2 was estimated to have 25.9 % CPC, and on average, a bee would have absorbed 0.13 mg per day of protein. Sub1 was anticipated to have the highest CPC at 41 %, and based on the calculations a bee would receive 0.32 mg per day of protein. Surprisingly, Sub2 showed considerably higher head weight than Sub1 and FB. This is contrary to the prediction that higher protein consumption would lead to higher head weight. Substitute No. 2 was formulated based on optimum health requirements for honey bees consisting the right balance of cellulose (fibre), vitamins, mineral, carbohydrate and protein. Vitamins are essential for the growth and development of honey bees; in particular B-group vitamins are required for brood rearing. It has been shown that a vitamin fortified feed can considerably increase the HG and

allow nurse bees to secrete royal jelly (Dietz 1975, 137-138; Herbert, Vanderslice and Higgs 1985, 29). This feed was able to stimulate significantly higher head weight than MixP, FB and Sub1 even though their consumption and CPC were higher. This agrees with the finding by Standifer *et al.* (1970, 909) that the CPC does not necessarily influence how well the HG will develop. From this observation it can be concluded that it was the actual composition of the Sub2 which stimulated good HG development. This also proved that a well balanced artificial feed such as Sub2 can be an effective substitute for pollen during period of pollen shortage to sustain hives or to strengthen hives.

Food consumption varied from day to day in this experiment, in the first two days fresh food was consumed in large amounts, but diminished over time. There are several plausible reasons for bees to consume more during the first two days, the first being the moisture loss in the food and the second being fresher therefore it has higher appeal.

There are some external influences on the consumption of feeds, such as continuous moisture loss which varied depending on the amount of syrup (or liquid) used to make the feeds. Over time, the feeds became drier and lighter, and therefore bees could consume the same volume of food (same protein intake) yet the measuring apparatus may show misleadingly lower weight because of moisture loss.

Another reason for declining consumption could be explained by the apparent behavioural differentiation in bees. There are disadvantages in having only HNB in cages in experiments because there are tendencies for temporal division of labour to re-establish normality in colonies (Robinson *et al.* 1989, 110). When large numbers of foragers or worker bees are removed from a colony, young nurse bees become precocious foragers (Huang and Robinson 1996, 147-148 and 1992, 11727). As nurse bees become foragers their lipid and protein stores become depleted (Toth *et al.* 2005, 4641) due to drastic reduction in their protein (pollen) intake notably after 10 days (Dietz 1975, 132). According to Haydak (1937, 258), wax workers and foragers feed almost exclusively on carbohydrate food such as nectar and honey.

During the cage experiment, this important social transformation must be factored in when considering the possible motive for decreasing feed consumption.

During the course of the experiment, some bees began pollen collection (foragers), while others circulated the cage and the entry (guard bees), and some were secreting wax. This fits with the theory of behavioural differentiation and as a result, some bees may have reduced their protein intake.

2.4.2. Nosema infection

The last aim of this experiment was to produce bees free from nosema infection. However spores were identified in half the samples. This suggests that isolating HNB from other bees in the apiary is not sufficient enough to prevent nosema disease. The infection in 7DAY bees were evidently less severe compared to MNB, so this method could still be implemented to reduce initial level of infection, but not to prevent it.

Pollen was found in the mid-gut of a 0-day-old bee, suggesting that prior to the experiment, the bees already had contact with pollen from the combs they hatched from. A recent study by Hige *et al.* (2007b, 77) showed that nosema spores were detected in bee bread and it was believed to be left behind by infected foragers that lick the pollen as they moisten the pellet. This is a highly probable route of infection.

It is also perhaps the inevitable programming of nurse bees which make the spread of *N. apis* so easy, because one of the first things HNB do after they hatch is to clean the nest (Sigg, Thompson and Mercer 1997, 7148; Stephen 1975, 333) which involves licking faecal matter and chewing up old wax (Gary 1975, 219). If the faecal matter was contaminated, the HNB would become infected. This is also an equally likely reason for infection. The infection could have also compromised the development of the HG.

2.5. CONCLUSION

This is the first experiment to prove that nutritionally balanced artificial feed such as Sub2, Sub1, and FB to be significantly better than the pollen at stimulating the HG development. Sub2 was the better feed of the three, because it stimulated higher head weights with less consumption. The materials used to make Sub1 and Sub2 are readily available for purchase, and they may prove to be more economical than purchasing pollen.

To further understand whether long term feeding of Sub2, FB and Sub1 could be more beneficial than pollen, a more detailed study should be conducted over a longer period of time. It would also be beneficial to test (gas chromatograph or staining technologies) whether cage-raised bees can indeed secrete royal jelly and to compare that capacity to hive-raised nurse bees. Results from such study will give further insights into how the HG works in absence of normal stimuli found in the hive.

There was no significant relationship between how much feed (protein) bees consume and how much their glands will grow except for PrSub and Sub1. This part of the experiment clearly showed that protein level is not the key factor that determines how well the HG develops. There are still many gaps about the nutritional requirement of bees which warrants more research.

Prior to doing this experiment, a preliminary experiment was carried out during which time *N. apis* spores could not be identified in samples. However, during the experiment, the same attempt to produce nosema-free nurse bees was unsuccessful because nosema was found in the bees. Therefore, it is clear that isolating HNB from the apiary bees is not sufficient in preventing the disease. In order to prevent the disease the comb would have to be decontaminated prior to hatching nurse bees or nurse bees must be immediately removed from the comb as they hatch which would be extremely time consuming. To produce nosema-free nurse bees on a commercial scale, it is therefore impractical to hand pick every nurse bees as they hatch. Currently there are no chemicals which can decontaminate the comb without

causing harm to the eggs, larvae and pupae, and no technologies to automatically remove hatching nurse bees. While it is not possible to produce nosema-free bees, the next step forward would be to find and breed naturally resistant bees or modify bees on a molecular level, namely to produce genetically resistant bees, and these bees might be acceptable to authorities in Japan as escort bees.

CHAPTER 3: LONGEVITY STUDY INTO THE ABILITY OF HATCHED NURSE BEES IN ESCORTING VIRGIN QUEEN BEES

3.1. INTRODUCTION

Japan's quarantine restriction against honey bees with *Nosemosis* or nosema has prompted interest into producing nosema-free escort nurse bees for queen export. After the suspension of Australia queen export to Japan in 2007, their fruit farmers have had to hand pollinate many of their crops because they could not buy enough honey bees (Kamiya 2009). In 2006, Japan was one of the markets that contributed to the 4 million dollars live bee sales in Australia (New South Wales Department of Primary Industry 2007), and as a result of the export suspension, beekeepers have suffered financial losses.

Nosema is a crippling disease of honey bees caused by *Nosema apis*, an intestinal protozoa that ruptures the intestine epithelial cells. The rupture causes swelling and constriction of the ventriculus which prevents nutrient absorption leading to malnutrition (Shimanuki and Knox 2000, 17). As a result, overall productivity of honey bee decreases, developing glands become deformed, and strong hives become weak (Furgala and Mussen 1978, 66; Gochnauer, Furgala and Shimanuki 1975, 638-639). Malone and Gatehouse (1998, 171-173) found a severe reduction in digestive proteolytic enzymes and protein digestion three days after infection. Young bees that become infected lose optimum digestion hence their hypopharyngeal glands (HG) do not receive the required protein needed for normal development.

When Hornitzky (2005) compared honey production of healthy hives with infected hives, he found a 24.5 kg difference. He also found that infected queens produce 12 % less compared to healthy queens and in some cases, depopulation would eventuate when the rate of death exceeds rate of birth. Assuming that a commercial beekeeper owns 300 infected hives and he

receives \$5 per kg on honey, and every honeyflow he loses 24.5 kg/hive, he would lose 7350 kg which would be equivalent to \$36,750 per honeyflow.

The problems with producing nosema-free bees are that bees can become rapidly infected through contact with infected bees, eating contaminated food or cleaning contaminated combs (Bailey and Ball 1991, 64-72; Fantham and Porter 1912, 163-189). One spore is sufficient to cause an infection and within 7-9 days the spores become mature and can replicate to 50 million spores in a short time (Bailey and Ball 1991, 64-72; Hornitzky 2005; Malone and Gatehouse 1998, 172). Currently there are no cures for nosema however chemicals such as Fumidil B and Protofil can reduce the severity (Chioveanu, Ionescu and Mardare 2004, 31; Fries and Camazine 2001, 199-202). Fumidil B inhibits the DNA replication of *N. apis* thus affecting the RNA synthesis while Protofil disintegrates the hyphae and cytoplasmic membrane (Hornitzky 2005; Morgan, Mandava and Ignoffo 1985, 65; Soković *et al.* 2009, 240-245).

Traditionally during export, each mated queen that is caged is escorted by several mature nurse bees (MNB) that can nurture and feed queens with royal jelly. This proteinous food is synthesized by a fully functional HG which MNB (hive-raised) typically have (Haydak 1970, 143). In a healthy hive where appropriate stimulation and pollen are present, newly hatched nurse bees (HNB) can fully develop their HG within 6-12 days (Huang and Otis 1989, 265). Standifer (1967, 419) and van der Steen (2007, 114) showed that when HNB are raised in isolation of the hive, they can still develop a functional HG when proteinous food is available to them.

The effect of eight different pollen and artificial feeds on the development of the HG have already been thoroughly investigated and presented in the previous chapter. This chapter will assess whether cage-raised HNB fed on 16 different pollen and artificial feeds can keep 100 % of the queens alive for more than 8 days.

3.2. METHODS

3.2.1. Experimental design

This experiment was divided into three parts. Method for caging and feeding HNB was described in Chapter 2.

Part 1: 0-day-old HNB which had no prior food were caged with newly hatched virgin queens in export cages (see Figure 3.1). They were fed Rg10%, Rg15%, Rg20% and Rg25%. Bees were kept in a thermal regulated room. The number of dead queens and the nurse bees were recorded daily for 10 days.

Part 2: caged 0-day-old HNB consumed either pure irradiated redgum or FeedBee® (FB) for 7 days. After 7 days, these nurse bees (will be referred to as 7DAY bees hereafter) were caged with newly hatched virgin queens. In the export cages (see Figure 3.1), 7DAY bees were fed Rg10%, Rg15%, Rg20%, Rg25%, FB10%, FB15%, FB20%, FB25% or IS as a control. MNB also served as another control. The number of dead queens and the nurse bees were recorded daily for 10 days.

Part 3: caged HNB consumed pure irradiated MixP for 7 days. And after 7 days, these 7DAY bees were caged with newly hatched virgin queens. In the export cages (see Figure 3.2), bees were fed MixP, Rg100%, RgS, Psup, Sub1, Sub2, FB, PrSub or IS as a control. The number of dead queens and the nurse bees were recorded daily for 10 days.

3.2.2. Feed preparation

The method for preparing the feeds can be found in Chapter 2. Part 1 and 2: Table 3.1 gives details of the ingredient used and the crude protein content (CPC) which is calculated based on the values described in Appendix 3. The dry material was combined with syrup to make into soft patties.

Table 3.1: The ingredients for different feeds and percentage crude protein content (CPC). *Rg10%* to *Rg25%*= feed containing 10% to 25% redgum, and *FB10%* to *FB25%*= feed containing 10% to 25% FeedBee®.

Name of feeds	Redgum (g)	FB (g)	IS (g)	CPC (%)
Rg10%	10		90	2.67
Rg15%	15		90	4.00
Rg20%	20		80	5.34
Rg25%	25		75	6.68
FB10%		10	90	3.58
FB15%		15	90	5.37
FB20%		20	80	7.16
FB25%		25	75	8.95

Part 3: MixP, Rg100%, RgS, Psup, Sub1, Sub2, FB, PrSub and IS were made with ingredients previously describe in Chapter 2.

3.2.3. Cages

Part 1 and 2: HNB were raised in nosema-free wooden cages hand built by Dr. Rob Manning (see Manning 2006, 67). American plastic Jz-Bz export cages were used (see Figure 3.1). These were obtained from JL Guilfoyle Pty. Ltd., Western Australia. For Part 3, wooden export cages were used. These cages are a traditional type of exporting queen cage used in Western Australia. Mostly used by a resident queen raiser and formerly used by the Department of Agriculture and Food, Government of Western Australia (see Figure 3.2).

Part 3: HNB were raised in large cages (see Chapter 2).



Figure 3.1: Jz-Bz export cages.



Figure 3.2: Wooden export cages. A= food on one end and B= piece waxed paper to cover the food.

3.2.4. Raising virgin queen bees

Part 1 and 2: new frames of comb were placed in strong hives 4 days prior to grafting for the queens to lay in. Twelve plastic queen cells were either glued onto wooden cell bars or metallic bars using bee wax and placed in strong hives a day prior to grafting. On grafting day, frames containing 1 to 2-day-old larvae that were prepared earlier were taken out of the hives. Using a standard plastic Chinese grafting tool, 1 to 2-day-old larvae was gently scooped up and transferred to plastic queen cells. When a bar of cells was full, they were immediately returned to the hives they were taken from. These hives were queen-right with queen below an excluder and uncapped brood placed either side of the queen cells in the super box above the excluder. When queens were due to hatch, queen catchers (stainless steel and plastic) were placed over the cells to prevent virgin queens from escaping (see Figure 3.3 and 3.4). For each part, 500 queen cells were grafted. All the items used for grafting were standard items purchased from JL Guilfoyle Pty. Ltd., Western Australia.



Figure 3.3: Left: queen cells on metallic bars ready to graft larvae into them. Right: stainless steel queen holders and one hand made plastic holders on frames with nurse bees. A= black queen cell cups on metallic bars, B= stainless steel spiral queen holders, and C= nurse bees attending to the queens inside the holders.

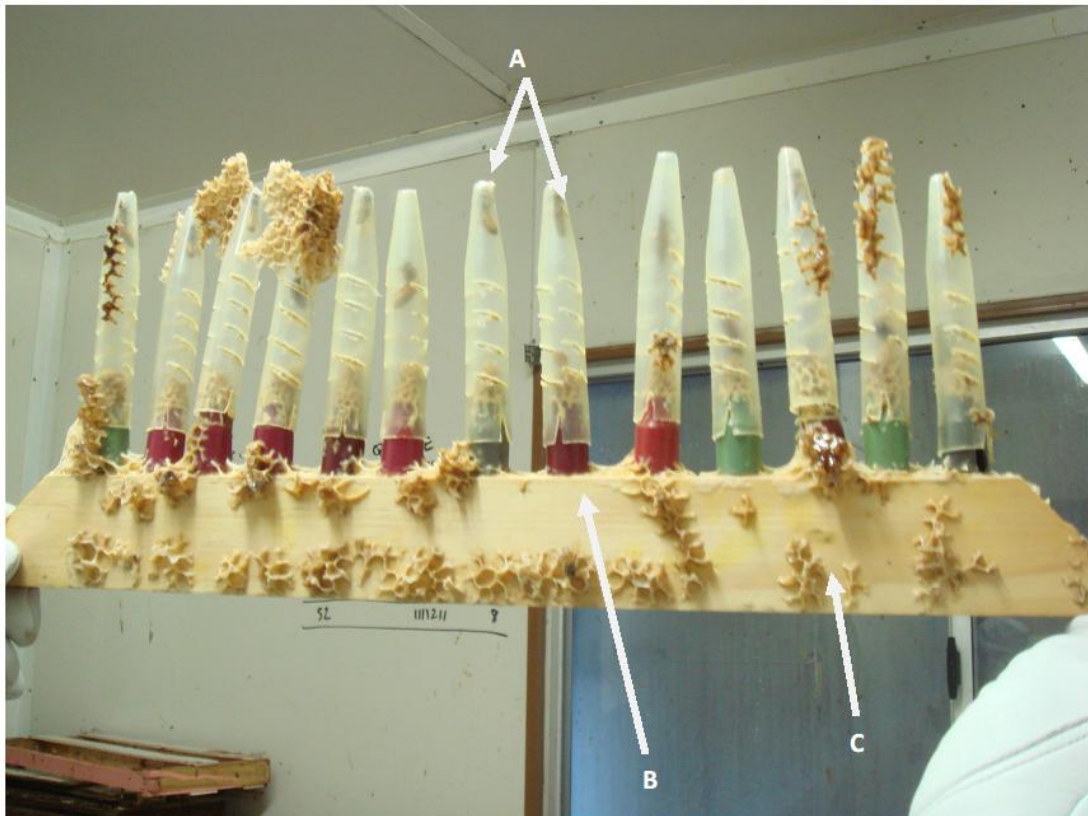


Figure 3.4: Plastic queen holders on a wooden bar. A= plastic queen holders each with a virgin queen inside holders. B= a queen cell cup, and C= a wooden bar. Note the bar is upside down for the purpose of the picture.

The plastic queen catchers were designed and made by the author and Dr. Rob Manning of Department of Agriculture and Food, Government of Western Australia using standard plastic test tubes. Tubes were cut at the end and on the side to allow ventilation.

Part 3: more than 700 queen cells were donated by a queen bee breeder.

3.2.5. Measuring survival

Each day for 10 days, the number of dead nurse bees and dead queens were recorded for survival analysis. The first and second part of this experiment was conducted from March to May 2009 and the third part was conducted from July to October 2009.

3.2.6. Statistical analysis

The “Survival” library in the R Statistical System was used to fit the survival or Kaplan-Meier curves to the longevity data of the queens. A log-rank test (compared to a Chi-squared distribution) was used to compare the survival curves, and estimates of the median survival day are computed for each group (Therneau 1999).

The survival function was defined over time t (the probability that a queen survives at least to the time t). T was the positive random variable with distribution function $F(t)$ and density $f(t)$.

The survival function $S(t)$:

$$S(t)=1-F(t)=P\{T>t\},$$

The hazard rate or hazard function was $\lambda(t)$:

$$\lambda(t)=f(t)/S(t)$$

The constant hazard indicated that, over each interval, a constant proportion of surviving queens were expected to die (Therneau 1999).

3.3. RESULTS

Part 1: the survival of virgin queens and HNB in escort cages was recorded and analysed, the median or day to which 50 % of the queen is still alive is presented in Table 3.2. The survival curves which compared the HNB control and redgum feeds are illustrated in Figures 3.5 and 3.6.

The median survival time for queens fed Rg15% was twice that of HNB fed IS and Rg10% whereas those fed Rg20% and Rg25% have median survival of 6 days which is three times longer than control and Rg10% but not significantly longer than Rg15%. Although Rg15% had median survival time of 4 days, the queens lived until the 8th day, whereas Rg20% and Rg25% queens lived until the 6th day.

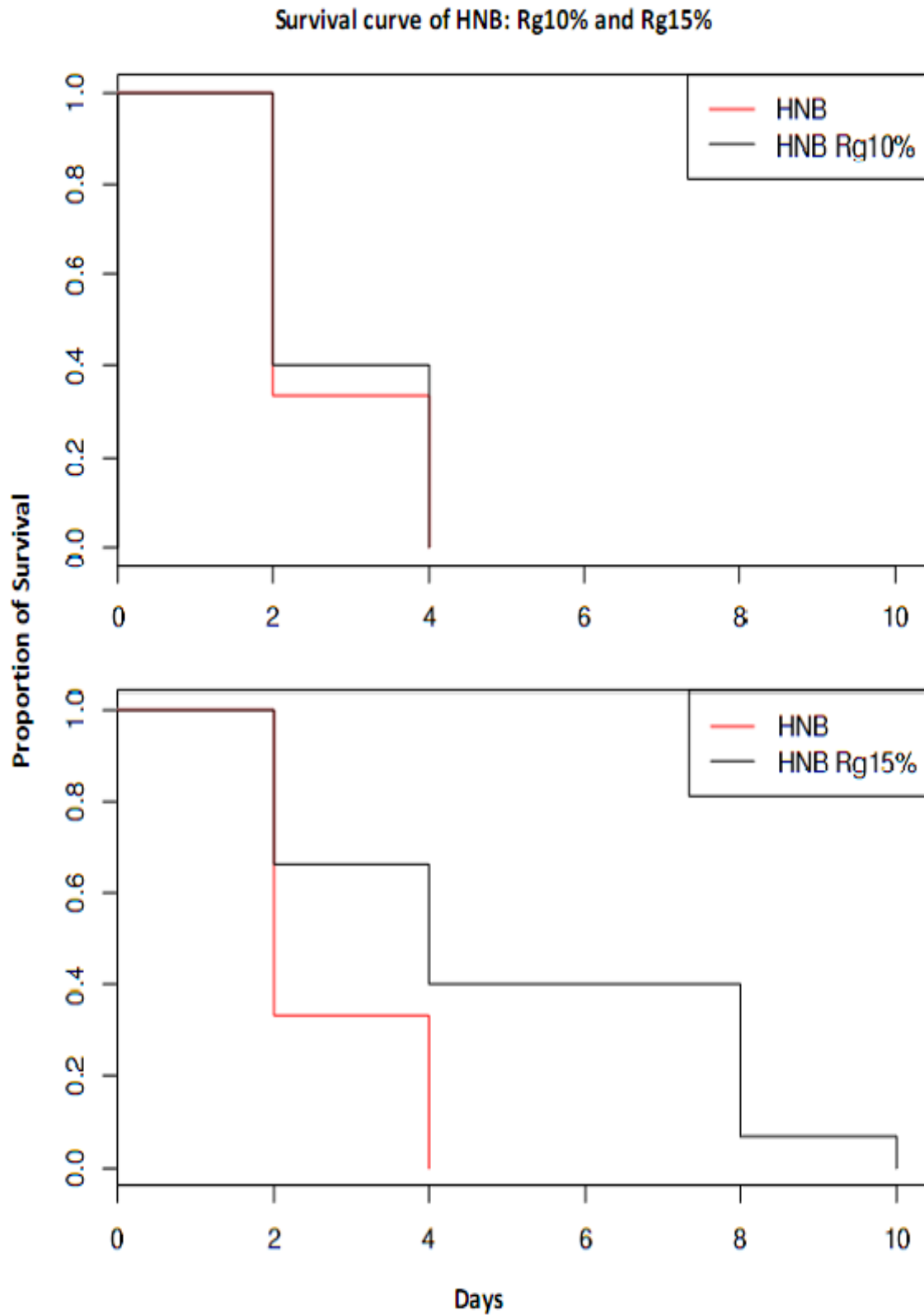


Figure 3.5: Survival curve comparing the hatched nurse bee (HNB) control and Rg10% and Rg15%. *HNB*= hatched nurse bees fed icing sugar, *Rg10%*= feed containing 10% redgum, and *Rg15%*= feed containing 15% redgum.

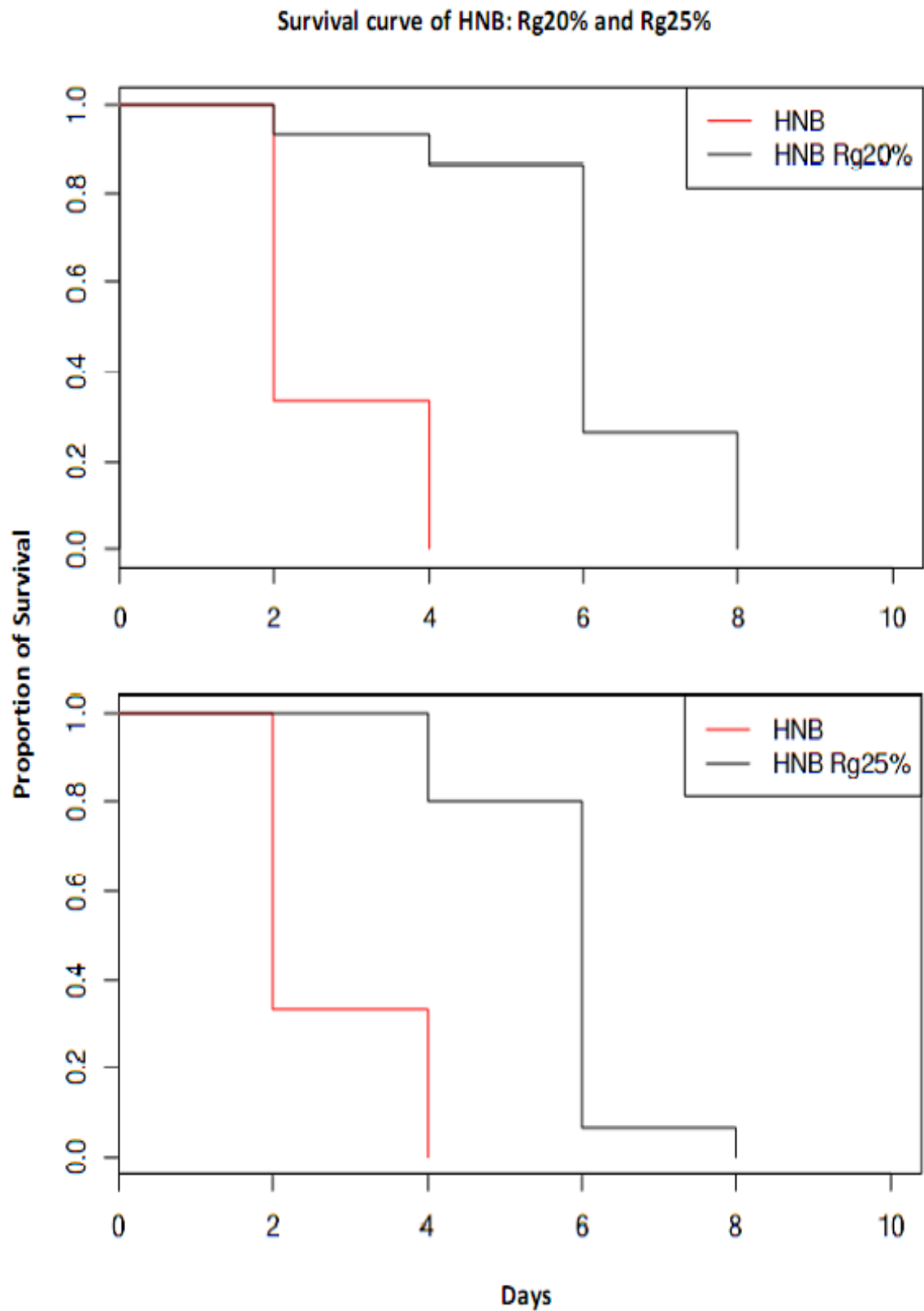


Figure 3.6: Survival curve comparing the hatched nurse bee (HNB) control and Rg20% and Rg25%. *HNB*= hatched nurse bees fed icing sugar, *Rg20%*= feed containing 20% redgum, and *Rg25%*= feed containing 25% redgum.

Table 3.2: Median survival day of caged hatched nurse bees (HNB) on redgum. *NA*= cannot be determined, *LCL*= Lower Confidence Limit, *UCL*= Upper Confidence Limit. Log-rank test= 41.6 on 4 degrees of freedom $P < 0.001$, *IS*= icing sugar (control), and *Rg10%* to *Rg25%*= feed containing 10% to 25% redgum.

Feeds	Total no. of queens	No. at start	No. of deaths	Median	0.95 LCL	0.95 UCL
IS	15	15	15	2	2	NA
Rg10%	15	15	15	2	2	NA
Rg15%	15	15	15	4	2	8
Rg20%	15	15	15	6	6	NA
Rg25%	15	15	15	6	6	6

Part 2: the survival of virgin queens and HNB, 7DAY bees and MNB in escort cages were recorded and analysed, the median or 50 % survival is presented in Table 3.3.

The median survival time for HNB control (IS) without prior feeding was twice as long as 7DAY bee control (IS) which was raised on pure redgum, and four times longer than 7DAY control (IS) was fed pure FB.

The median survival time for HNB control, MNB on Rg10%, Rg25% and FB10% were above 6 days and they were not significantly different to each other, but are significantly longer than all the other feeds (at or below 4 days). Although Parts 1 and 2 of the experiment were conducted in summer, the median survival time for HNB controls in this part of the experiment was four times longer than HNB controls in the first part.

Table 3.3: Median survival day of caged hatched nurse bees (HNB), caged 7-day-old bees (7DAY) bees and hive raised mature nurse bees (MNB) on redgum and FB feeds. NA= cannot be determined, LCL= Lower Confidence Limit, and UCL= Upper Confidence Limit, IS= icing sugar (control), Rg10% to Rg25%= feed containing 10% to 25% redgum, and FB10% to FB25%= feed containing 10% to 25% FeedBee®. ^a HNB fed on IS with no prior feeding, ^b Raised on pure redgum prior to caging, and ^c raised on pure FB prior to caging. Log-rank test= 87.7 on 14 degrees of freedom $P < 0.001$.

Feeds	Total no. of queens	No. at start	No. of deaths	Median	0.95 LCL	0.95 UCL
<u>HNB</u>						
IS ^a	17	17	13	8	4	NA
<u>7DAY</u>						
IS ^b	10	10	9	4	4	NA
IS ^c	10	10	10	2	2	NA
Rg10%	15	15	15	4	4	4
Rg15%	15	15	15	4	2	4
Rg20%	15	15	15	4	2	NA
Rg25%	15	15	15	4	2	NA
FB10%	15	15	15	2	2	NA
FB15%	15	15	15	2	2	6
FB20%	15	15	15	4	2	6
FB25%	15	15	15	4	2	NA
<u>MNB</u>						
Rg10%	7	7	5	8	8	NA
Rg25%	7	7	7	6	4	NA
FB10%	7	7	6	6	6	NA
FB25%	6	6	6	4	4	NA

Part 3: the survival time of both virgin and mated queens and 7DAY bees in escort cages were recorded and analysed, the median or 50% survival is presented in Table 3.4 and 3.5. The median survival time of 7DAY bee control (IS) with mated queens was significantly longer than the control with virgin queens. The median survival time of 7DAY bee control (fed on IS) with virgin queens was about three times longer than 7DAY bees fed on pollen and artificial feeds, while 7DAY control with mated queens was four times longer.

The medial survival time for queens escorted by HNB and 7DAY bees fed Rg10% and Rg15% were not significantly different, however queens escorted by HNB fed Rg20% and Rg25% had significantly longer median survival time by 2 days.

Table 3.4: Median survival of 7-day-old (7DAY) bees on pollen feeds. *NA*= cannot be determined, *LCL*= Lower Confidence Limit, and *UCL*= Upper Confidence Limit. ^a 7DAY bees caged with mated queens. Log-rank test = 118 on 5 degree of freedom $P < 0.001$.

Feeds	Total no. of queens	No. at start	No. of deaths	Median	0.95 LCL	0.95 UCL
IS	16	16	16	5.5	4	7
IS ^a	10	10	8	8.5	7	NA
MixP	35	35	35	2	2	2
Psup	34	34	34	2	2	2
Rg100%	35	35	35	2	2	2
RgS	30	30	30	2	2	2

In general, 7DAY bee controls that were raised on MixP, pure redgum or FB did not improve the survival of the virgin queens they were escorting compared to HNB that was caged with virgin queens without prior feeding. Virgin queens escorted by MNB had significantly longer survival compared to HNB and 7DAY bees on all 16 different feeds. Mated queens escorted

by 7DAY bees had significantly longer survival than virgin queens escorted by 7DAY bees. For 7DAY bees, the presence of natural (pollen feed) or artificial protein (artificial feed) did not increase survival of virgin queens in export cages.

Table 3.5: Median survival of 7DAY bees on artificial feeds. *NA*= cannot be determined, *LCL*= Lower Confidence Limit, and *UCL*= Upper Confidence Limit. ^a 7DAY bees caged with mated queens. Log-rank test = 119 on 5 degrees of freedom $P < 0.001$.

Feeds	Total no. of queens	No. at start	No. of deaths	Median	0.95 LCL	0.95 UCL
IS	16	16	16	5.5	4	7
IS ^a	10	10	8	8.5	7	NA
Sub1	25	25	25	2	2	2
Sub2	25	25	25	2	2	2
FB	25	25	25	2	2	2
PrSub	28	28	28	NA	NA	NA

3.4. DISCUSSION

The aim of this experiment was to determine whether HNB and 7DAY bees raised in isolation of the hive could be used as escorts for queens during export, and whether they could keep 100% of the queen bees alive for more than 8 days. Exporting queens from Australia to another country can take more than a week. When escorted by MNB a mated queen can survive for up to a month (pers. com. Dr. Rob Manning, Department of Agriculture and Food, Government of Western Australia, March 2, 2009). The preliminary experiment showed that 100 % of the virgin queens ($N=16$) escorted by MNB were still alive after 8 days. Ideally, all queens should survive for at least 8 days. Usually, only mated queens are exported, however in this experiment virgin queens were used because they are more vulnerable than mated

queens (author's preliminary observation), and if HNB or 7DAY bees can keep them alive for more than 8 days, it proves that HNB and 7DAY bee could keep mated queens alive for longer.

Part 1: newly hatched bees are born without *Nosemosis* (Bailey and Ball 1991, 68; Higes *et al.* 2007b, 212), thus using them as escorts is a novel way of exporting queens to overcome the Japanese quarantine restriction against bees with *Nosemosis* (see Chapter 2 for information on HNB and nosema infection). At 0-day-old, the HG of HNB is undeveloped and non-functional (Deseyn and Billen 2005, 53), and by providing protein for HNB, they will be able to develop their HG. In a hive HNB that consume pollen with higher protein content will generally produce more royal jelly (Haydak 1937, 258-262). In theory, HNB fed with Rg25% should keep virgin queens alive the longest, which was in agreement with the results, except that Rg20% was also as efficient. Contrary to expectations, the highest surviving group (Rg25%) could not keep 100 % of the queens alive for more than 8 days.

Huang and Otis (1989, 264-275) suggest that for the first three days of a bees' life, their HG are under structural development during which the gland physically cannot secrete royal jelly. It is only after 5 days that nurse bees begin secreting royal jelly (Cruz-Landim and Hadek 1969, 121; Painter and Bieseke 1966, 1425-1416). If this was true, HNB would not be able to keep the queens alive at all, unless they are capable of feeding themselves. To investigate this possibility, in both Part 2 and 3, caged HNB were fed different pollen feeds for 7 days prior escorting queens.

Part 2: in this part of the experiment, virgin queens escorted by HNB (control) survived 2 to 4 times longer than 7DAY bees fed IS or other feeds. This indicates that raising the escort bees in isolation with either pure redgum or FB is actually not beneficial to the survival of the queens. There are a number of possible scenarios which could account for these observations. First, 7DAY bees may not be capable of secreting royal jelly because their glands are not fully functional during the experiment. Second, they may not synthesize royal jelly because they lack appropriate stimulation and they may be less inclined to take care of virgin queens.

The first two possibilities were discussed in detail in Chapter 2. Queens synthesize and secrete a blend of volatile, non-volatile and aromatic compounds to produce queen mandibular pheromone or QMP. This is a powerful attractant of workers that is responsible for eliciting retinue (antennating, licking, grooming, and feeding of the queen) behaviour of 1 to 12-day-old nurse bees (Wossler and Crewe 1999, 343-349; Wossler *et al.* 2006, 1044). Beggs *et al.* (2007, 2460) proved that the key amine responsible for eliciting retinue behaviour in nurse bees is the homovanillyl alcohol in QMP. Although virgin queens can secrete QMP, the quantity is virtually undetectable in 1-day-old virgin queens (Beggs *et al.* 2009, 2464; Keeling *et al.* 2003, 4486; Kocher *et al.* 2009, 1010). The homovanillyl alcohol and other compounds in QMP increase with age of a virgin queen or when she becomes mated, making her more attractive to nurse bees (Sasaki, Irokawa and Sata 1989, 13; Strauss *et al.* 2008, 1527; Wossler and Crewe 1999, 349). In this experiment, only newly hatched or 1-day-old virgin queens were used, this could explain why nurse bees do not want to feed her.

During this experiment when virgin queens were caged with 7DAY escort bees the queens were heard to be “pipping”, which may indicate some alarm or preparation for fighting between the queens and the nurse bees. Wossler *et al.* (2006, 1045-1046) reported hostile behaviour of nurse bees towards virgin queens such as stinging and balling. Likewise a review by Gary (1975, 249) suggested for the first few hours after emerging, virgin queens are ignored, and when they turn 1-day-old, they can be either be accepted or be attacked and chased around the hive until they becomes mated. Hefetz and Katzav-Gozansky (2004, 46) found that nurse bees are even repelled by the smell of decyl ecanoate found in virgin queen faeces and will sting any bee smeared with this compound, including the virgin queens.

In this part of the experiment, virgin queens escorted by HNB (control) had a median survival time that was longer than bees fed with food containing solid protein which lived only for 2 to 4 days. Standifer *et al.* (1971, 231) recorded similar survival time for isolated virgin queens fed 7.5 % protein gumweed pollen which averaged 2.7 days while virgin queens fed dextrose-levulose syrup and royal jelly lived for 7.4 days. In a normal hive, queens feed exclusively on royal jelly which is high in readily digestible aqueous protein (Dietz 1975,

132). If indeed virgin queens were not attended to or fed by the nurse bees in their cages, they would rely on themselves to feed. Both FB and redgum feed contain solid proteins, and queens may not be able to digest pollen grains or solid protein particles found in them. This could explain why virgin queens that had access to pure carbohydrate feed survived longer than queens with feed fortified with protein. However this does not explain why virgin queens escorted by 7DAY bees fed on IS died much earlier than when she was escorted by HNB fed on IS.

During this experiment, 7DAY bees were seen to attack the virgin queens while HNB were calm and fighting was never observed. In several cases, dead virgin queens escorted by 7DAY bees were found with her sting ejected, indicating that she died fighting. A combination of this volatility and negligence towards the virgin queens may have resulted in a shorten life span. However this explanation is still not adequate, because it does not account for why MNB (similar age but raised in hives) kept their virgin queens alive up to twice as long. This clearly indicates fundamental difference between the retinue capacities of cage-raised bees fed redgum and FB and those that are hive-raised (MNB).

“Single-cohort colonies” or colonies with only a single age or type of bees adapt to different roles (Robinson *et al.* 1989, 110). In this case, all the HNB bees did not remain nurse bees; instead some became foragers and guards over time. This might be a problem because it means that some of the 7DAY bees caged with queens were not nurse bees at all. Evidences from this part of the experiment suggest that the lack of normal stimuli that are present in a normal hive have detrimental effects on the behavioural development of HNB. Such stimuli include the presence of brood and being fed by older nurse bees as described in Chapter 2.

Part 3: virgin queens escorted by 7DAY bee controls (IS) in this part of the experiment had similar median survival time as 7DAY bee controls described in Part 2. However mated queens escorted by 7DAY bee controls survived twice as long as virgin queens under the same condition. Like Part 2, virgin queens were also prone to being attacked by 7DAY bees that escorted them. Contrary to this, 7DAY bees were very calm in the presence of mated

queens, and fights were never observed. This is no surprise as mated queens can secrete high levels of homovanillyl alcohol which attract and evoke full retinue behaviour of nurse bees (Wossler and Crewe 1999, 349). Wossler *et al.* (2006, 1047) found that mated queens have 11 times higher homovanillyl alcohol compared to 1-day-old virgin queens. Although 7DAY bee controls kept 50 % of their mated queens alive after 8.5 days, it is shorter than MNB (fed IS) which kept 100 % of their mated queens alive for 8 days. The fact that the 7DAY bee controls were not aggressive towards the mated queens may be the key reason to longer median survival when compared with 7DAY bee controls with virgin queens. Evidence from this part of the experiment once again reinforces the theory that lack of normal stimuli that are present in a normal hive can be detrimental on the behavioural development of nurse bees.

The sample size (queens) was greater than previous studies such as by Hefetz and Katzav-Gozansky (2004), Kocher *et al.* (2009), Sasaki, Irokawa and Sata (1989), Standifer *et al.* (1971), and Wossler *et al.* (2006) which used 5-25 queens for each variable they investigated. Despite this, the survival analysis was did not determine the Upper Confidential Limit which restricted what can be compared in terms of significance levels.

Overall, regardless of the feed, HNB, 7DAY and MNB cannot keep 100% of the virgin or mated queens alive for more than 8 days, with an exception of mated queens caged with MNB (author's preliminary finding).

3.5. CONCLUSION

The first part of the experiment began by comparing the ability of HNB to escort virgin queens without prior feeding. The virgin queens escorted by HNB which had access to IS had a median survival time of 2-days which is only half the time when compared to Rg20% and Rg25%. Part 2 and 3 was dedicated to understand whether raising bees in isolation for 7 days prior caging would increase median survival for queens. Evidence revealed that virgin queens escorted by all the 7DAY bees that were fed food fortified with solid protein (pollen or artificial feed) had much lower median survival time than all the 7DAY bee controls fed only

a pure carbohydrate feed. There are no studies into the queens' ability in digesting solid protein such as pollen, but from this experiment it seems that if they consume food fortified with solid protein, they die prematurely.

In general HNB accepted virgin queens more readily compared to 7DAY bees and MNB. Although 7DAY bees and MNB were of similar age, 7DAY bees was raised in isolation of the hive (in cages) and were not exposed to the appropriate stimuli required for normal retinue behaviour, therefore they are less capable of tending to the virgin queens. The 7DAY bees accepted mated queens more readily than they did virgin queens; this is thought to be provoked by higher levels of homovanillyl alcohol present in the QMP of mated queens.

In conclusion, both HNB and 7DAY bees were unable to keep 100 % of the virgin or mated queens alive for more than 8 days, with an exception of mated queens caged with MNB (author's preliminary finding). The prior feeding of HNB did not help increase longevity or median survival time of virgin queens, and raising nurse bees in cages have detrimental effects on retinue behavioural developments. The best environment to raise any living being is always their natural environment. For bees, it is their hives where they could learn, receive appropriate care and stimuli which help them fully develop their physiques and skills. To produce bees capable of tending to queens and keeping them alive during export, nurse bees needs to be hive-raised.

CHAPTER 4: HEAT THERAPY ON LIVE NURSE BEES TO INACTIVATE *NOSEMA APIS*

4.1. INTRODUCTION

Nosema infection of bees is highest in spring, and in summer the infection becomes drastically reduced (Doull and Cellier 1961, 280-287; Furgala and Mussen 1978, 70; Hornitzky 2005 and 2008b). There have been several theories into why this happens. Matilla and Otis (2006, 709) believed that the high numbers of emerging nurse bees and their interaction with infected individuals increase the nosema infection. Bailey and Ball (1991, 70) suggested that the massive spring clean up of infectious faeces (in colder climate) in the hive contributes higher nosema infection. Others like Burnside and Revell (1933, 603) and Manning (1993, 288) have found higher temperatures in the environment retarded the multiplication.

Heat therapy has been applied effectively to kill or inactive microsporidia in arthropods. Boohene, Geden and Becnel (2003, 1146-1152) found that by heating wasp (*Muscidifurax raptor*) pupae for 45 minutes at 50 °C inactivated all the *Nosema muscidurafacis* spores which causes nosema disease. When these wasp pupae were heated for 60 and 75 minutes, 3 or 5 hours at 45 °C, 85-100 % were cured. For defence, honey bees are capable of heating up to 46 °C during heat-balling (Ono, Okada and Sasaki 1987, 1031) and experimentally can be heated up to 51.8 ± 0.42 °C (Ken *et al.* 2005, 494).

The biotic potential of *N. apis* spores is 33 °C (Martín-Hernández 2009, 2556), and at 37 °C replication stops (Burnside and Revell 1933, 606). Hive equipments heated for 15 minutes at 60 °C (The World Organisation for Animal Health 2009) and 24 hours at 49 °C (Cantwell and Shimanuki 1969, 53; Moeller 1978) kills *N. apis* spores. No one has investigated the effect of heat therapy on the viability of *Nosema apis* in live honey bees, thus the aim of this

experiment is to see whether incubating nurse bees at elevated temperatures could inactive *N. apis* spores whilst keeping the bees alive.

4.2. METHODS

4.2.1. Experimental design

Infected hive-raised mature nurse bees (MNB) were brushed into large cages (refer to Chapter 2) and incubated at variable temperatures and times as described in Table 4.1. Bees that were incubated for more than 24 hours were given 50 % w/w sugar solution or syrup. Bees were all incubated in a Day-night thermostat controlled incubator from Lindner & May Pty. Ltd., Brisbane (serial: 521).

As a positive control, hatched nurse bees (HNB) were fed spores extracted from infected hive-raised MNB that was incubated for 3 days at 32 °C. As a negative control, HNB that are nosema-free are fed sugar syrup. All the HNB that were fed spore extracts and sugar solution were incubated for 6 days at 32 °C. Twenty HNB were used for each treatment. The viability was assessed by *in vitro* germination of *N. apis* spores extracted from samples and by feeding extracted spores to HNB.

Table 4.1: Mature nurse bee (MNB) heat treatments. ^a Positive control.

Temperature (°C)	Time (hours)	No. of MNB incubated
32.0 ^a	72.0	240
40.0	24.0	245
40.0	47.0	261
40.0	72.0	241
42.0	3.00	220
50.0	0.75	241

4.2.2. Assessing nosema infection

After the heat treatment, all dead bees were removed from each treatment and the live bees were frozen at -20 °C for 48 hours. All the abdomens were removed from bees from each treatment and spores were purified according to Rice (2001). Initially, the spores were subject to *in vitro* germination (Rice, 2001); however, no germination could be observed even on the positive control. It was then decided that the spores be fed to HNB. For each treatment, 20 HNB (collected as they hatched from their frames, so these bees are definitely nosema-free) were feed purified spores, each dose containing approximately 500,000 *N. apis* spores.

Spore concentrate was mixed with syrup and fed by pipetting directly into the bees' mouth piece. After 6 days, dead bees were removed and were then frozen at 4 °C for 48 hours before the abdomens were removed. *Nosema apis* spores were examined microscopically following Cantwell (1970, 222-223) where 1 ml of distilled water was added to each abdomen. If one or more spores were identified, it meant the spores were still viable.

4.3. RESULTS

During the heat therapy, only bees that were incubated at 40 °C for 47 hours and 72 hours died. At 47 hours, 56 % of the bees died and at 72 hours all of the bees died. Some bees died during the 6 day incubation period, and these bees were removed at the end of the experiment.

After 6 days, all HNB abdomens were found to contain spores except for the negative control. This means, at 6 days, there was more spores than what was fed in the beginning.

Table 4.2: Mortality of mature nurse bee (MNB) and the presence of spores in HNB. *ND*= not determined, and ^a Positive control.

Temperature (°C)	Time (hours)	Mortality (%) of incubated MNB	<i>N. apis</i> found in HNB
32.0 ^a	72.0	0.00	Yes
32.0 ^b	72.0	ND	No
40.0	24.0	0.00	Yes
40.0	47.0	56.0	Yes
40.0	72.0	100	ND
42.0	3.00	0.00	Yes
50.0	0.75	0.00	Yes

4.4. DISCUSSION

The aim of this experiment is to see whether incubating nurse bees at elevated temperatures inactivated *N. apis* spores whilst keeping bees alive. Spores were found in abdomens of the HNB, this means that MNB subject to treatments (described in Table 4.2) did not inactivate *N. apis* spores. Results from this experiment cannot prove whether the elevated temperature in summer caused the drastic reduction in infection, but it did show that bees can sustain high temperatures for a fairly long time.

Fantham and Porter (1912, 163-189) described that active *N. apis* spores will begin germination in the chyle stomach of a bee when their cell wall is broken down by the digestive enzymes. When the spore germinates, the polar filaments are released allowing the spore to latch onto the epithelial cells of the stomach where it initiates spore production, and by 6 days, mature spores are formed (Gochnauer, Furgala and Shimanuki 1975, 639). And hence if *N. apis* spores were inactive, the digestive enzymes would have broken down the cell walls and so when it reached the abdomen, no visible mature spores should have been found.

So, in theory, if spores were found in the abdomen 6 days after artificial infection, it means the spores were not inactivated and indeed have multiplied into mature spores.

In a very short time, each bee had to ingest and digest 500,000 spores and it is possible that not all the spores were digested and consequently were intact and passed to the ventriculus and rectum. After this experiment was carried out, Fenoy *et al.* (2009, 6886) published a study which effectively used Sytox green and 4', 6-diamidino-2-phenylindole to stain *Nosema ceranae* and could accurately identify whether the spores were viable. Viewing *N. apis* spores in the abdomen is an inadequate way of determining spore viability, an accurate and quantitative method should be used, such as the viability staining by Fenoy *et al.* (2009, 6886).

4.5. CONCLUSION

This is the first experiment to show that honey bees can be heated for up to 50 °C for 45 minutes without mortality. To improve heat therapy, future experiments should consider conjunctive use of chemotherapy as well as the viability staining techniques to quantitatively evaluate the number of viable and non-viable mature spores.

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APPENDIX 1

The most noticeable stage in the life history of *Nosema apis* is the spore and this is where their life cycle begins. The primary infection begins when bees consume the spore allowing it to travel to the chyle stomach, where the acidic digestive juices begin the process of breaking down the cell wall of the spore. Upon contact to the stomach juices, the sporoplasm will press onto the vacuole and the polar filament (Figure 1.3) is ejected with tremendous force (spore germination). The filament acts as a temporary fixture to the gut wall (Fantham and Porter 1912, 163-189).

It then follows several migratory sequels taking the spore through gut cells as well as into haemocoelic fluid. Assuming it enters the epithelial cells of the gut, it engages in spore germination, growth and multiplication (Fantham and Porter 1912, 163-189). There are many reasons why germination is initiated but it is mostly due to the changes in the physical and chemical nature of the environment the spore is exposed to (Undeen 1990, 223). In the case of *N. apis* spores, varying levels of *in vitro* germination can be achieved by dehydration of spores in air, followed by the rehydration with neutral distilled water or phosphate buffered saline at pH 7.1 or solution of 0.5 M NaCl with 0.5 M NaHCO₃ and pH of 6 (Weidner *et al.* 1999, 210-211).

There is a general consensus on germination, however, scientists remain much divided about their interpretations of what they believe is the life cycle of *N. apis* after germination, some more intricate than others (Jacobs 1977, 79-83). Ultimately, the objective of an infection of this kind is for the spore to multiply into more spores, and then spread it to continue their life cycle. In order for that to happen, the sporoplasm is subject to complete alteration which involves inimitable reconstruction of the membrane topology (Keeling and Fast 2002, 93-95).

After the cell wall of mature spores is degraded and the filaments are released, it undergoes transformation to become planonts. The planonts replicate and eventually becomes meronts

via schizogony. Meronts elongate and soon forms diplokaryon (autogamy). After a few more modifications, elongated meronts transform into mature spores via sporogony (Fantham and Porter 1912, 163-189; Jacobs 1977, 79-83).

Sporogony is a process whereby the spore wall thickens and organelles (polar filaments, endoplasmic reticulum and ribosomes) develop (Didier 1998, 1-2). The replication of more spores inside the epithelial cells continues to increase of the osmotic pressure, and when it reaches the maximum threshold, the pressure will rupture the cell wall. When the cell ruptures, it releases spores and digestive acidic into the ventriculus causing inflammation and swelling (Shimanuki and Knox 2000, 17).

A portion of the released spores will remain in the lumen, while the remainder will be expelled in faeces which mark a renewed cycle (Bailey and Ball 1991, 64-72). See Figure A1 for a diagram of *N. apis* replication.

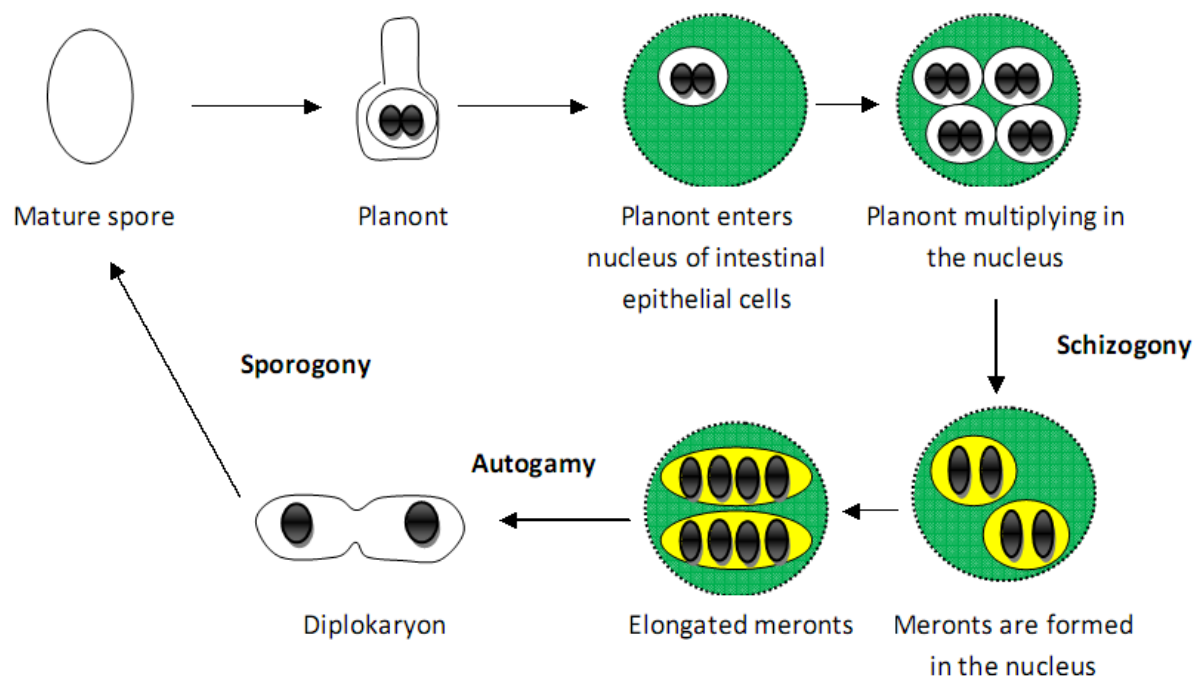


Figure A1: *N. apis* replication.

Self infections manifest by the repeat of the parasites life cycle from the remainder of the vegetative spores in the lumen, hence the continuous deterioration of the bee over time (Gajger *et al.* 2009). The replication of spores is rapid, only taking 7-9 days to become fully developed. A typically infested bee would have 30 to 50 million spores (Bailey and Ball 1991, 64-72; Malone and Gatehouse 1998, 172), where only 1 spore is sufficient to cause an infection (Hornitzky 2005).

APPENDIX 2

Table A1: Comparison of the two commonly used chemotherapy agents. ¹ Cantwell and Shimanuki 1970, 263; ² Chioveanu, Ionescu and Mardare 2004, 31; ³ Didier 1997, 1541; ⁴ Goetze and Zeuttschel 1959, 219-220; ⁵ Hornitzky 2005; ⁶ Kochansky and Nasr 2004, 301; ⁷ Manning 1993, 287; ⁸ Moffett, Lackett and Hitchcock 1969, 886-889; ⁹ Morgan, Mandava and Ignoffo 1985, 65; ¹⁰ MSDS for fumagillin 2009; ¹¹ Rice 2001; ¹² Soković *et al.* 2009, 240; and ¹³ Williams *et al.* 2008, 342.

	Fumagillin	Thymol
Mode of Action	Inhibit DNA replication of <i>N. apis</i> ⁵ ; and affect RNA synthesis ⁹	Disintegrate fungal hyphae and cytoplasmic membrane releasing the cellular components ^{10, 12}
Commercial product with this active ingredient	Fumidil B	Protofil ²
Other uses	Treat microsporidiosis in AIDS patients and <i>Encephalitozoon hellem</i> and <i>E. intestinalis</i> ³ ; <i>N. ceranae</i> ¹³	Suppress <i>Varroa jacobsoni</i> and <i>Acarapis woodi</i> ; and inhibit growth of <i>Escherichia coli</i> and <i>Salmonella typhimurium</i> ¹¹
Origin	Natural antibiotic secreted by <i>Aspergillus fumigatus</i> ⁶	From <i>Thymus vulgaris</i> , <i>Thyme</i> , Horsemint and others ¹¹
LD50	Oral: 2000 mg/kg of rat body weight ¹⁰	Oral: 980mg/kg of rat body weight ¹¹
Detection in honey	0.01 mg/kg in unfed colonies; 0.07 mg/kg in fed colonies ⁷	Yes: detected above 1.1 mg/kg of sugar solution fed to bees ¹⁰
Effects on infection	Significantly lowers infection ^{1, 4, 6, 8}	Significantly lowers infection ¹¹

APPENDIX 3

Mix pollen is calculated based on 25 % protein content. FeedBee® contains 35.8 % (from package). Table A1 lists the main ingredient in feed and their amino acid content which was used to calculate and estimate the protein content in each feed. The calculation is based on the total amino acid content (essential and non-essential). Amino acid content for micellar milk protein, soy protein isolate and whey protein concentrate were derived from packaged labels.

Table A2: Protein content used for calculating the protein in each feed. ^a Yamada and Sgarbieri (2005, 3933), and ^b Manning (2001).

Amino acids	Amino Acids (g) per 100 g of ingredient				
	Brewer's yeast ^a	Micellar milk protein	Irradiated Redgum ^b	Soy protein isolate	Whey protein concentrate
Essential					
Arginine	0.00	6.20	1.90	15.0	5.70
Histidine	4.70	5.50	1.00	5.12	2.90
Lysine	8.90	12.3	2.10	12.4	14.8
Tryptophan	1.30	2.70	0.80	2.76	4.60
Phenylalanine	0.00	10.1	1.20	10.2	5.50
Methionine	2.70	2.50	0.70	2.56	3.40
Threonine	5.20	3.50	1.00	7.46	8.60
Leucine	6.70	17.2	1.90	16.0	19.5
Isoleucine	4.70	9.80	1.20	9.65	8.80
Valine	5.40	11.7	1.40	9.85	8.1
Non-Essential					
Others	0.00	18.5	14.6	9.00	8.00
Total	39.6	100	26.7	100	100

APPENDIX 4

Figure A2 to A5 shows the median head weight of MNB fed on IS (control), and 7DAY bees fed MixP, Rg100%, RgS, Psup, Sub1, Sub2, FB, and PrSub.

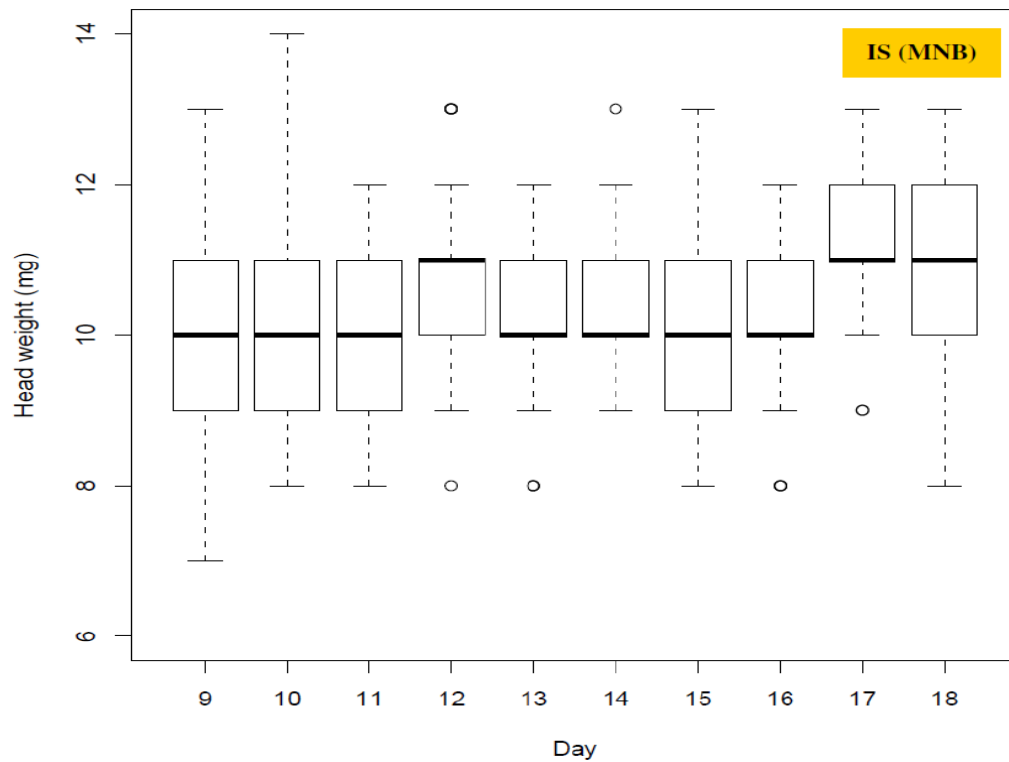


Figure A2: Median head weight (mg) mature nurse bees (MNB) fed IS from days 9-17. *IS*= icing sugar, *dark line*= median head weight, *box*= lower 25 % and upper 75 % quartiles, *whiskers*= 1.5 x interquartile range, and *dots*= outliers outside the whiskers ($N=50/\text{day}$).

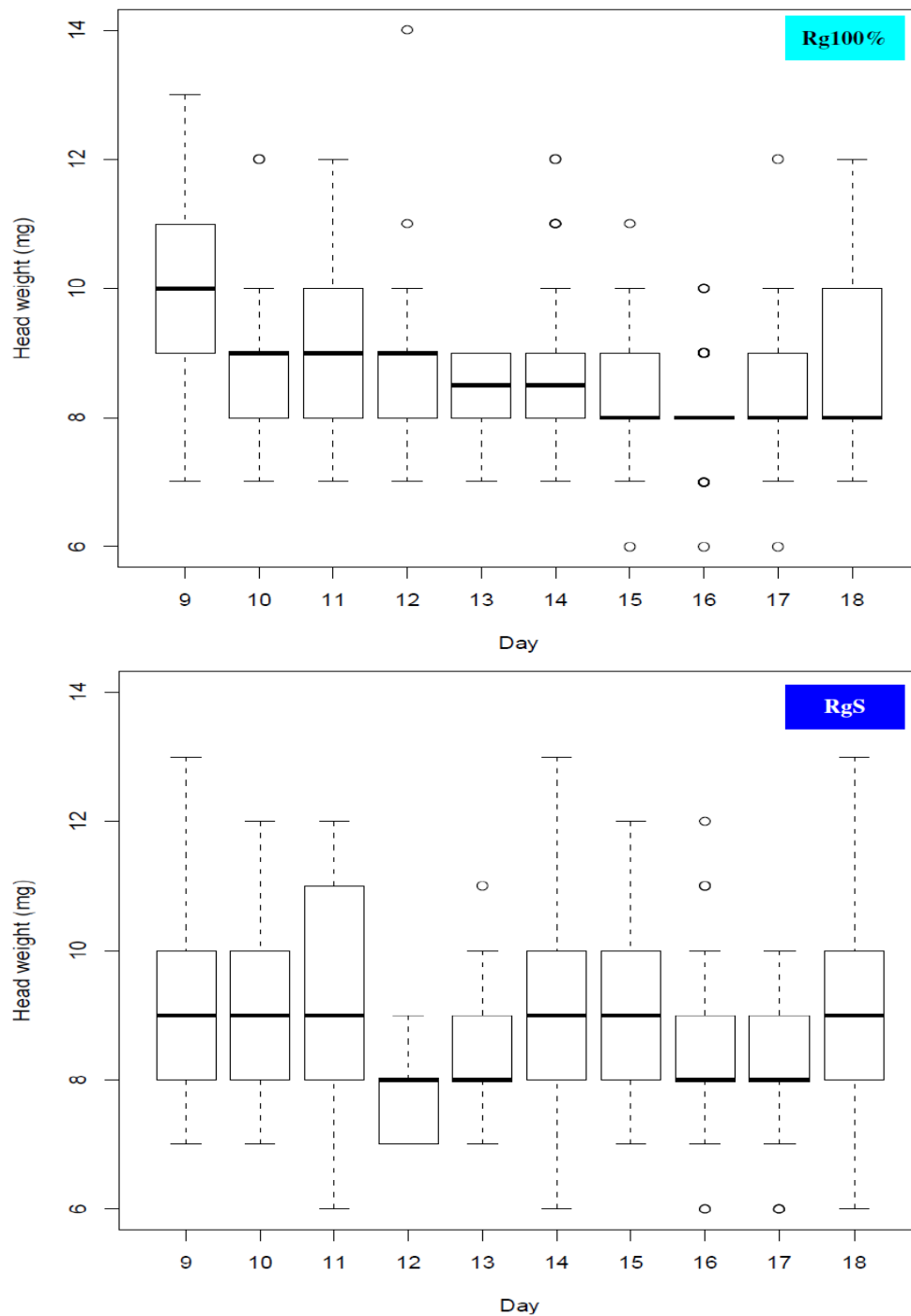


Figure A3: Median head weight (mg) 7-day-old (7DAY) bees fed Rg100% and RgS. from days 9-17, *Rg100%*= redgum pollen, *RgS*= redgum and soya isolate, dark *line*= median head weight, *box*= lower 25 % and upper 75 % quartiles, *whiskers*= 1.5 x interquartile range, and *dots*= outliers outside the whiskers ($N=50/day$).

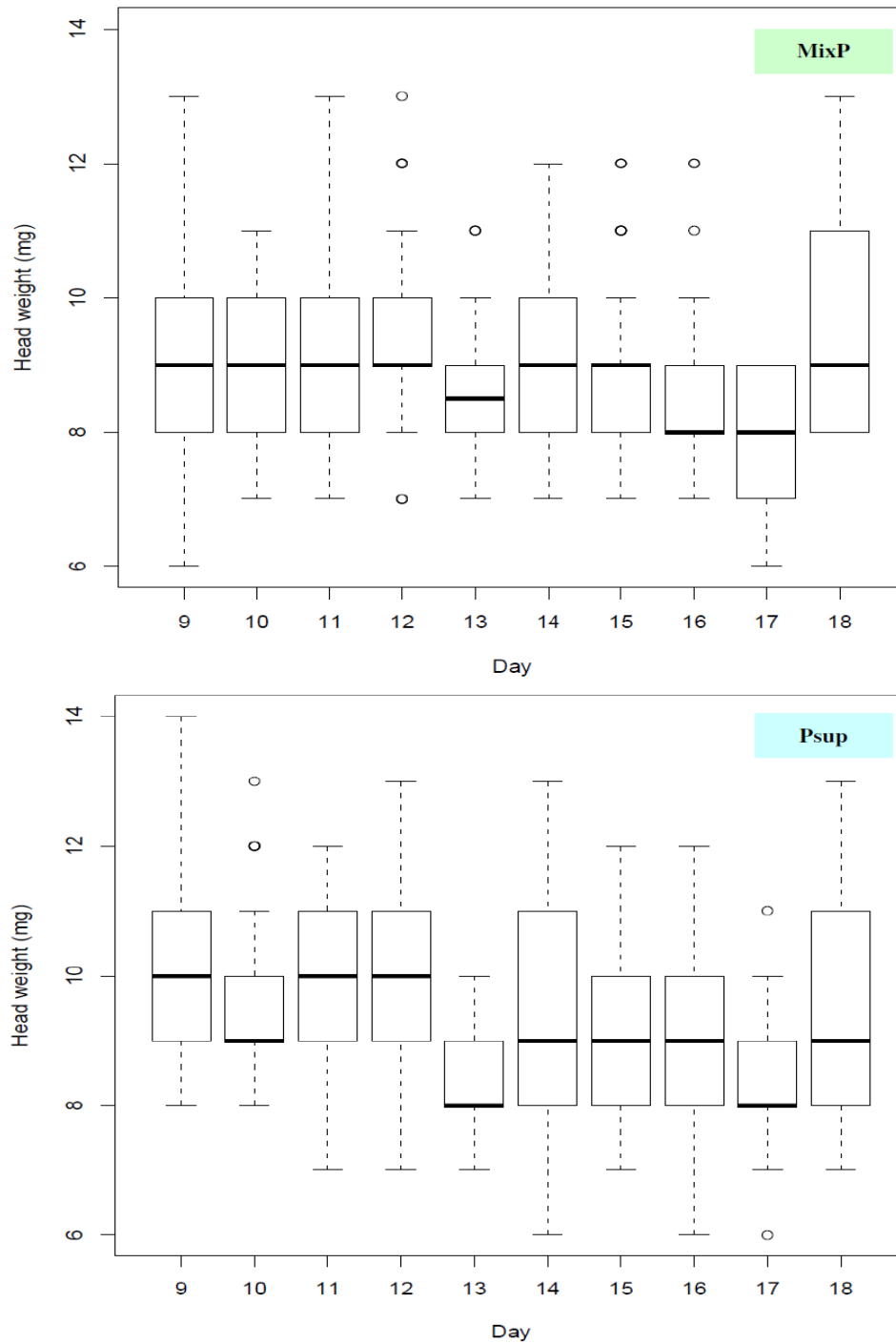


Figure A3: Median head weight (mg) of 7-day-old (7DAY) bees fed MixP and Psup from days 9-17. *MixP*= mix pollen, *RgS*= redgum and soya isolate, dark *line*= median head weight, *box*= lower 25 % and upper 75 % quartiles, *whiskers*= 1.5 x interquartile range, and *dots*= outliers outside the whiskers ($N=50/day$).

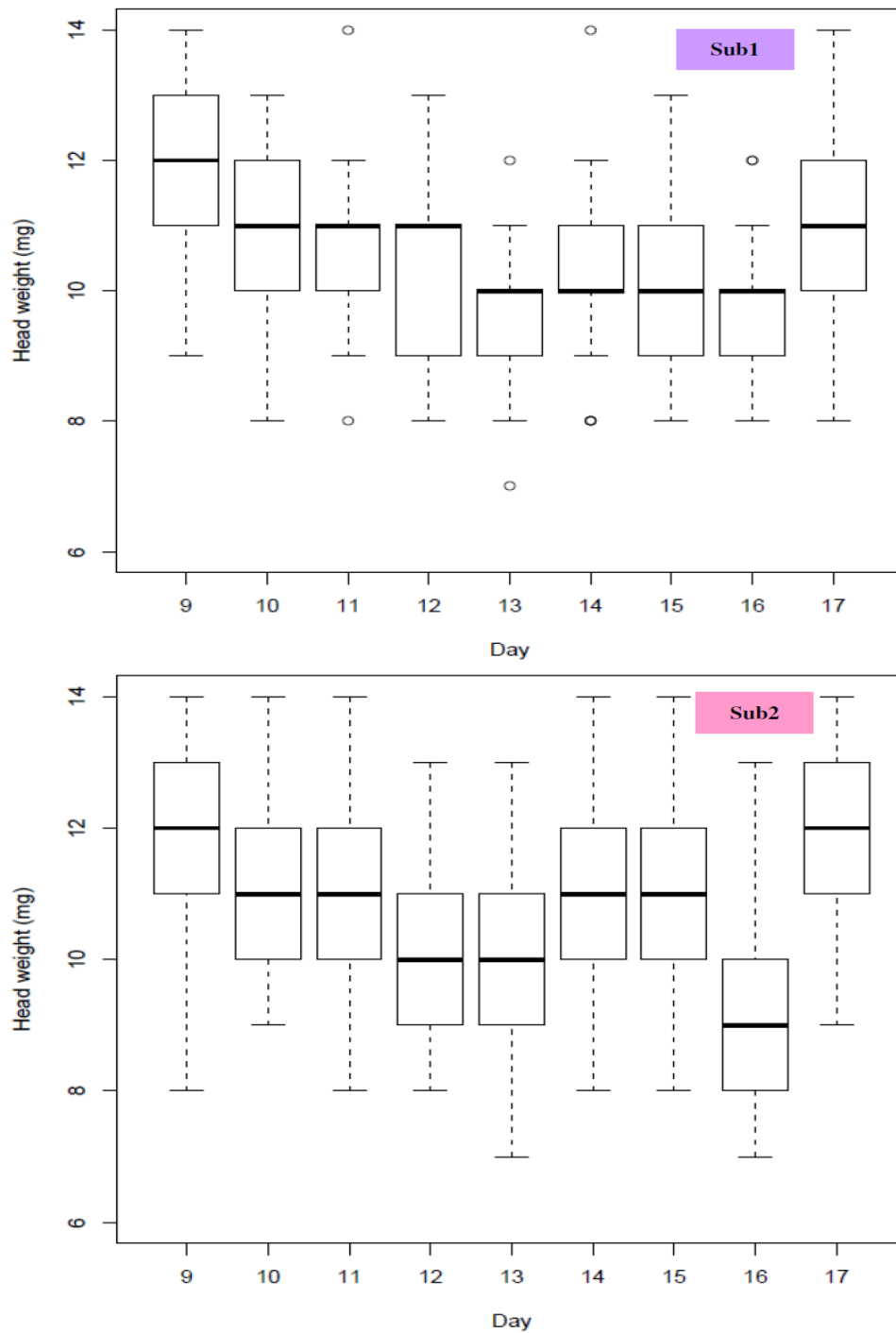


Figure A4: Mean head weight (mg) of 7-day-old (7DAY) bees fed Sub1 and Sub2 from days 9-17. *Sub1*= Substitute No. 1, *Sub2*= substitute No. 2, dark *line*= median head weight, *box*= lower 25 % and upper 75 % quartiles, *whiskers*= 1.5 x interquartile range, and *dots*= outliers outside the whiskers ($N=50/\text{day}$).

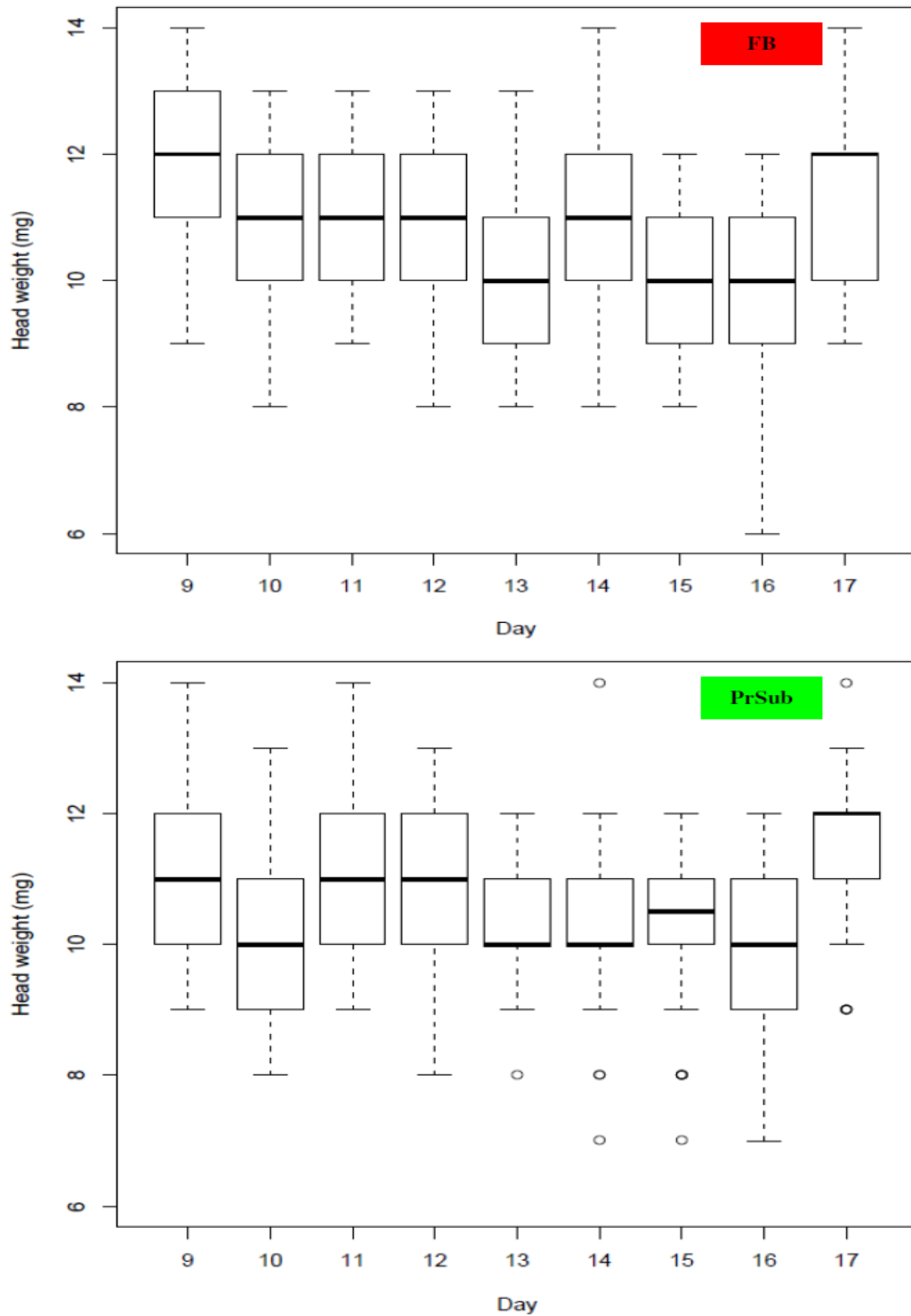


Figure A5: Mean head weight (mg) of 7-day-old (7DAY) bees fed FB and PrSub from days 9-17. *FB*= FeedBee®, *PrSub*= pollen substitute, dark *line*= median head weight, *box*= lower 25 % and upper 75 % quartiles, *whiskers*= 1.5 x interquartile range, and *dots*= outliers outside the whiskers ($N=50/day$).

APPENDIX 5

From standard statistical tables, the cut-off value for an absolute value of the correlation coefficient to be significant ($P < 0.05$) with $N=9$ pairs is 0.67. Any correlations outside this range are regarded as not significant (see Figure A7 and A8).

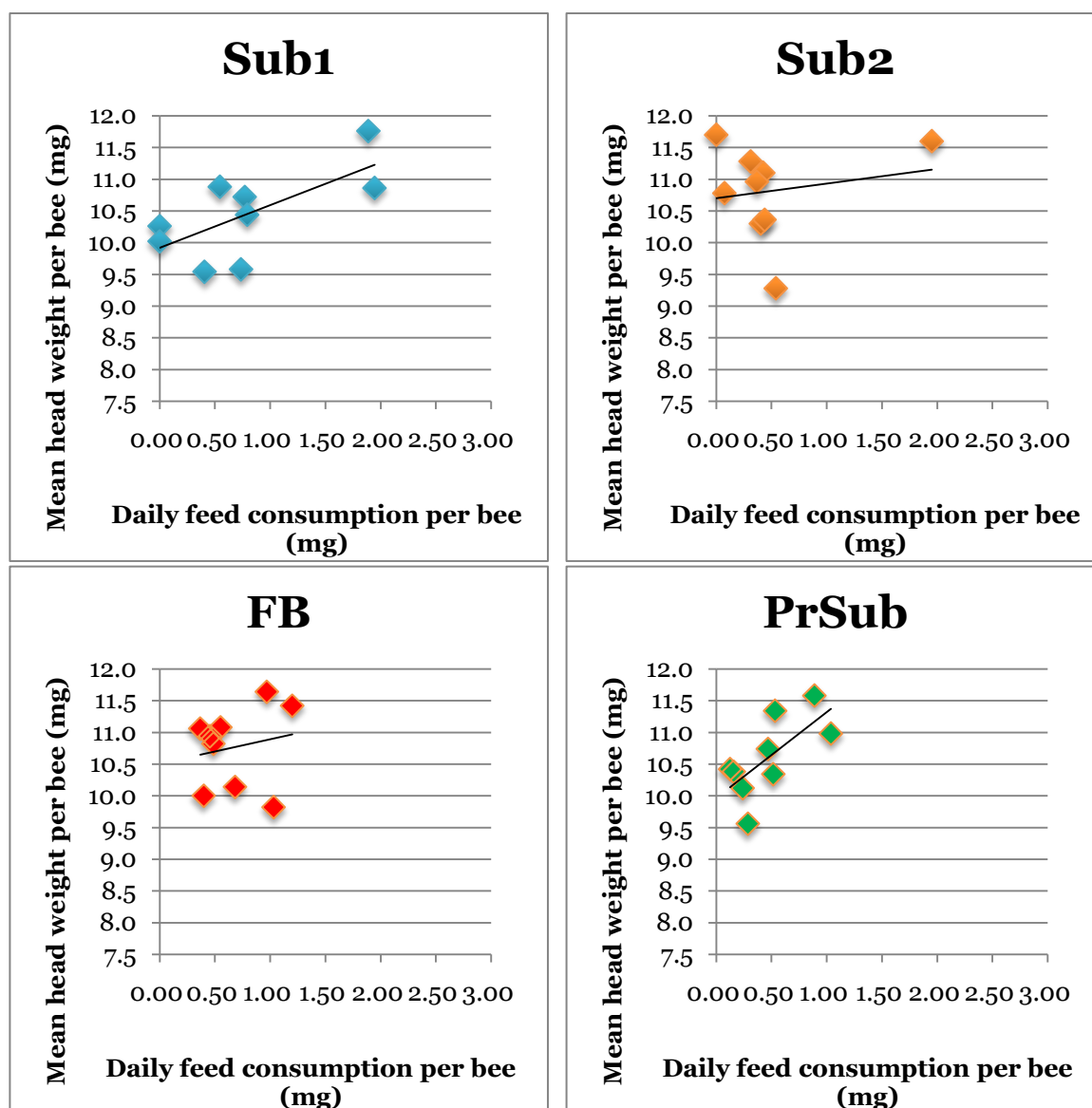


Figure A6: Mean head weight per bee and feed consumption per bee correlation. *Sub1* ($r = 0.68$, $P < 0.05$), *Sub2* ($r = 0.18$, NS), *FB* ($r = 0.19$, NS) and *PrSub* ($r = 0.69$, $P < 0.05$). NS= not significant, FB= FeedBee®, PrSub= pollen substitute, and Sub1= Substitute No. 1, and Sub2= substitute No. 2.

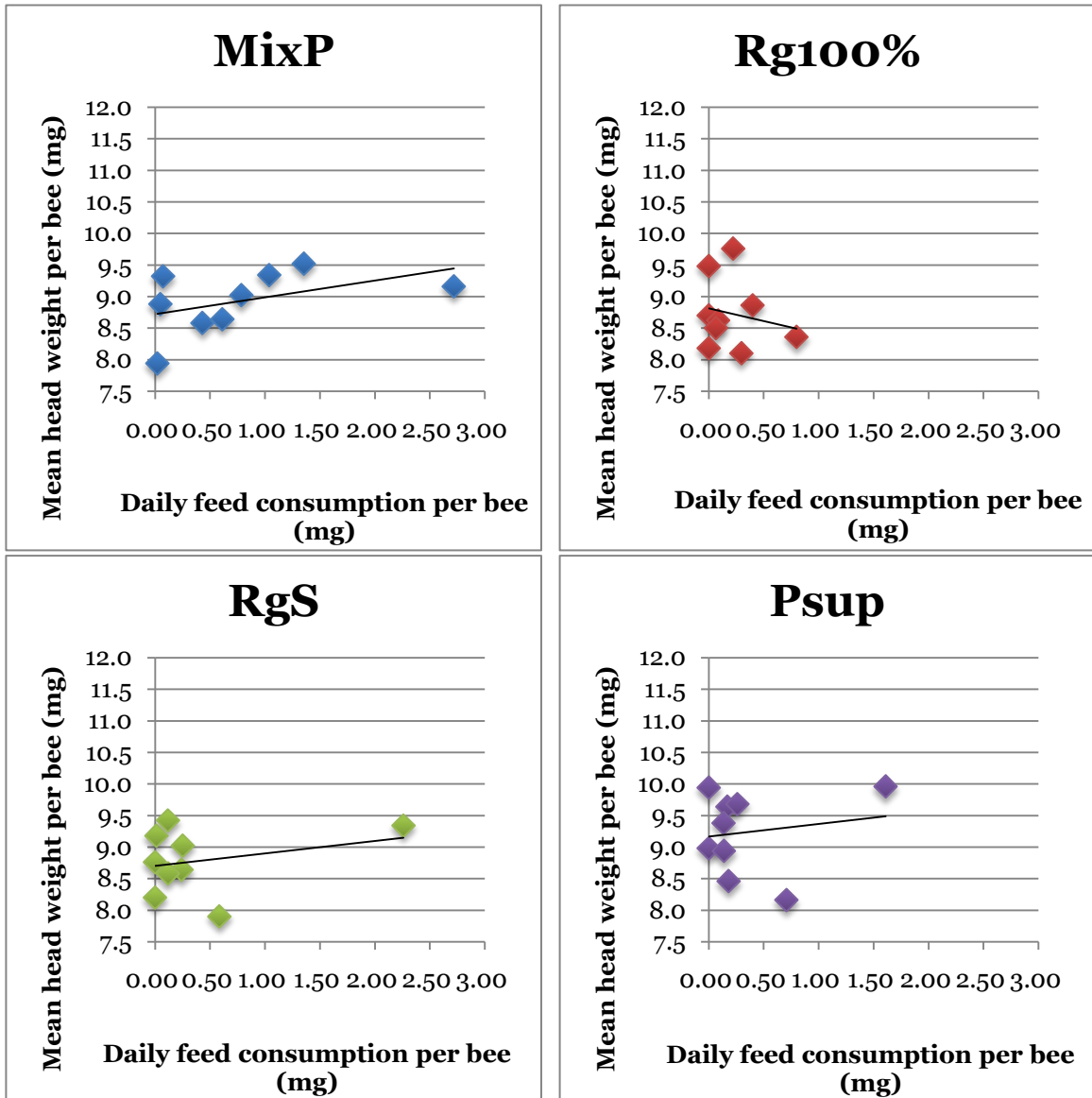


Figure A7: Mean head weight per bee and feed consumption per bee correlation. *MixP* ($r = 0.47$, NS), *Rg100%* ($r = -0.19$, NS), *RgS* ($r = 0.28$, NS) and *Psup* ($r = 0.16$, NS). NS= not significant, *Psup*= pollen supplement, *MixP*= mix pollen, *Rg100%*= redgum pollen, and *RgS*= redgum and soya isolate.